CHARLES UNIVERSITY, PRAGUE, 1ST FACULTY OF MEDICINE
LABORATORY FOR STUDIES OF MITOCHONDRIAL DISORDERS
DEPARTMENT OF PEDIATRICS

STRUCTURAL-FUNCTIONAL CORRELATIONS OF HYDROXYMETHYLBILANE SYNTHASE

PhD thesis

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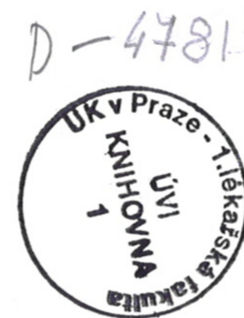

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Prague 2009

ACKNOWLEDGEMENTS

This study was carried out during the years of 2003-2009 at the Laboratory for Studies of Mitochondrial Disorders, Department of Pediatrics, 1ST Faculty of Medicine, 'Charles University, Prague, Czech Republic.

I would like to thank to all those who gave me the opportunity, mental support, and assistance to complete this thesis and, particulary, to the following people:

Firstly to my supervisor, Prof. MUDr. Pavel Martásek, DrSc., for all the experience I gained while working in his lab, and for introducing me to the Porphyria world.

To all my colleagues from Laboratory for Study of Mitochondrial Disorders, esspecially to Mgr. Danka Zahoráková and Ing. Petra Lelková, for everyday intellectual and mental support and constantly cheering me up.

To all from abroad I worked with, esspecially to Xiaoye Schneider-Yin and Elisabeth I. Minder from Central Laboratory, Triemli Hospital, Zurich, Switzerland and to Rivka Mamet and Nili Schoenfeld from National Laboratory for the Biochemical Diagnoses of Porphyrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel, for excellent and very beneficial cooperation.

To my family for the inspiration and the great exemplar in both professional and personal life.

And most importantly, to my husband for his endless support, patience and tolerance, and always keeping me busy outside of my proffessional life.

I appreciate the participation of the patients and their relatives in the study. This research was supported by the Ministry of Education, Sport and Youth of the Czech Republic, and The Granting Agency of the Charles University; contract grant number: GA UK 10/2004, GAUK 257540 54007, MSM0021620806, 1M6837805002. I would also like to thank to American Journal Experts (AJE) for proof-reading of this thesis.

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LIST OF ABBREVIATIONS

AA	Amino acide
AIP	Acute intermittent porphyria
ALA	5-aminolevulinic acid
ALAD	Aminolevulinic acid dehydratase
ALAS1	Hepatic ubiquitous aminolevulinic acid synthase
ALAS2	Erythroid aminolevulinic acid synthase
ATP	Adenosine triphosphate
CAR	Constitutively active receptor
cDNA	Coding deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CEP	Congenital erythropoietic porphyria
CPOX	Coproporphyrinogen oxidase
CYP	Cytochrome P450
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DPM	Dipyrromethane
EEG	Electroencephalography
EPI	European Porphyria Initiative
EPP	Erythropoietic protoporphyria
FECH	Ferrochelatase
GABA	Gamma-aminobutiric acid
gDNA	Genomic deoxyribonucleic acid
GST	Glutathione S-tranferase
HCP	Hereditary coproporphyria
HMB	Hydroxymethylbilane
HMBS	Hydroxymethylbilane synthase
HOXG	Heme oxygenase
HRM	High-resolution melting
IRP	Iron-regulatory protein
Km	Michaelis constant
LS	Lichen sclerosus
mRNA	Messenger ribonucleic acid
NA	Nucleic acide
NO	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis
PBG	Porphobilinogen
PBGD	Porphobilinogen deaminase
PCR	Polymerase chain reaction
PCT	Porphyria cutanea tarda
PDB	Protein database
PPOX	Protoporphyrinogen oxidase
PXR	Pregnane xenobiotic receptor
RNA	Ribonucleic acid
SDS	Sodium dodecylsulfate
URO	Uroporphyrin
UROD	Uroporphyrinogen decarboxylase
UROS	Uroporphyrinogen III synthase
Vmax	Maximal velocity
VP	Variegata Porphyria

INTRODUCTION

Background

The porphyrias are a group of predominantly inherited disorders in heme biosynthesis in which a specific spectrum of accumulated and excreted porphyrins and heme precursors are associated with characteristic clinical features. With the exception of the first enzymatic step, seven deficiencies linked to seven enzymes in the heme biosynthesis pathway have been described. Porphyrias can be divided into hepatic or erythropoietic categories, depending on the anatomical origin, and acute or cutaneous groups, depending on the clinical presentation. The two types of clinical expression can occur separately or together: acute life-threatening neurovisceral attacks, which are associated with the overproduction of aminolevulinic acid (ALA) and porphobilinogen (PBG); and cutaneous symptoms that result from photosensitisation caused by porphyrins.

The acute intermittent porphyria (AIP) is an autosomal dominant disorder, classified as acute hepatic porphyria. It is characterised by a deficiency of hydroxymethylbilane synthase (HMBS), the third enzyme in heme biosynthesis. Where an affected allele is present, the activity of the enzyme is decreased by about 50% in all tissues. The prevalence of symptomatic disease varies from 1 to 10 per 100 000, but due to frequent misdiagnosis and low penetrance the rate may be much higher (Badminton and Elder, 2002; Meyer, et al., 1972; Strand, et al., 1970).

The clinical manifestation of the disease is associated with an acute neurological syndrome characterised mainly by acute attack (Albers and Fink, 2004; Meyer, et al., 1998). Acute attacks are manifested by a wide variety of clinical features including autonomic neuropathy with arrhythmias, central nervous system impairment, peripheral motor neuropathy with muscular weakness and paresthesia or less common sensory symptoms, and the most common clinical presentation, a severe pain caused by neurovisceral crises, usually of abdominal or back origin (Meyer, et al., 1998; Nordmann and Puy, 2002).The porphyric manifestation usually occurs in the third decade in life, though in rare cases it can manifest before puberty or after menopause (Elder, et al., 1997). Clinical expression is, in general, highly variable and ~90% of AIP heterozygotes remain asymptomatic throughout life (Elder, et al., 1997; Petrides, 1998). Symptoms may occur recurrently, lasting from a few days to a few weeks, or occur as a single sporadic incident.

Human HMBS is determined by a single gene located on chromosome 11 (Meisler, et al., 1980). The HMBS gene was characterised in 1986 (Raich, et al., 1986), which enabled further studies at the molecular level. The gene is divided into fifteen exons and spans approximately 10 kb of DNA (Yoo, et al., 1993).

Hydroxymethylbilane synthase (also known as porphobilinogen deaminase or uroporphyrinogen I synthase, EC 4.3.1.8 or EC 2.5.1.61) is a monomeric protein with a dipyromethane (DPM) cofactor in a single catalytic active site (Louie, et al., 1992). cDNAs encoding the 42-kD housekeeping and 40-kD erythroid-specific isoenzymes have been isolated and characterised (Grandchamp, et al., 1987). Relatively high amino-acid sequence conservation has been found, amounting to at least 32% to the proteins from bacteria, fungi, plants and mammals. Crystallographic structures of *E. coli* HMBS and recently of human housekeeping HMBS have been determined (Louie, et al., 1992; Song, et al., 2009).

The diagnosis of AIP is made on the basis of characteristic clinical symptoms, such as elevated levels of urinary porphyrin precursors ALA and PBG and a decreased erythrocytic HMBS activity. Diagnosis has been aided in recent years by the identification of the causal mutations in the HMBS gene. Biochemical analyses remain the preferred choice in diagnostic laboratories because of their simplicity and rapidity, however identification of the causal mutation provides the ultimate proof of AIP occurrence.

Over 300 mutations in the HMBS gene have been identified (http://www.hgmd.cf.ac.uk) (Stenson, et al., 2009). Mutations are equally distributed within the HMBS gene and no prevalent site for mutation has been identified.

Purpose of the study

The purpose of this study was first to perform molecular analysis of the AIP patients. Once a mutation is detected in a patient, molecular testing is offered to family members. In each affected family, this becomes an important tool for individualised medicine, allowing for careful drug prescription; in addition, it is very important for the asymptomatic carriers to be warned of precipitating factors, thus avoiding an acute attack.

The proper DNA diagnostics can be achieved by a combination of a robust and effective pre-screening method and a confirmatory DNA sequencing step. We decided to establish a new generation pre-screening method, which will be highly sensitive and relatively time- and

cost-effective. Our method of choice was high-resolution melting (HRM) analysis using the LightScanner instrument.

Another important aspect of this project was to study the molecular heterogeneity of AIP in relation to the HMBS protein. We aimed at characterisation of the impact of the HMBS gene mutation on the structure and function of the enzyme, and demonstration of how this aids the interpretation of clinical, biochemical and genetic data in establishing an AIP diagnosis. To demonstrate this, we used expression and characterisation of mutant HMBS enzymes in the prokaryotic system together with the use of predictive computer-assisted structure-function correlation studies.

Initiation of the study

The idea for this study originated in a laboratory with clinical, diagnostic and scientific experience in most types of porphyria. At the beginning of this study in 2003, only a few AIP-causing mutations had been identified within Czech porphyric families. For the molecular diagnosis, the traditional, but time-consuming method of denaturing gradient gel electrophoresis (DGGE) was performed. Furthermore, very little was known about the exact detrimental effects of specific AIP-causing mutations on the enzyme structure.

The study began in 2003 at the Laboratory for Studies of Mitochondrial Disorders, Department of Pediatrics, 1ST Faculty of Medicine, Charles University, Prague, Czech Republic. In 2007, we initiated collaborations with Porphyria centres abroad, namely Central Laboratory, Triemli Hospital, Zurich, Switzerland and National Laboratory for the Biochemical Diagnoses of Porphyrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel.

REVIEW OF THE LITERATURE

Heme and hemoproteins

Heme serves as a prosthetic group for a wide range of proteins, either covalently or non-covalently bound to the protein itself; the biological functions diverge with heme type. The core structure of a heme molecule is tetrapyrrole, a substance significantly called 'pigment of life' (Leeper, 1989) since it gives the specific colour to green leaves and red blood.

The heme compound contains four pyrrole rings connected in a cyclic fashion. The four pyrrole rings are interconnected through one-carbon (methine or methylene) bridges to form the protoporphyrin. The macrocycle is a highly conjugated system and therefore deeply coloured. Since tetrapyrroles tend to incorporate metals to form complexes, heme consists of iron. The iron in the heme substance is capable of undergoing oxidation and reduction reactions.

Recent protein databank surveys identifies at least several hundred metalloproteins with heme as their subunit, all involved in diverse basic cellular functions. Among the most significant functions, hemoproteins act in: transportation of and sensing of diatomic gases such as oxygen, carbon monoxide and nitric oxide (White and Marletta, 1992); electron transfer through cytochromes in mitochondrial transport chain, performing roles of detoxification and drug metabolism (in the enzyme system of cytochromes P450); and play roles in synthesis of regulatory or signalling molecules such as guanylate cyclase (Stone and Marletta, 1996) and nitric oxide synthase (White and Marletta, 1992). Another important role of hemoproteins is its role in the oxidative stress response where hemoproteins are partially responsible for the destruction of invading bacteria and virus, in the biosynthesis of the thyroid hormones and in the metabolism of tryptophan. Moreover, heme acts as a regulatory molecule by mediating gene expression at the level of transcription (Hach, et al., 1999; Ogawa, et al., 2001; Reddy, et al., 1996; Schmitt, 1999), translation (Chen and London, 1995), protein targeting (Lathrop and Timko, 1993), protein stability (Qi, et al., 1999; Wang, et al., 1999) and cell differentiation (Nakajima, et al., 1999).

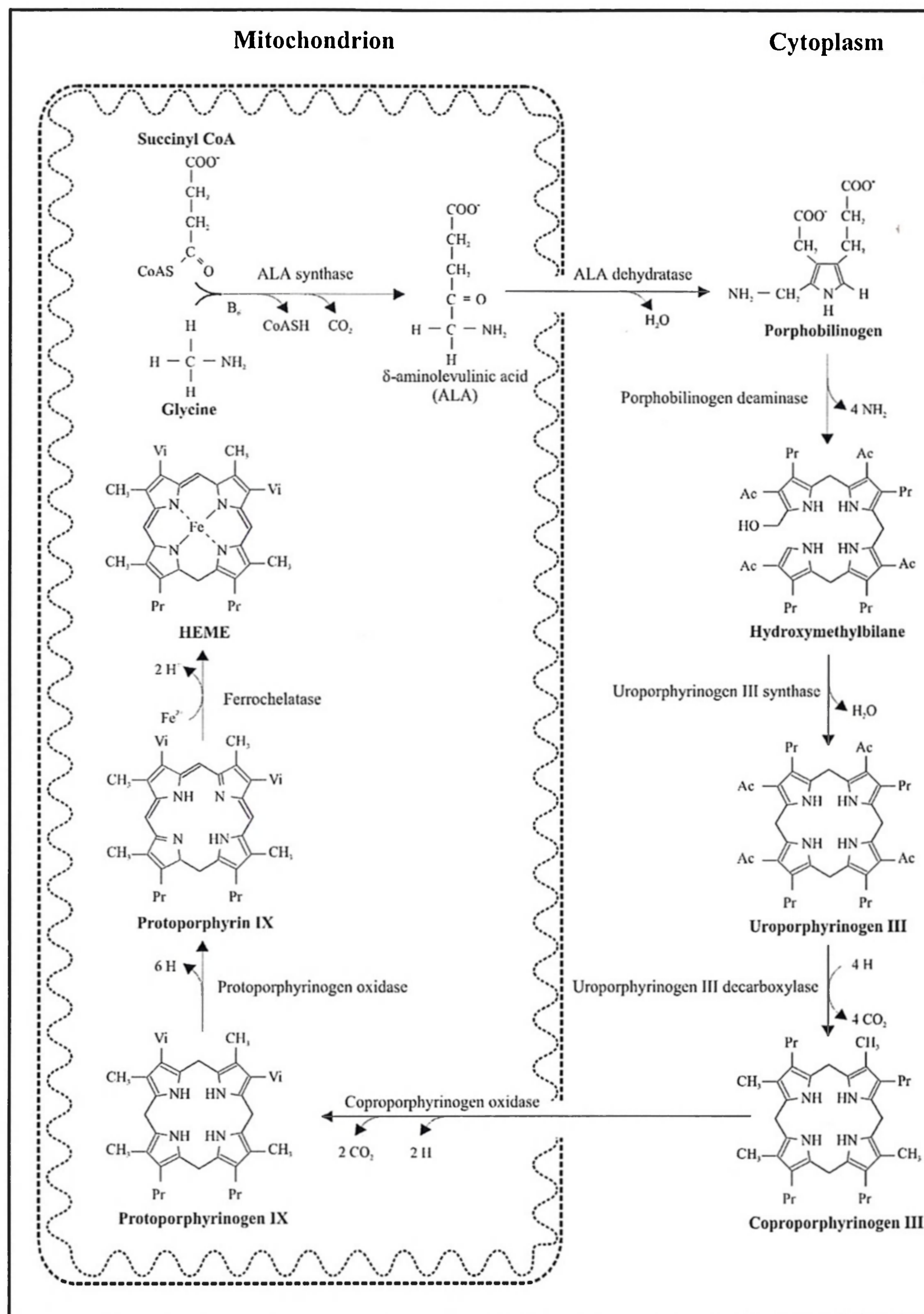

Figure 1 The heme biosynthetic pathway in mammals

Intermediate products and enzymes involved in the synthetic cascade of the heme, which takes place in the mitochondria and cytosol. Ac= - CH_2COOH, Pr= $-CH_2CH_2COOH$, Vi= $-CH=CH_2$ **(Mustajoki, 1999)**

Heme biosynthesis

Heme biosynthesis is widely distributed process in eukaryotic cells although considerable quantitative variations exist between different cell types. The synthesis takes place in all living cells, but predominantly in the liver and bone marrow. The major fraction is synthesized in erythroid cells, about 85%, where the heme is utilized in hemoglobin formation (Kauppinen, 2005). In the liver, 80% of heme is used for covering great demand for microsomal cytochromes P450.

There are eight enzymatic steps in the heme biosynthesis. The initial enzymatic step take place in the mitochondrion, the following four enzymes operate in the cytosol and the final three steps are directed back into mitochondria, or more precisely, into the inter-membrane space and into the inner membrane. Enzymes in the heme pathway are encoded by nuclear genes, all which have been widely studied and characterized (Anderson, 2001). Initial four enzymes of the biosynthesis contain tissues-specific promoters. Two different transcripts are presented, one being ubiquitous, the other one being erythroid-specific. For the first enzyme, aminolevulinic acid synthase (ALAS), isoenzymes are encoded by two different genes (Bishop, et al., 1990). For aminolevulinic acid dehydratase (ALAD), hydroxymethylbilane synthase (HMBS) and uroporphyrinogen III synthase (UROS), different transcripts are synthesized via tissue-specific promoters and the use of alternative splicing (Anderson, 2001).

The biosynthetic cycle begins with condensation and decarboxylation of two amino acids, glycine and succinyl-CoA, coming from the citric acid cycle (Krebs cycle), to form D-aminolevulinic acid (5-ALA or δ-ALA). This reaction is the rate-limiting step of the overall synthesis as the enzyme responsible for this reaction, ALAS, is highly sensitive to intracellular iron and heme levels and, therefore, strictly regulated by its concentration. The following reaction, catalyzed by ALAD, combines two molecules of ALA to give porphobilinogen (PBG), the first pathway intermediate which contains a pyrrole ring. In the next step, initiated by elimination of the amino group, the porphyrin ring is formed by condensation of four molecules of PBG, in the reaction running under control of HMBS. The reaction proceeds through deamination into hydroxymethylbilane (HMB). HMB can be non-enzymatically converted into uroporphyrinogen I and, in the second step, to coproporphyrinogen I. Enzymatically, UROS hydrolyses the linear tetrapyrrole HMB creating the macrocyclic asymmetric tetrapyrrole uroporphyrinogen III. Decarboxylation of

all four acetyl side chains of uroporphyrinogen III, converting them to methyl groups, is catalyzed by uroporphyrinogen decarboxylase (UROD), forming coproporphyrinogen III as the next porphyrin intermediate. Following the oxidative decarboxylation of propionyl side chains, converting them to vinyl groups, it is catalyzed by coproporphyrinogen oxidase (CPOX), and forms protoporphyrinogen IX. Final enzyme of this porphyrin conversion, protoporphyrinogen oxidase (PPOX), adds more double bonds in oxidative reaction, yielding protoporphyrin IX. In the last biosynthetic step, Fe^{2+} is inserted into protoporphyrin IX via ferrochelatase (FECH) to generate heme (**Figure 1**).

Heme degradation

Heme degradation is of the same importance as the biosynthesis, for being involved in homeostasis of cellular heme pool. The main part of heme catabolism proceeds in two steps, ultimately yielding bilirubin, carbon monooxide, and iron as the ferric ion (Fe^{3+}). In the first step, heme is oxidized to biliverdin by the two isoforms of endoplasmic reticulum enzyme heme oxygenase (HOXG). In the second reaction, biliverdin is converted to bilirubin by biliverdin reductase (BVR). While the substantial reaction is the first step, crucial for the regulation is the fine maintenance of the unsteady balance between induction and repression of HOXG, driven by heme level in case of induction and, by hypoxia, in case of repression. Moreover, HOXG, the rate-limiting step in heme catabolism, appears to play an important role in a number of neurodegenerative disorders, such as Alzheimer disease and aging (Atamna, et al., 2002).

The mechanism of heme biosynthesis control

Due to the special importance of this molecular compound, cellular heme levels are tightly regulated to maintain a balance between heme biosynthesis, demands for heme as a molecular system component, and heme catabolism. Two main tissues are characteristic for its high demand for heme: liver and erythroid bone marrow. Therefore, regulation of heme biosynthesis is split and proceeds in two different tissue-dependent manners (May, et al., 1995).

In liver, the rate-limiting regulatory enzyme of heme synthesis is inducible hepatic ubiquitous ALAS1, which operates in mitochondria initiating the synthesis. The metabolic

activity of this enzyme is determined by the regulatory DNA-binding nuclear proteins affecting ALAS1 gene transcription, and by the post-translational control (Thunell, 2006). Heme, the end-product of this pathway, plays a role as a feedback inhibitor. When the free heme pool decreases under the critical level due to increased utilisation of heme in hemoprotein synthesis, or due to accelerated catabolism through the activation of HOXG, ALAS1 expression is induced (Thunell, 2006). The known feed-back control mechanisms of ALAS1 by heme are as follows: regulation of the translocation of ALAS1 to the mitochondria (Hayashi, et al., 1980), control of the post-translational level of this enzyme by reducing its mRNA-stability (Hamilton, et al., 1991), and repression of the transcription of the housekeeping gene for ALAS1 (Kolluri, et al., 2005). These mechanisms of heme feed-back control are of therapeutic importance; infusion of stabilised exogenous heme, heme arginate or hematin, can abort attacks of porphyria in patients with an inborn error of metabolism in this process, by reducing transcription of ALAS1.

In erythrocytes, the erythroid ALAS2 is expressed only during erythroid differentiation. In the process of erythroid cell differentiation, which is under the general control of a hormone erythropoietin (Spivak, 1986), the highly up-regulated heme synthesis is coordinated with globin synthesis to give rise to hemoglobin. The erythropoietin hormone interacts with specific cell receptors and activates the transcriptional factor GATA-1, a key regulatory element in erythroid differentiation (Orkin, 1992). The GATA-1 factor in turn stimulates transcription of the ALAS2 gene. A further mechanism of ALAS2 translational control is based on the availability of iron, which is mediated by a cell associated transferrin (Hemmaplardh and Morgan, 1974). Translation of the ALAS2 mRNA is prevented by binding of the iron-regulatory protein (IRP) to the iron-responsive element in the 5`prime untranslated region of mRNA. Iron enters the cell in a complex with the transferrin via transferrin receptor pathway. High levels of cellular iron ions converts IRP to its non-RNA binding form and translation of mRNA can then start. The precursor protein of ALAS2 is translocated into mitochondria where it begins heme biosynthesis (Ponka, 1997) (**Figure 2**). In erythroid cells, in contrast to housekeeping ALAS1 protein, ALAS2 is induced by heme (May, et al., 1995).

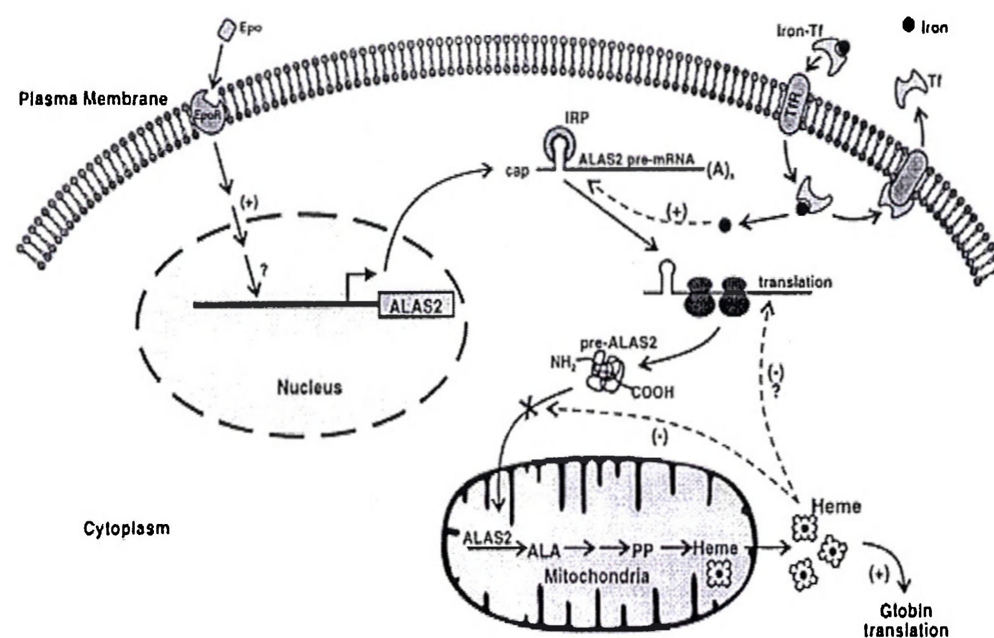

Figure 2 Regulation of the heme biosynthesis in erythropoietic cells

The regulation of ALAS2 expression in erythroid cells is under general control of erythropoietin (Epo). The transcription of the ALAS2 gene is stimulated through the erythropoietin receptor (EpoR). Translation of ALAS2 mRNA is prevented by the binding of the iron regulatory protein (IRP). It is relieved by iron entering as a complex with transferrin (Tf) via the transferrin receptor (TfR). Following translation, the precursor protein (pre-ALAS2) is imported into mitochondria where it becomes functionally active. ALA= aminolevulinic acid, PP= protoporphyrin **(Sadlon, et al., 1999)**

There has been great improvement in our understanding of the biosynthetic regulation of the heme synthesis pathway, while the connection between some of the endogenous and exogenous stimulatory factors and the mechanism of heme biosynthesis activation has been deciphered. The heme produced by the hepatic ALAS1 is mainly utilised in the synthesis of catalytic proteins. The major fraction of the hepatic hemoprotein pool is a group of cytochromes P450 (CYP). CYPs are responsible for the metabolism of steroid hormones, drugs and other xenobiotics, which are clearly proven accelerating factors (Guengerich, et al., 1995). The identification of the DNA-binding proteins constitutively active receptor (CAR) and pregnane xenobiotic receptor (PXR), which mediate the transcription of CYP genes, explain the concerted transcription of these genes and the ALAS1 gene (Podvinec, et al., 2004). Characterisation of this CAR-PXR transduction mechanism further enhances our understanding of the impact of steroid hormones and/or xenobiotics on the heme biosynthetic pathway. The transcriptional modulation of ALAS1 induction by these and/or other nuclear factors may explain some other biological observations such as the fasting induction of ALAS1, and multiple kinds of stress induction (Handschin, et al., 2005; Thunell, 2006) **(Figure 3)**.

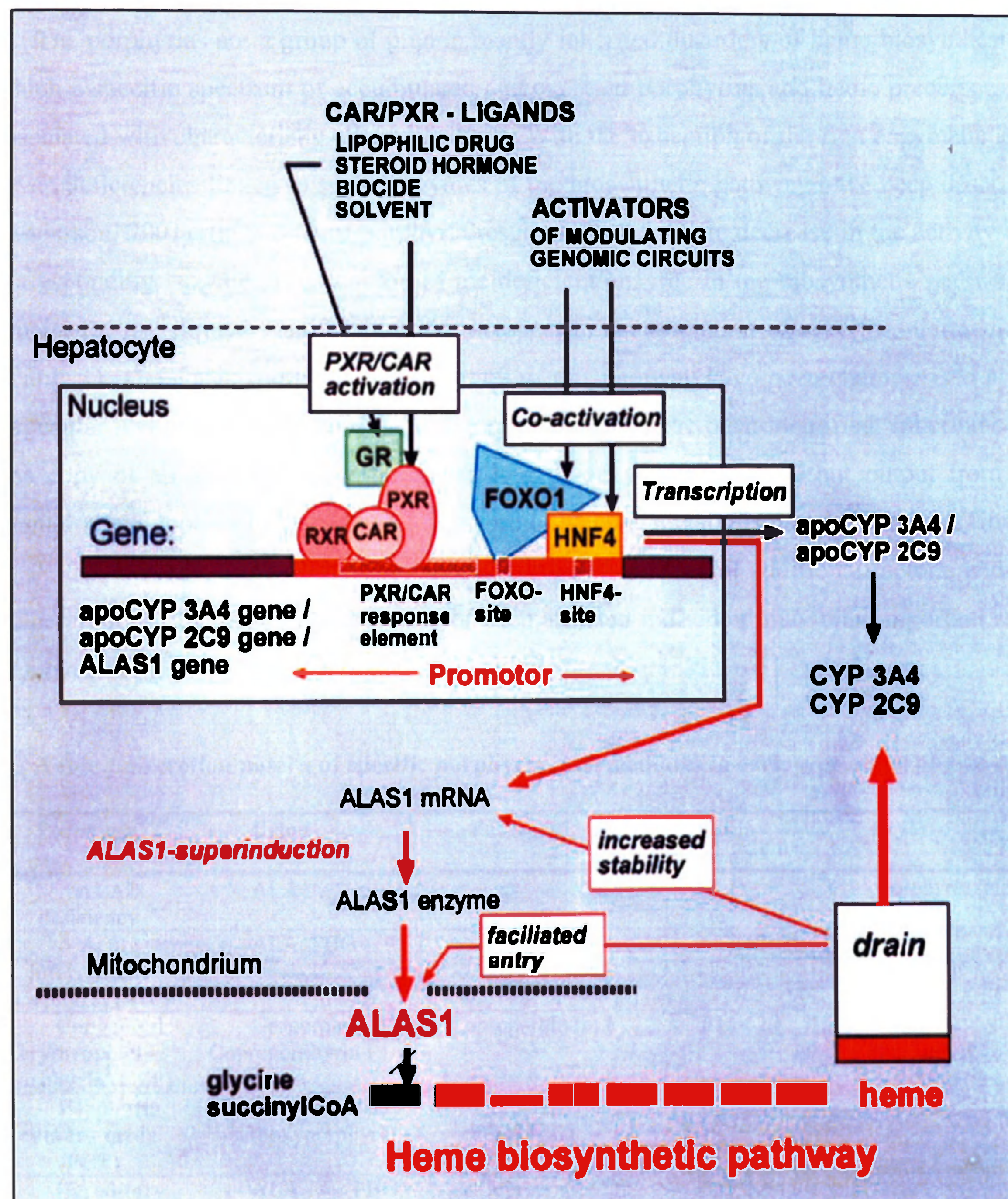

Figure 3 The CAR-PXR transduction mechanism and the superinductive ALAS1 response

Transcriptions of the genes for ALAS1 and apoCYP take place consertedly. Nuclear receptors are activated by several exogenous and endogenous ligands. Co-activators of the nuclear receptors modulate the response of the target genes GR= glucocorticoid receptor, RXR= 9-cis retinoic acid xenobiotic receptor, FOXO= forkhead box class O, HNF= hepatocyte nuclear factor (Thunell, 2006).

Porphyrias

The porphyrias are a group of predominantly inherited disorders of heme biosynthesis in which a specific spectrum of accumulated and excreted porphyrins and heme precursors are associated with characteristic clinical features. With the exception of the first enzymatic step, seven deficiencies linked to seven enzymes of the biosynthetic pathway have been described (Anderson, 2001). Each type of porphyria results from a specific decrease in the activity of a corresponding enzyme. The position of the deficient enzyme in the biosynthetic pathway is crucial for the clinical manifestation with respect to accumulation of specific intermediates (**Table 1**). The genes coding for the enzymes of this pathway have been characterised at the molecular level (Anderson, 2001) and several mutations have been identified. Inheritance of one copy of an affected allele can cause loss of enzymatic activity, but output from the normal allele appears to be sufficient for normal cellular metabolism in individuals (Gouya, et al., 2004). The porphyric manifestation is triggered by several additional factors, and the vulnerability of the genetic background of each affected individual may play important roles in the resulting porphyric phenotype.

Table 1 Excretion patern of specific porphyrin intermediates in each type of porphyria

Porphyria	Urine	Faeces	Plasma (peak, nm)	Erythrocytes
ALAD deficiency	ALA	-	-	Protoporphyrin (zinc)
Acute intermittent porphyria (AIP)	ALA, PBG	-	615-620	-
Congenital erythropoietic porphyria (CEP)	Uroporphyrin I, Coproporphyrin I	Coproporphyrin I	615-620	Protoporphyrin (zinc and free), Uroporphyrin I
Porphyria cutanea tarda (PCT)	Uroporphyrin, 7-carboxyporphyrin	isocoproporphyrin, 7-carboxy and 5-carboxyporphyrin	615-620	-
Hereditary coproporphyria (HCP)	ALA, PBG, Coproporphyrin III	Coproporphyrin III	615-620	-
Variegata Porphyria (VP)	ALA, PBG, Coproporphyrin III	Protoporphyrin, Coproporphyrin III	624-627	-
Erythropoietic protoporphyria (EPP)	normal	-	626-634	Protoporphyrin (free)

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Most porphyrias are autosomal dominant conditions, only two of them share an autosomal recessive mechanism of inheritance. Low clinical penetrance is an important feature of all of the autosomal dominant porphyrias (Anderson, 2001; Gouya, et al., 2004).

Porphyrias can be divided into hepatic or erythropoietic types depending on the anatomical origin, and further divided into acute or cutaneous types depending on the clinical presentation. Two types of clinical expression can occur separately or in combination: acute life-threatening neurovisceral attacks, which are associated with the overproduction of ALA and PBG; and/or cutaneous symptoms that result from photosensitisation caused by porphyrins (Albers and Fink, 2004; Anderson, 2001; Meyer, et al., 1998).

Porphyrias are panethnic and their prevalence varies from 0.5 to 10 per 100 000 in different population (Anderson, 2001). All types of porphyria are summarised in **Table 2**.

In 2001, in an attempt to improve the accessibility and sharing of information about porphyrias for both the porphyria specialists and patients, the European Porphyria Initiative (EPI) platform was founded by experts from different European countries (Deybach, et al., 2006). The EPI web page (www.porphyria-europe.com) is constantly updated and important information is made available.

Table 2 Classification of porphyrias

Porphyria	Deficient enzyme	Classification	Inheritance	Clinical	Mutations (HGMD)	Symptoms
ALAD deficiency	ALAD	acute	autosomal recessive	hepatic	12	neurovisceral
Acute intermittent porphyria	HMBS	acute	autosomal dominant	hepatic	309	neurovisceral
Congenital erythropoietic porphyria	UROS	cutaneous	autosomal recessive	erythro-poietic	38	photosensitivity
Porphyria cutanea tarda	UROD	cutaneous	sporadic / autosomal dominant	hepatic	103	photosensitivity
Hereditary coproporphyria	CPO	acute + cutaneous	autosomal dominant	hepatic	45	photosensitivity/ neurovisceral
Porphyria variegata	PPOX	acute + cutaneous	autosomal dominant	hepatic	149	photosensitivity/ neurovisceral
Erythropoietic protoporphyria	FECH	cutaneous	autosomal dominant	erythro-poietic	120	photosensitivity

Acute intermittent porphyria

With the exception of South Africa and Chile, acute intermittent porphyria (AIP, MIM # 176000) represents the most frequent type of acute porphyria throughout the world (Hift and Meissner, 2005). This predominantly inherited autosomal dominant disorder, classified as acute hepatic porphyria, is characterised by a deficiency of HMBS, the third enzyme in heme biosynthesis. In such cases, the activity of the enzyme is decreased by about 50% in all tissues (Meyer, et al., 1972). A few cases of homozygous and compound heterozygous conditions have been reported, showing a HMBS activity as low as 2% of normal activity. Such cases manifest early in life, often during childhood, and are made obvious by a more severe clinical phenotype (Beukeveld, et al., 1990; Hessels, et al., 2004; Llewellyn, et al., 1992).

Based on biochemical analyses of enzyme activities in erythroid and non-erythroid tissues, AIP is classified as a classic or non-erythroid variant. In the classic variant, both isoforms are deficient, with half-normal activities in all tissues. But in about 5% of examined families, normal enzyme activity in erythrocytes and half-normal activity of the housekeeping enzyme was found, representing non-erythroid variant of AIP (Hessels, et al., 2004; Puy, et al., 1998; Whatley, et al., 2000).

The prevalence of symptomatic disease varies from 1 to 10 per 100 000, but due to frequent misdiagnosis and low penetrance this number may be much higher (Badminton and Elder, 2002; Meyer, et al., 1972; Strand, et al., 1970).

Expression of the disease is highly variable. It is determined in part by environmental, metabolic, and hormonal factors that share the ability to induce hepatic ALAS, and thereby to increase the production of porphyrin precursors, ALA and PBG.

Over 300 mutations in HMBS gene are known (http://www.hgmd.cf.ac.uk) (Stenson, et al., 2009). These mutations include one mutation in the promoter region, missense, nonsense, splicing and frame-shift mutations and in-frame deletions and insertions. Mutations are equally distributed along the HMBS gene and no particularly sensitive site for mutations has been identified. Regarding the prevalence of AIP in the Czech Republic, no statistical data exist. To date, 14 different mutations have been identified in the Czech and Slovak population: c.70G>A (p.Gly24Ser) (Puy, et al., 1997; Rosipal, et al., 1997), c.76C>T (p.Arg26Cys) (Kauppinen, et al., 1995), c.77G>A (p.Arg26His) (Llewellyn, et al., 1993), c.87+5G>T (r.spl?) (Luchinina, et al., 2005), c.158_159insA (p.Ile54HisfsX12) (Puy, et al.,

1997; Rosipal, et al., 1997), c.331G>A (p.Gly111Arg) (Gu, et al., 1993), c.518G>A (p.Arg173Gln) (Delfau, et al., 1990), c.610C>A (p.Gln204Lys) (Ulbrichova, et al., 2009), c.675delA (p.Ala226ProfsX28) (Ulbrichova, et al., 2009), c.750A>T (p.Glu250Asp) (Ulbrichova, et al., 2009), c.771+1G>T (r.spl?) (Rosipal, et al., 1997), c.799G>A (p.Val267Met) (Puy, et al., 1997; Rosipal, et al., 1997), c.899_900delinsTGCCTGCATCTG (p.His300LeuFsX10) (Douderova and Martasek, 2009) and c.965_966insA (p.Asn322LysfsX36) (Ulbrichova, et al., 2006) (**Table 3, Figure 4**). Some of them are described in this thesis.

Table 3 Mutations found in Slavic population

Mutation NA	Exon	Mutation AA	References
c.70G>A	3	p.Gly24Ser	(Puy, et al., 1997; Rosipal, et al., 1997)
c.76C>T	3	p.Arg26Cys	(Kauppinen, et al., 1995)
c.77G>A	3	p.Arg26His	(Llewellyn, et al., 1993)
c.87+5G>T	IVS3	(r.spl?)	(Luchinina, et al., 2005)
c.158_159insA	4	p.Ile54HisfsX12	(Puy, et al., 1997; Rosipal, et al., 1997)
c.331G>A	7	p.Gly111Arg	(Gu, et al., 1993)
c.518G>A	10	p.Arg173Gln	(Delfau, et al., 1990)
c.610C>A	10	p.Gln204Lys	(Ulbrichova, et al., 2009)
c.675delA	12	p.Ala226ProfsX 28	(Ulbrichova, et al., 2009)
c.750A>T	12	p.Glu250Asp	(Ulbrichova, et al., 2009)
c.771+1G>T	12	(r.spl?)	(Rosipal, et al., 1997)
c.799G>A	13	p.Val267Met	(Puy, et al., 1997; Rosipal, et al., 1997)
c.899_900delinsT GCCTGCATCTG	14	p.His300LeuFsX 10	(Douderova and Martasek, 2009)
c.965_966insA	15	p.Asn322LysfsX 36	(Ulbrichova, et al., 2006)

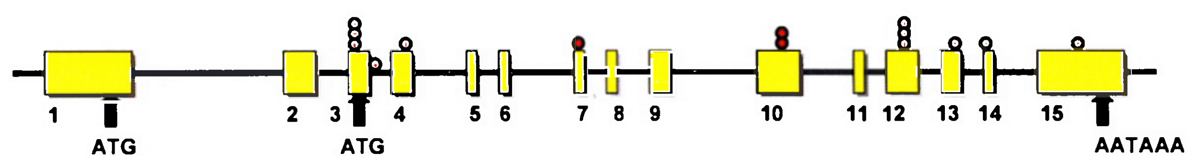

Fig 4 Schematic representation of the HMBS gene with mutations found in Slavic population

Localization of mutations in exons and intron, marked by red circle, clearly demonstrates wide heterogeneity of AIP and the fact that no DNA variation hot spot site for mutations within HMBS gene in Slavic population was found as mutations are evenly dispersed throughout the gene. ATG= translational start site, AATAAA= polyadenylation signal.

Clinical manifestation

The manifestation of the disease is associated with an acute neurological syndrome characterised mainly by an acute attack (Albers and Fink, 2004; Meyer, et al., 1998). The porphyric manifestation usually occurs in the third life decade, though there is the rare occurrence before puberty or after menopause (Elder, et al., 1997). Clinical expression, in general, is highly variable and ~90% of AIP heterozygotes remain asymptomatic through life (Elder, et al., 1997; Petrides, 1998). Individual gene carriers differ from each other in both biochemical and clinical manners. Symptoms may be recurrent and last from a few days to a few weeks, or they may occur as a single sporadic incident in a lifetime.

Acute attacks are manifested by a wide variety of clinical features including: autonomic neuropathy with arrhythmias, central nervous system impairment, peripheral motor neuropathy with muscular weakness and paresthesia or less common sensory symptoms, and most commonly a severe pain caused by neurovisceral crises, usually of abdominal or back origin (Meyer, et al., 1998; Nordmann and Puy, 2002). This general symptom is often associated with nausea, vomiting and constipation. Complications usually include convulsion, hypertension, seizure, tachycardia and hyponatraemia. Hypomagnesaemia can also occur representing another electrolyte abnormality accompanying this disorder (Liamis, et al., 2007). Among psychiatric disturbances, symptoms widely range from minor behavioural changes such as irritability, restlessness, insomnia, anxiety or depression to more severe agitation, hysteria, psychosis with hallucinosis or even delirium (Crimlisk, 1997; Millward, et al., 2001; Regan, et al., 1999). An unmistakable symptom of an acute attack, which can often lead to the correct diagnosis, is the occurrence of red or dark-coloured urine resulting from high concentrations of porphyrins and porphyrin precursors.

Acute attacks can be potentially life-threatening, especially when respiratory paralysis occurs (Goldberg, 1959). In patients with severe AIP, hepatocellular cancer may be a potential and serious complication (Kauppinen and Mustajoki, 1988). The chronic complications include the development of renal disease which eventually leads to kidney failure (Andersson, et al., 2000).

Triggering factors of an acute attack

Clinical presentation appears to require additional factors that affect the heme pathway by increasing demand for heme, by causing an additional decrease in enzyme activity, or by combination of both these factors. This is made possible by the mechanism probably common to all triggering factors, namely the ability of multiple agents to activate the nuclear receptors or their co-activators, responsible for initiating transcription of the ALAS1 gene (Thunell, 2006). These factors include genetic and environmental conditions. The environmental factors can be divided into two main groups: exogenous and endogenous agents. Of the exogenous factors, porphyrinogenic drug are of great importance, since AIP was determined to be pharmacogenetic (Moore and Hift, 1997; Tschudy, et al., 1975). Many common medications which have an effect on either induction of hepatic cytochrome P450, or destructive effect on heme or on inhibition of heme synthesis can provoke an acute attack (McColl and Moore, 1981). Lists of safe and unsafe drugs and recommendations in managing the use of hormonal contraception (Andersson, et al., 2003) and local anaesthetics (Jensen, et al., 1995) are available on the website of a platform European Porphyria Initiatives (www.porphyria-europe.com) (Deybach, et al., 2006). Other exogenous agents include tobacco, cannabis, alcohol, organic solvents, terpenes, biocides, infection, major surgery, restricted carbohydrate and calorie intake and various kinds of stress, possibly including oxidative stress (Albers and Fink, 2004; Anderson, et al., 2005; Bonkovsky and Barnard, 2000; Bonkovsky, et al., 1992; Hift and Meissner, 2005; Kauppinen and Mustajoki, 1992; Moore and Hift, 1997; Thunell, et al., 1992). Of the endogenous factors, the use of steroid hormones and the fluctuations in female sex hormones are particularly important, and together explain the higher frequency of disease manifestation in woman (Andersson, et al., 2003). Although it appears that an individual's genetic background can influence susceptibility to acute attacks, the genes involved have not yet been identified.

Pathogenesis of porphyric neuropathy

The symptomatology of porphyric neuropathy consistently includes histological changes in peripheral and autonomic nerves. These changes involve demyelisation, axonal disruption, axonal vacuolisation and degeneration, central chromatolysis, muscle denervation, decreased

motor nerve conduction velocities, reddish fluorescence in white matter and EEG abnormalities (Albers and Fink, 2004; Lin, et al., 2008; Pischik and Kauppinen, 2009).

Despite our advanced understanding of the molecular and biochemical basis of AIP, the mechanism of the development of transient neurovisceral symptoms of AIP remains unclear. Many theories concerning the pathophysiology have been proposed (Meyer, et al., 1998). At present, we believe that more than one mechanism can give rise to neurovisceral symptoms.

One of the main hypotheses suggests the potential depletion of heme that develops in liver and possibly in neuronal tissue during an acute attack. This may impair critical cell processes dependent on hemoprotein formation (Bonkovsky, 1993; Watson, 1975). It has been revealed that certain drugs have the ability to increase demand in hepatic heme by inducing the biosynthesis of cytochrome P450 enzymes that utilise heme as a prosthetic group, in some cases by as much as 40-50-fold in the liver (Albers and Fink, 2004). Another supporting theory is that some of the cerebral manifestations of the disease are due to decreased functioning of signalling molecules such as nitric oxide (NO) produced by nitric oxide synthase or cGMP due to lack of guanylate cyclase, which are another hemoproteins (Kupferschmidt, et al., 1995). The decreased production of NO may explain some of the cerebral manifestations such as vasospasm (Kupferschmidt, et al., 1995; Sze, 1996). Other neurological effects are suggested to be caused by the energy production level in the brain, since heme is an essential component of the mitochondrial electron transport chain and critical to aerobic metabolism and adenosine triphosphate (ATP) production (Yeung Laiwah, et al., 1987). Interestingly, there is growing evidence of an additional globin protein containing heme called neuroglobin. Together with hemoglobin and myoglobin, known for transport of oxygen in blood and muscle respectively, this new protein has a high affinity for oxygen. It is possible that impairment of heme metabolism may lead to an aberrant neuroglobin pathway (Burmester, et al., 2000).

Other findings suggest that some of the neurological symptoms may be secondary effects of heme deficiency. In the case of decreased function of the hepatic tryptophan pyrrolase, a heme-dependent enzyme which controls tryptophan degradation, tryptophan levels in blood increase, and tryptophan is then quickly converted into serotonin and may cause an unbalance of neuro-transmissional processes in the brain (Litman and Correia, 1985). In agreement with this idea, some tryptophane metabolite excretion in the urine of patients with a porphyria attack has been detected, disappearing after administration of heme-arginate (Puy, et al., 1993). This hypothesis on heme depletion in the system is supported by the fact that simple

compensation of the absent heme in the form of heme-arginate is sufficient to control the condition of an acute attack (Kordac and Martasek, 1986; Mustajoki, et al., 1986).

Nevertheless, the leading hypothesis postulates that the symptoms of acute hepatic porphyria are caused by the neurotoxicity of high levels of accumulated ALA and PBG precursors, primarily in neuronal tissues. Under special circumstances and in combination with the deficient HMBS enzyme, heme biosynthesis accelerates through the induction of ALAS1 transcription. Under these conditions, the aberrant enzyme may become rate-limiting. Furthermore, due to negative feedback control, a reduced heme pool may cause additional induction of ALAS1. This may result in the pathological accumulation of metabolites at the impaired biosynthetic step (**Figure 5**).

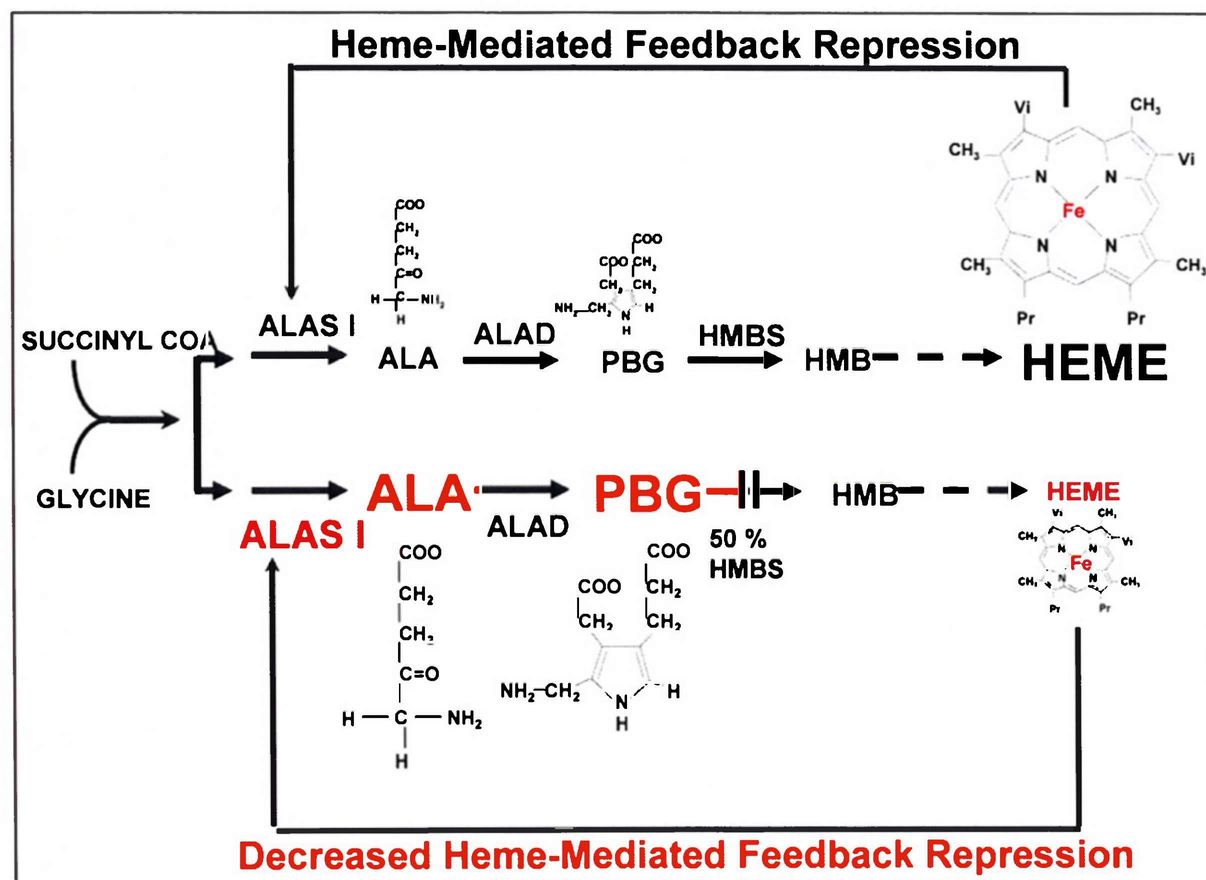

Figure 5 The mechanism of an acute attack

In the presence of HMBS deficiency, availability of the heme from the regulatory heme pool in hepatocytes decreases. It leads to the acceleration of the synthesis due to the heme-mediated feedback represion and, therefore, to the porphyrin precursor overload; Pr= $-CH_2CH_2COOH$, Vi= $-CH=CH_2$ (scheme according to (Anderson, 2001).

It has been well documented that the acute attacks of AIP are accompanied by an increase in porphyrin precursors, ALA and PBG, which accumulate in different tissues such as liver, kidney and cerebrospinal fluid (Aarsand, et al., 2006; Anderson, 2001; Kauppinen, et al., 1995; Kauppinen and von und zu Fraunberg, 2002; Miyagi, et al., 1971; Solis, et al., 2004; Sweeney, et al., 1970; Thunell, 2000; Thunell, et al., 2000). The neurotoxicity of ALA may have a structural basis. One theory suggests that due to similarities in structure, ALA competes with the inhibitory neurotransmitter gamma-amino-butiric-acid (GABA) and excitatory glutamic acid for receptor binding in synaptic plasma membranes (Albers and Fink, 2004; Brennan and Cantrill, 1979; Meyer, et al., 1998; Yeung Laiwah, et al., 1987) (**Figure 6**). This could explain some of dysfunctions in the central nervous system. ALA, undergoing metal-catalysed oxidation, can also mediate lipid peroxidation and induce iron release from ferritin in the liver (Oteiza, et al., 1995). This and other similar principles may be responsible for oxidative damage (Hermes-Lima, et al., 1992). In addition, autoxidation of ALA generates reactive oxygen species (oxygen radicals) that produce oxidative damage on the membranes of the CNS (Batlle, 1993). In the peripheral nervous system it has been suggested that the neuropathic effects of ALA are attributable to its pro-oxidant properties which damage myelinating Schwann cells (Felitsyn, et al., 2008). This hypothesis on neurotoxicity is supported by the fact that other disorders, such as ALAD deficiency, tyrosinaemia type I and lead poisoning all resemble the neurological symptoms of acute porphyria, and share the symptom of increased levels of ALA. On the other hand, this underestimates the possible toxicity of PBG since no increase in PBG levels was observed in such cases (Anderson, 2001). There have been several studies involving the administration of ALA to humans (Dowdle, et al., 1968; Mustajoki, et al., 1992) and to rodents (Edwards, et al., 1984), which failed to reveal any effects linked to these symptoms. These findings are further supported by the observation that many patients excrete constantly excessive amounts of PBG and ALA and still remain asymptomatic (Harper P, 2003). However, direct administration of ALA to cultured cells of various origins and species have demonstrated neurotoxic effects (Meyer, et al., 1998). Moreover, from the observation of a special case of a homozygous AIP patient, the results of full-scale neurological and neuroradiological findings suggest that the neurological manifestations result from porphyrin precursor toxicity (Solis, et al., 2004).

A mouse model of AIP, in which a partial deficiency of HMBS was created, was produced by homologous gene targeting. This animal exhibits some of the neurologic symptoms, such as impaired motor function, ataxia, and increased levels of ALA in plasma

and brain, as well as decreased heme saturation of liver tryptophan pyrrolase. The study of this mouse model supports the hypothesis that nerve damage results from heme depletion as opposed to the neurotoxic effect of porphyrin precursors (Johansson, et al., 2003; Lindberg, et al., 1999; Lindberg, et al., 1996). Clearly, many details of pathogenesis of porphyric neuropathy still remain unresolved and await further explanation.

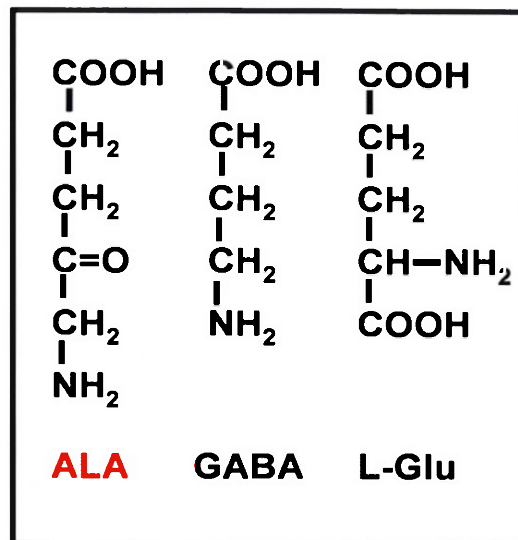

Figure 6 Structural similarity between aminolevulinic acid (ALA) and gamma-aminobutiric acid (GABA) and glutamic acid (Glu)

Diagnosis

Since acute porphyric attack can be potentially life-threatening, prompt and proper diagnosis of AIP heterozygotes is crucial to prevent attacks in both symptomatic and asymptomatic carriers. However, the precise diagnosis of AIP can be precluded by several eventualities. At an early stage, consideration of porphyria as a potential diagnosis is crucial, since life-threatening aspects of acute attacks arise mainly from misdiagnosis followed by inadequate treatment or late diagnosis. Diagnosis is not always transparent, as patients suffering from one of the acute porphyries often develop nonspecific clinical features mimicking other diseases which are more common. Such cases with similar symptoms include appendicitis, pancreatitis, gallstones, encephalitis, and Guillain-Barre syndrome above all (Crimlisk, 1997; Poblete Gutierrez, et al., 2001). Moreover, correct diagnosis can be complicated by the low penetrance of the disease, since only approximately 10% of those who inherit AIP will develop symptoms (Elder, et al., 1997; Petrides, 1998).

Traditionally, the diagnosis of porphyria is made on the basis of clinical symptoms, characteristic biochemical findings and enzyme assays. Once acute porphyria is suspected based on clinical symptoms, specific types should be distinguished, as different types of porphyria often reveal overlapping clinical and/or biochemical findings. This is especially true in case of AIP, HCP and ALAD deficiency (Kauppinen, 2005). Since accumulated heme precursors differ in precursor type and concentration level according to type of porphyria, the measurement of urinary, fecal, and plasma porphyrins, enzymatic activity in erythrocytes and the analysis of the excretion of and the fluorescence pattern of the plasma porphyrins should be performed (Bonkovsky and Barnard, 1998; Deacon and Elder, 2001; Sassa, 2006).

Of the biochemical findings, the marked increase in urinary PBG accompanied by an increase in ALA levels and some other porphyrins are common features for AIP, HCP, and VP. These precursors, essential for the diagnosis, are highly elevated during an acute attack and high excretion of these metabolites is often detected in the asymptomatic phase. During acute attacks, the first measurement of PBG is generally increased from 25 to 100 times the upper limit of the normal control (Anderson, et al., 2005). In AIP patients, the urinary porphyrins, uro- and coproporphyrin, are usually moderately increased, predominantly uroporpyrin I and III. In the plasma and fecal porphyrin levels, the excretion is usually normal or slightly increased only. In clinically asymptomatic carriers, only about one-third present a slight increase in excretion of PBG (two to five times the normal level), but others can show normal excretion of this precursor (Nordmann, et al., 1999). This means that asymptomatic AIP carriers are rarely detected through the measurement of urinary and fecal porphyrin precursors. The porphyrin measurement results often display a high variability primarily due to significant overlap between measurements made in patients, in clinically asymptomatic carriers, and in normal control individuals. The results of the porphyrin analysis, therefore, are not always conclusive (Bonaiti-Pellie, et al., 1984; Grandchamp, et al., 1996; McColl, et al., 1982; Pierach, et al., 1987). Nowadays, the biochemical detection of porphyrin precursors have been implemented as the first step in analysis of clinical syndromes and of the severity of the disease, e.g. in detecting acute or latent phase (Sassa, 2006).

Measurement of erythrocyte HMBS activity is another component of laboratory diagnostic tools. In most of the AIP patients, HMBS activity is approximately half of the normal activity (Meyer, et al., 1972). Some complications in the measurement of HMBS activity arise from the fact that a measurement of normal erythrocyte activity does not completely exclude AIP, since this enzymatic activity is normal in the case of the non-

erythroid variant of AIP (Gross, et al., 1996; Mustajoki, 1981). Another complication is that the range for values measured is wide and overlaps (Erlandsen, et al., 2000; Gross, et al., 1997). Moreover, the enzymatic activity in erythrocytes is highly age-dependent (Anderson, et al., 1977), and, in some cases, during an acute attack this activity can be normal (Kostrzewska and Gregor, 1986). Therefore, measurement of erythrocyte HMBS activity is still used as a diagnostic tool, but has a lower diagnostic value in screening for AIP in patients with acute symptoms (Anderson, 2001).

Due to inaccuracies in biochemical diagnostic tools, in recent years, several DNA-based screening molecular techniques have been included in the clinical diagnostic process as the final step to confirm the gene carrier status (Frank and Christiano, 1998; Grandchamp, et al., 1996; Kauppinen, 2004; Sassa, 2006). The search for the disease-causing mutation in each affected family is an important tool for individualised medicine, allowing for careful drug prescription and acute attack prevention. The molecular techniques are not only important in diagnostics, but also increase the deep insight into genes and their function in the field of science.

Treatment

An acute porphyric attack requires immediate intervention because it is known to be a life-threatening event and may result in serious neurological damage. Due to modern therapeutic options, the mortality rate nowadays is low.

In management of the porphyric attack, which usually requires hospitalisation, treatment is focused on a specific cure of symptoms and complications. This involves disease-specific therapy, and identifying and removing precipitating factors of an acute attack.

Of the specific therapies, the administration of intravenous heme followed by large amounts of carbohydrates is considered the most powerful treatment (Anderson, et al., 2005; Anderson, 2001; Bonkovsky and Barnard, 2000; Handschin, et al., 2005; Li, 2005; Stein and Tschudy, 1970; Watson, et al., 1978). This restores the regulatory heme pool and therefore suppresses hepatic ALAS1 induction and reduces the overproduction of ALA and PBG precursors. In an event of an acute attack, the heme therapy should be initiated as soon as possible (Mustajoki and Nordmann, 1993).

There are two types of heme derivates available for use to suppress severe acute attacks - hematin (Panhematin®, Abbott Laboratories) and heme-arginate (Normosang, Orphan

Europe). Since the early 1970's acute attacks have been treated with hematin (heme hydroxide) preparations (Dhar, et al., 1975). This lyophilised substance is preferentially used by the American population. But, due to its low stability and the evidence of some side effects such as transient anticoagulant effects and thrombophlebitis, a new compound - heme-arginate - with improved qualities, was introduced in Finland and is currently widely used across Europe and South Africa (Bonkovsky, 1993; Goetsch and Bissell, 1986; Mustajoki, et al., 1986; Tenhunen and Mustajoki, 1998; Tenhunen, et al., 1987). Heme-arginate, which is composed of human hemin and L-arginate as an additive, was created to improve both stability and solubility. In addition, no significant changes in coagulation, fibrinolysis and thrombophlebitis or other side effects were observed (Badminton and Elder, 2002; Mustajoki and Nordmann, 1993; Tenhunen and Mustajoki, 1998). When administered, it is used as a short time infusion (15-20 min) in a dosage of 3 mg per kg of bodyweight a day over a period of four days (Mustajoki and Nordmann, 1993). It is not recommended for use of longer than seven days, since long-term prophylactic use of heme-arginate for those having multiple recurrent attacks is being discussed (Tenhunen and Mustajoki, 1998).

However, one disadvantage of the repeated treatment of heme derivates is the synchronous induction of a crucial enzyme in the heme degradation pathway, heme oxygenase. This may reduce the efficacy of the treatment; therefore some trials of inhibitors of heme oxygenase have been performed. Preliminary observations are promising, but there is still great concern about the drugs' side effects (Elder and Hift, 2001).

In parallel with specific heme therapy, the monitoring of neurologic complications, respiratory function and electrolyte imbalance, identification of and removal of precipitating factors, as well as symptomatic treatment should not be underestimated. Symptomatic treatment usually involves the use of drugs such as narcotic analgesics for abdominal and extremity pain, and phenothiazine for nausea, vomiting and acute psychiatric conditions. Drugs should be selected with great respect to the porphyrinogenicity of such substances. Approximately 1000 therapeutic drugs categorised with regard to porphyrinogenicity are presented on the internet (http://www.drugs-porphyria.org) (Thunell, et al., 2007).

For some patients with severe recurrent attacks and no means of accessible treatment, liver transplantation may be an option (Seth, et al., 2007; Soonawalla, et al., 2004).

Of similar importance as the accurate treatment of acute attacks is the general knowledge to prevent acute attacks and genetic counselling within the families. The detailed procedure of treatment and diagnosis of porphyria has been summarised in several reviews (Anderson, et al., 2005; Kauppinen, 2005).

Gene

Human HMBS is determined by a single gene located on chromosome 11 (Meisler, et al., 1980), assigned to the locus to the long arm in the segment 11q24.1-q24.2 (Namba, et al., 1991). HMBS gene is divided into 15 exons ranging from 39 to 438 bp and 14 introns ranging from 87 to 2913 bp in length and, spans approximately 10 kb of DNA (Chretien, et al., 1988; Yoo, et al., 1993). All exon/intron boundaries conformed to the GT/AG rule (Breathnach and Chambon, 1981) are consist with the 5' and 3'consensus sequence for splice junctions of transcribed genes (Yoo, et al., 1993). Six Alu repetitive elements were identified in or adjacent to the HMBS gene, which makes this 10-kb gene relatively Alu-rich, as these repetitive elements occur on the average every 4kb across the genome (Hwu, et al., 1986; Yoo, et al., 1993).

The HMBS gene was the first gene ever described to be a single gene having dual purpose of being encoded by mRNAs transcribed from two promoters, a housekeeping and an erythroid-specific (Grandchamp, et al., 1987; Chretien, et al., 1988). The housekeeping promoter is in the 5' flanking region and its transcript is encoded by exons 1 and 3 through 15. The erythroid-specific promoter is located 3 kb downstream from the housekeeping promoter in the first intron and its transcript is encoded by exons 2 trough 15 (Grandchamp, et al., 1987; Gubin and Miller, 2001; Chen, et al., 1994) (**Figure 7**).

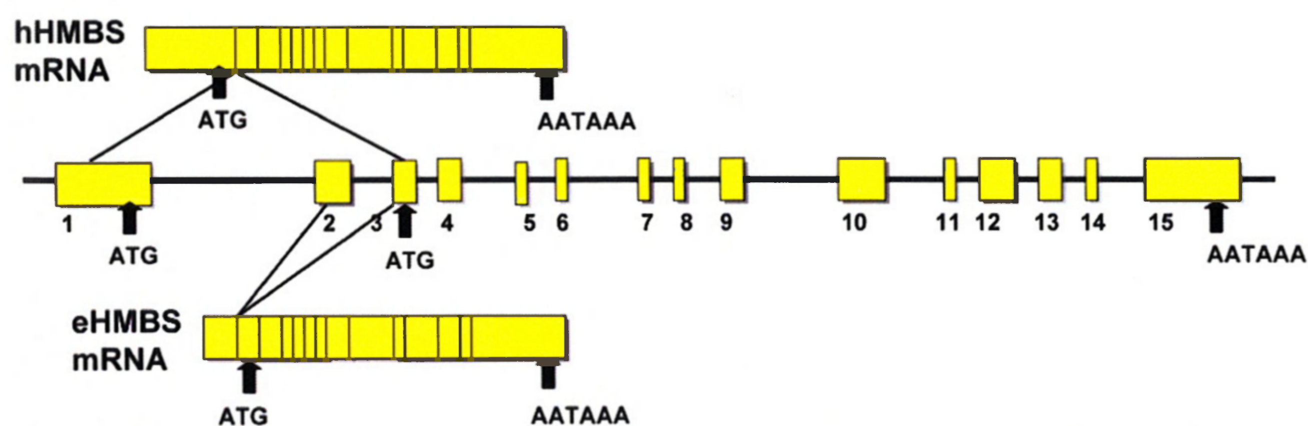

Figure 7 Genomic organization of the HMBS gene and alternative splicing of the transcripts

Tissue-specific expression of the HMBS gene is under control of two separate promoters: housekeeping promoter (hHMBS) and erythroid-specific promoter (eHMBS). The housekeeping transcript is encoded by exons 1 and 3 through 15. The erythroid-specific transcript is encoded by exons 2 trough 15. ATG= translational start site, AATAAA= polyadenylation signal.

The housekeeping promoter has certain features characteristic of housekeeping promoters, while comparison of the human beta-globin genes with the erythroid-specific promoter of HMBS gene suggests a modular organization, which might be common to a set of erythroid-specific promoters (Chretien, et al., 1988; Mignotte, et al., 1989a; Raich, et al., 1989). Several putative transcriptional factor binding sites for both promoter regions have been suggested based on sequence similarities (Yoo, et al., 1993). The sequence of housekeeping promoter region of human HMBS gene is GC rich and displays neither the classical TATA box, nor the CAAT box (Benoist, et al., 1980). Computer-assisted analysis for housekeeping gene region revealed several putative binding sites for a variety of transcriptional factors including AP1, AP4, thyroid responsive element, core enhancer element, SP1 element binding sites and CAC box (Yoo, et al., 1993). All the housekeeping genes bearing this modular organization (no TATA and CAAT, but cluster of SP1 binding sites) are either regulated during the cell cycle (Farnham and Schimke, 1986) or subjected to the feedback regulation by a product of the biosynthetic pathway in which they are involved (Luskey, et al., 1983).

The structural organization of the erythroid promoter of HMBS gene is very similar to that of beta-globin gene promoters, containing several separate sequence elements necessary for efficient transcription. There are at least four known motives common for beta-globin promoter region and erythroid promoter region of HMBS gene being very similar, i.e. ATAAAA box, CAAT box, CAAC box and CAC box (Chretien, et al., 1988). Furthermore, erythroid-specific trans-acting factors, two NF-E1 binding sites and one NF-E2 binding site, were identified (Frampton, et al., 1990; Chretien, et al., 1988; Mignotte, et al., 1989a; Mignotte, et al., 1989b). Nuclear factor NF-E1, which is now termed as GATA-1, is erythroid-specific trans-acting factor that recognizes sequence in erythroid promoter (Mignotte, et al., 1989a; Mignotte, et al., 1989b). A sequence with matching to the consensus CAC box (CACCC motif) was found close to the GATA-1 binding site in erythroid-specific promoter region of HMBS. It was suggested that CAC-GATA-1 association is probably involved in erythroid-specific initiation, since no consensus TATA box was found (Frampton, et al., 1990; Mignotte, et al., 1989a). Another trans-acting factor NF-E2, erythroid-specific enhancer binding protein, was suggested to cooperate together with GATA-1 in correct regulation of this promoter in erythroid cells (Mignotte, et al., 1989a; Mignotte, et al., 1989b). Computer-assisted analysis showed other putative regulatory elements like NF-1, AP1, AP4 and topoisomerase binding site (Yoo, et al., 1993). The expression of beta-globin and HMBS mRNA are among the earliest events characterizing

erythroid cell differentiation and, their structural similarities indicate that both genes can be activated in a coordinate manner during this process (Fibach, et al., 1979; Mignotte, et al., 1989a).

Moreover, during the studies of erythroid cell gene expression patterns, another distinct erythroid-specific HMBS mRNA was found and the existence of this transcript was confirmed in primary tissue (Gubin and Miller, 2001). This alternative erythroid-specific transcript is encoded by exons 2 trough 15. This finding suggests that the erythroid-specific mRNAs contain an alternate 5' region and that the erythroid-specific cell development is accomplished at the genomic level via multiple mechanisms, including shared patterns of gene organization, transactivation, and RNA maturation (Gubin and Miller, 2001). The existence of this second erythroid HMBS isoform has not been reported elsewhere after originally published, despite extensive analyses of this gene over years. The linkage between the system and function of this third transcript of HMBS gene should be further investigated.

Protein

Hydroxymethylbilane synthase (also known as porphobilinogen deaminase or uroporphyrinogen I synthase, EC 4.3.1.8 or EC 2.5.1.61), is the third enzyme of the heme biosynthetic pathway. This monomeric protein with a single catalytic active site (Louie, et al., 1992) is organised into three domains approximately equal in size (Louie, et al., 1996). cDNAs encoding the 42-kD housekeeping and 40-kD erythroid-specific isoenzymes have been isolated and characterised (Grandchamp, et al., 1987; Raich, et al., 1986). The housekeeping isoform of the protein consist of 361 amino acids, with an additional 17 amino acid residues at the N-terminus compared to the erythroid variant of 344 amino acids (Grandchamp, et al., 1987; Raich, et al., 1986). Both isoforms catalyse the same reaction. HMBS from several different species has been studied and its enzymatic kinetic properties have been identified. For HMBS isolated from human erythrocytes, optimal pH at 8.2 and Km 6 μM was determined (Anderson, 1980; Jordan and Shemin, 1973; Miyagi, et al., 1979).

In the HMBS reaction, the enzyme cooperates together with uroporphyrinogen III cosynthase to form uroporphyrinogen III from PBG (**Figure 8**). At the same time, the enzyme catalyses the formation of its dipyrromethane (DPM) prosthetic group. In the first catalytic cycle, the apo-protein catalyses step-wise head-to-tail polymerisation of six units of PBG into an enzyme-bound straight-chain haexapyrrole, before a hydrolytical cleavage event

releases the tetrapyrrole intermediate hydroxymethylbilane (Battersby, et al., 1979). The intermediate is rapidly converted into uroporphyrinogen III, closing the tetrapyrrole ring. A DPM cofactor, which is not subjected to catalytic turnover, remains covalently bound to the enzyme to form the active holo-enzyme (Jordan, et al., 1988; Shoolingin-Jordan, 1995). The apo-enzyme of HMBS is unstable and heat-labile while the holo-enzyme is much more stable and resistant to heat (Awan, et al., 1997).

The catalytic reaction proceeds through four covalent enzyme-intermediate complexes, ES, ES_2, ES_3 and ES_4, which are sequentially generated during the course of the tetrapolymerisation. These are stable to various degrees and are isolable (Lander, et al., 1991; Warren and Jordan, 1988). There is evidence that the relative movement of the domains may be important during the catalytic cycle to accommodate the intermediates (Lander, et al., 1991; Warren and Jordan, 1988). Only the monopyrrolic porphobilinogen, and not di- or tripyrroles, can be efficiently used as a substrate (Louie, et al., 1996).

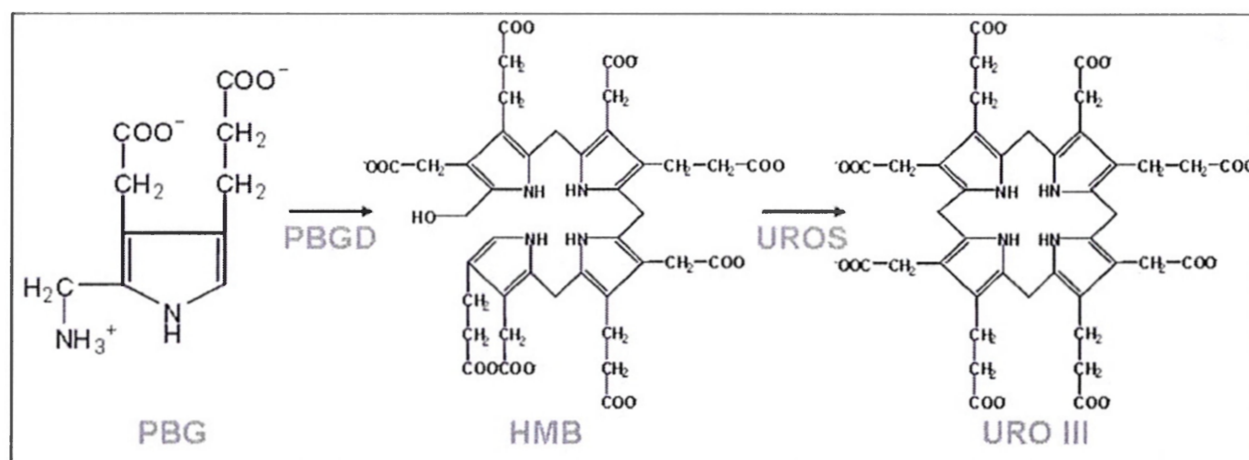

Figure 8 Enzymatic reaction of the hydroxymethylbilane synthase

In rapid conversion of porphobilinogen (PBG) into the uroporhyrinogen III (URO III), two enzymes cooperate. The first enzyme, hydroxymethylbilane synthase (HMBS), catalyzes the condensation of four molecules of PBG to form a linear unstable tetrapyrrole, hydroxymethylbilane (HMB). HMB is then enzymatically converted by uroporphyrinogen III synthase (UROS) to a cyclic tetrapyrrole, uroporphyrinogen III.

Relatively high amino acid sequence conservation is found in HMBS and its homologs, amounting to at least 32% in the proteins from bacteria, fungi, plants and mammals. It has been reported that the *E. coli* and human HMBS amino acid sequences have 43% identity and more than 60 % similarity (Brownlie, et al., 1994; Jordan and Warren, 1987; Shoolingin-Jordan, et al., 2003). Several crystallographic structures of *E. coli* HMBS have been

determined (PDB 1gtk, 1pda (Helliwell, et al., 2003; Louie, et al., 1996; Louie, et al., 1992)) (**Figure 9**).

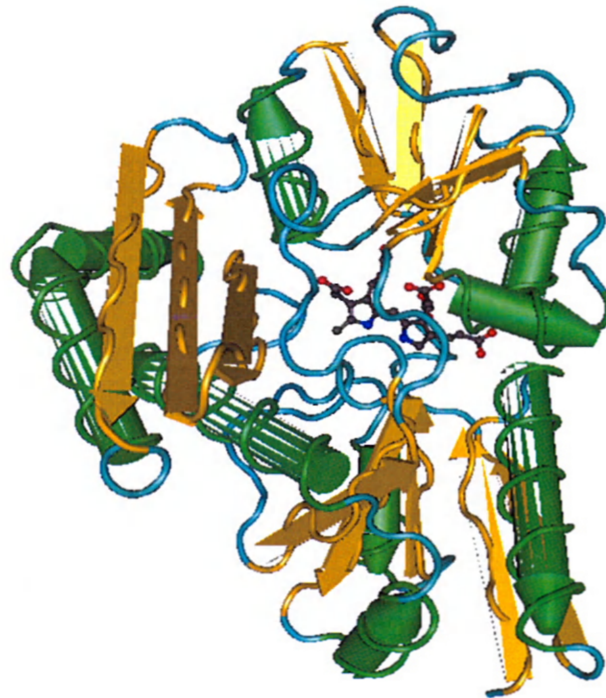

Figure 9 3D structure of the HMBS enzyme (PBD ID 1pda, (Louie, et al., 1992))

The monomeric protein with a single catalytic active site is organized in three domains approximately equal in size. The active site cleft contains the dipyrromethane cofactor.

Recently, the crystallographic structures of human housekeeping HMBS and human mutant protein Arg167Gln HMBS have been determined (PDB 3ecr and 3eq1; (Gill, et al., 2009; Song, et al., 2009)).

The best resolution attained so far for a three-dimensional structure of wild-type HMBS is a resolution of 1.66 Å for *E. coli* HMBS (Helliwell, et al., 2003) and of 2.2 Å for the human enzyme (Song, et al., 2009). Both the *E. coli* and human HMBS molecules have approximate dimensions of 57 x 43 x 32 Å, and have overall similar topology and other features in common (Gill, et al., 2009; Louie, et al., 1992). Approximately 38% of residues have been assigned a helical conformation, 25% an extended conformation, 8% turn, and 29% coil (Louie, et al., 1996). The polypeptide chain is folded into three domains of approximately equal size, each containing beta-strands and alpha-helices in secondary structure as well as a discrete hydrophobic core (Louie, et al., 1996; Louie, et al., 1992). Domain 1 consist of residues 20-115 and 214-238 (residues 3-99 and 200-217 in *E. coli*) and domain 2 consist of

116-213 (residues 105-193 in *E. coli*). These two domains are broadly similar in possessing the same structure of a doubly-wound, mainly parallel beta-sheet of five strands. The alpha-helical segments pack against each face of the beta-sheets. The C-terminal domain 3 comprising residues 239-356 (222-307 in *E. coli*) is an open-faced triple-stranded antiparallel beta-sheet with three alpha-helical segments covering one of the faces (Gill, et al., 2009; Louie, et al., 1992). Domains are connected by polypeptide chain connections. Besides the polypeptide-chain connections, there are only a few direct interactions between domains (Louie, et al., 1996). The active site cleft containing the DPM cofactor occurs at the large positively charged cleft region between domains 1 and 2 (Jordan and Warren, 1987).

Amino acid sequences from a wide range of organisms have been determined, and the 58 invariant residues (18% of the total protein) have been identified (Brownlie, et al., 1994). The majority of the invariant residues are clustered immediately around the active-site cleft. These are involved in the catalysis of the bond-making reactions, and in the formation of direct interactions with the DPM cofactor and the porphobilinogen substrate. Furthermore, the extensive network of hydrogen-bonding in this area may be of importance in stabilising the protein fold (Louie, et al., 1996). Two of these invariant amino acids are at the hot spot; the first is the cysteine Cys261 (Cys242 in *E. coli*) which is responsible for the covalent thioether bond of the DPM cofactor located on the loop of domain 3 and placed at the mouth of a deep cavity formed between the structurally related domains 1 and 2 (Brownlie, et al., 1994; Jordan and Warren, 1987; Shoolingin-Jordan, et al., 2003). The second invariant residue aspartate Asp99 (Asp84 in *E. coli*) creates hydrogen bonds with the cofactor, and was labelled as the key catalytic residue (Louie, et al., 1992). The acetate and propionate side groups of the DPM cofactor forms extensive salt bridge and hydrogen bond interactions with the HMBS protein and with bound water molecules. The basic side chains of Arg149, Arg150, Arg173, Ser96, Ser146, Ser147, Lys98, Asp99, Ala189 and Gly218 (Arg11, Arg131, Arg132, Arg149, Arg155, Lys83, Ser81, Ser129, Thr127 in *E. coli*) residues are, in particular, involved in these interactions.

In the human HMBS enzyme crystal structure, the DPM cofactor was found in the native reduced conformation with a hydrogen from a sulphate ion bonded to Arg26 and Ser28 at the proposed binding site (Gill, et al., 2009; Louie, et al., 1996). Analyses of the potential hinge region, which may assist in the opening and closing of the active site, revealed three amino acids Ser96, His120 and Lys238 as potential hinge residues (Song, et al., 2009).

The substrate is thought to interact with the positively charged side chains of Arg11, Arg149 and Arg155 at the binding site (Lambert, et al., 1994). The overall structure

possesses a large number of other ion pairs that may contribute to the considerable heat stability of the enzyme (Brownlie, et al., 1994; Louie, et al., 1992).

The main difference between the human and *E. coli* enzyme structures is that both the ubiquitous and erythroid human enzymes contain a 29-residue insertion in domain 3 and some other insertions in the loop regions as opposed to the *E. coli* structure (Brownlie, et al., 1994; Gill, et al., 2009). Although the insertion is located at a distance from the active site, it is expected to play a role in modulating enzyme conformational, which is associated with enzymatic action. There are only 148 identical residues between the human and *E. coli* structure, partly located around the active site and partly distributed evenly across the protein structure. Despite the many differences between the two structures at the amino acid level, the similarity in overall structure is achieved by subtle substitutions in the case of all substantial changes in side chains accompanied by compensatory changes and adjustments in adjacent residues (Gill, et al., 2009).

Structure-function correlations

To predict the impact of pathological mutations on the protein structure and function of human HMBS, the homology between human HMBS and the *E. coli* enzyme was used to build a structural model (Brownlie, et al., 1994). Now, the discovery of the human structure further facilitates an understanding of the structural correlations. No prevalent site for HMBS mutations has been determined; mutations are equally dispersed throughout the enzyme structure. Of these mutations, over 120 of them are missense/nonsense single base changes that result in one amino acid substitution or in the formation of a premature stop codon and subsequent protein truncation (http://www.hgmd.cf.ac.uk) (Stenson, et al., 2009). These mutations are of special interest, since single amino acid change can provide information on the functional or conformational importance of the wild-type residue.

Pathological mutations can be divided into three broad groups according to their molecular basis. The first group represents a change of amino acid that impacts on protein folding and stability. Such residues are usually located in close proximity to the hydrophobic core and in conformational restricted areas. The second group consists of residue changes with an effect on the binding, reaction and assembly of the DPM cofactor. If the DPM cofactor, important not only for the enzyme function, is absent in the enzyme structure, its stabilising ability is missing and the unstable apo-enzyme is therefore rapidly degradated.

Finally, the last group consists of mutations that affect the catalytic or substrate binding residues and thereby result in inactive proteins (Gill, et al., 2009; Song, et al., 2009).

LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by capital letters (A-J).

A. **Ulbrichova D**, Flachsova E, Hrdinka M, Saligova J, Bazar J, Raman CS, Martasek P. 2006. *De Novo* mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study. Physiol Res 55 Suppl 2:S145-54. (IF2006 2.09)

B. Flachsova E, Verma IC, **Ulbrichova D**, Saxena R, Zeman J, Saudek V, Raman CS, Martasek P. 2007. A new mutation within the porphobilinogen deaminase gene leading to a truncated protein as a cause of acute intermittent porphyria in an extended Indian family. Folia Biol (Praha) 53(6):194-201. (IF2007 0.6)

C. Schneider-Yin X[1], **Ulbrichova D[1]**, Mamet R, Martasek P, Marohnic CC, Goren A, Minder EI, Schoenfeld N. 2008. Characterization of two missense variants in the hydroxymethylbilane synthase gene in the Israeli population, which differ in their associations with acute intermittent porphyria. Mol Genet Metab 94(3):343-6. (IF2008 2.63)

[1]Both authors contributed equally to this work.

D. **Ulbrichova D**, Hrdinka M, Saudek V, Martasek P. 2009. Acute intermittent porphyria--impact of mutations found in the hydroxymethylbilane synthase gene on biochemical and enzymatic protein properties. FEBS J 276(7):2106-15. (IF2008 3.14)

E. **Ulbrichova D**, Schneider-Yin X, Mamet R, Saudek V, Martasek P, Minder EI, Schoenfeld N. 2009. Correlation between biochemical findings, structural and enzymatic abnormalities in mutated HMBS identified in six Israeli families with acute intermittent porphyria. Blood Cells Mol Dis 42(2):167-73. (IF2008 2.75)

F. **Ulbrichova Douderova D**, Martasek P. Detection of DNA Variations in the Polymorphic Hydroxymethylbilane Synthase Gene by High-Resolution Melting Analysis. *In press in Anal Biochem 2009* (IF2008 3.09)

G. **Ulbrichova Douderova D**, Schneider-Yin X, Lautenschlager S, Saudek V, Hofbauer GFL, Dziunycz PJ, Martasek P, Minder EI. **Lichen sclerosus et atrophicus in a patient carrying a novel hydroxymethylbilane synthase mutation**. *Prior to submission*

Report of the novel mutation identification

H. **Ulbrichova D**, Kurt I, Zeman J, Martasek P. 2008. Gene symbol: HMBS. Disease: Porphyria, acute intermittent. Hum Genet 124(3):315. (IF2008 4.04)

I. **Ulbrichova Douderova D**, Mamet R, Munter G, Martasek P, Schoenfeld N. Gene symbol: HMBS. Disease: Porphyria, acute intermittent. *Sent to Hum Genet 2009* (IF2008 4.04)

J. **Ulbrichova Douderova D**, Zeman J, Martasek P. Gene symbol: HMBS. Disease: Porphyria, acute intermittent. *Sent to Hum Genet 2009* (IF2008 4.04)

AIMS OF THE STUDY

In general, the aim of this study was to enable the proper molecular diagnosis of AIP patients at the DNA level in order to investigate the structural-functional consequences of mutations at the protein level.

The specific aims were as follows:

- The molecular diagnosis of newly diagnosed AIP patients and the molecular diagnosis of affected families using an established method.
- The optimisation of the next generation diagnostic method, High-Resolution Melting, using the LightScanner instrument for detection of DNA variations in the HMBS gene.
- The expression, purification and biochemical characterisation of human mutant HMBS enzymes with introduced pathological mutations of interest in the prokaryotic (*E. coli*) system.
- The structure-function correlation studies - the assessment of detrimental effects of DNA variations on enzyme function.

RESULTS AND DISCUSSION

Publication A

De Novo mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical ' study *(Ulbrichova D et al., 2006)*

The patient, a 15-year-old boy, was hospitalised while having his first acute attack, characterised by severe abdominal pain, hyponatraemia, cognitive failure, hypertension, tachycardia and subsequent neurological symptomatology, accompanied by the highly elevated porphyrin precursors ALA and PBG in urine, all clinical features typical of AIP.

The prescreening molecular method of denaturing gradient gel electrophoresis (DGGE) revealed a characteristic pattern indicating DNA variation in the HMBS gene. DNA sequencing confirmed the presence of a novel heterozygous mutation, the c.965_966insA. The molecular screening of the HMBS gene in family members was performed, but failed to reveal any DNA variation. This was in agreement with the fact that all of the other five family members from two generations were asymptomatic. Therefore, we suggest that 966insA is a *de novo* mutation since nonpaternity was excluded by DNA microsatellite analysis.

The mutation c.965_966insA is localised in exon 15 of the HMBS gene. At the protein level, it results in a frameshift and production of a STOP codon after expression of 36 completely different amino acids compared to the original sequence (p.Asn322LysfsX36). The truncated mutant protein consists of 357 amino acids as opposed to the normal 361 amino acids. Usually, the truncation is expected to lead to an unstable and inactive protein.

To investigate the impact of this small insertion mutation on the protein structure and to further study the functional consequences, we decided to express wild-type and mutant protein in the prokaryotic system. For the construct preparation, we used cDNA sequences obtained by reverse transcription of total RNA extracted from peripheral leukocytes. We cloned the cDNA for the HMBS protein into the pGEX-4T-1 expression vector. We used the *E. coli* BL21 (DE3) expression system. We then introduced the insertion mutation into the construct using site-directed mutagenesis. The HMBS proteins were expressed as GST-fusion proteins and purified by affinity chromatography using Glutathione Sepharose 4B columns. We performed the residual activity measurement.

In agreement with the premise that protein truncation would lead to instability, the SDS-PAGE analyses of the mutant protein revealed several bands, in contrast to a single homogenous band in the case of wild-type protein. Wild-type as well as mutant HMBS enzymes were similar in size, measuring approximately Mr 68 kDa with the GST-tag and Mr 42 kDa without the GST-tag. The purified mutant enzyme had a relative activity level of 0.18% of level achieved by wild-type enzyme. This result is in good agreement with the observation that the erythrocytic HMBS activity in the AIP-affected individual is decreased to about 50% of the healthy average, as the patient was heterozygous for the mutation.

We designed the mutant protein structure using the computer-assisted structure prediction program, using the 3D structure of *E. coli* HMBS as a template. The c.965_966insA mutation is localised in the $\beta 3_3$ sheet of domain 3. Due to the incurred frameshift, part of the third enzyme domain has a different formation; two helices $\alpha 2_3$ and $\alpha 3_3$ of domain 3 are completely missing compared to the wild-type protein. In the wild-type protein, the C-terminal helices protect the beta-strands from being exposed to solvent. This is in agreement with the severely decreased stability of the mutant HMBS.

In summary, the *de novo* mutation c.965_966insA (p.Asn322LysfsX36) was found in a young patient with AIP. Due to a truncated protein sequence with an abnormal C-terminus domain, this small insertion mutation c.965_966insA leads to an almost complete loss of the enzymatic function and decreases the stability of the protein. These results further broaden our understanding of the detrimental effects of mutations in a case of relatively early AIP manifestation. This case is of particular interest as the identification of a *de novo* mutation is a rare event.

In this study, we cooperated with Second Pediatric Department in Košice, Slovak Rep., and Laboratory of Structural Biology in Houston, USA. I contributed to this study by the cooperation on the identification of the proband´s mutation in the HMBS gene using DGGE analysis and direct sequencing as well as by the DNA screening of his family members. I performed protein expression, purification, protein analyses and enzyme activity measurements.

Publication B

A new mutation within the porphobilinogen deaminase gene leading to a truncated protein as a cause of acute intermittent porphyria in an extended Indian family *(Flachsova E et al., 2007)*

Our laboratory was contacted by a 50-years-old proband from Nepal who suffered from severe abdominal pain accompanied by dark urine. After considering the possibility of having acute intermittent porphyria, he, as a non-health professional, searched the internet for help with treatment and molecular diagnostic confirmation of his clinical status. He arranged to send samples to us- either blood or gDNA - from himself and from 15 members of his family.

Molecular testing revealed a novel heterozygous mutation c.972_973insG in exon 15 of the HMBS gene. Analysis of the protein sequence indicated that following the insertion mutation, four amino acids were different. After this, a premature stop codon occurred, leading to a prematurely truncated protein in which 44 amino acids of the C-terminus of HMBS was missing. Usually, and as observed in our previous study, the truncation was predicted to result in an unstable and inactive protein.

To further investigate the detrimental effects of this small insertion mutation on the protein structure, we decided to express the mutated gene in the prokaryotic system and to perform biochemical testing of the purified enzyme.

In agreement with our previous study results, the SDS-PAGE analyses of the mutant protein displayed again several bands, suggesting high protein instability. The purified mutant enzyme had an activity level 0.5% of the average wild-type level.

Using the computer-assisted structure prediction, the 3D structure of the mutant protein was designed. The c.972_973insG mutation is located in domain 3 in close proximity to the terminal helix, which is much more independent of the rest of the structure and points towards the solvent. The mutations in the penultimate helix or its removal destabilise the whole C-terminal domain; in turn, the N-terminal domain cannot fold in a stable unity and therefore the whole protein is destabilised. Even point mutations in the penultimate helix can lead to AIP, underlining the importance of this helix for enzyme stability.

After DNA screening in the proband's family members, the same mutation was subsequently found in 12 of them. In agreement with the observation that most AIP carriers are without symptoms throughout their life, 7 out of the 12 were asymptomatic.

In summary, a novel mutation c.972_973insG within the HMBS gene was identified in 12 members of an extensive Indian family from Nepal. This mutation results in a truncated and

highly unstable protein with loss of enzymatic function. The uniqueness of this case lies in the fact that the proband diagnosed himself based on information from the internet. This highlights the importance of the accessibility of online information especially in the case of rare diseases. Molecular screening within the proband's family was used for genetic counselling.

In this study, we cooperated with Department of Genetic Medicine, Sir Ganga Ram Hospital, New Delphi, India and Laboratory of Structural Biology in Houston, USA. I contributed to this study by the protein expression, purification, protein analyses and enzyme activity measurements.

Publication C

Characterization of two missense variants in the hydroxymethylbilane synthase gene in the Israeli population, which differ in their associations with acute intermittent porphyria *(Xiaoye Schneider-Yin et al., 2008)*

In this study, we report on mutational analysis and *in vitro* characterisation of HMBS variants identified in two individuals who were suspected of having AIP.

The first patient, a 17-year-old Ashkenazi Jewish female, had been experiencing menstruation-related recurrent episodes of severe abdominal pains accompanied by vomiting, tachycardia and hypertension for 3 years. Biochemical analyses failed to show elevations in urinary PBG and ALA levels during acute and latent phases. However, erythrocyte HMBS activity showed an average activity that was 60% of normal. The patient was treated with either glucose or heme-arginate (Normosang, Europe) for acute attacks. While lacking full AIP-confirming biochemical evidence, the diagnosis of acute porphyria was questionable.

The second patient, a 30-year-old Ashkenazi Jewish female, was suffering from recurrent acute attacks of abdominal pain, hyponatraemia and urinary retention for 12 years. Biochemical analyses showed increased urinary ALA and PBG, and reduced erythrocyte HMBS activities about 50% of normal. She was successfully treated with glucose.

After molecular screening of the HMBS gene, two novel heterozygous mutations c.176C>T in exon 5 and c.643G>A in exon 11 was identified separately in each subject. At the protein level, the mutation c.176C>T leads to an amino acid substitution p.Thr59Ile and the mutation c.643G>A leads to an amino acid substitution p.Val215Met. Both p.Thr59Ile

and p.Val215Met were absent in the HMBS gene of 50 non-porphyric Ashkenazi Jewish subjects.

We expressed both variant proteins in the prokaryotic system and we performed biochemical testing of the purified mutant enzymes. Recombinant p.Thr59Ile and p.Val215Met mutant enzymes had residual activity of 80.6% and 19.4%, respectively, compared to that of the wild-type enzyme.

While the clinical symptoms, *in vitro* and *in vivo* biochemical analyses, as well as the changes in Km, Vmax and thermostability observed in the mutant protein of the patient who carried p.Val215Met all suggested a causal relationship between p.Val215Met and AIP, the association between the p.Thr59Ile substitution and AIP is less obvious. In view of the results we received, despite the normal urinary excretion of ALA and PBG during acute attacks, the high residual activity (80.6%) of the recombinant enzyme and the similarities between p.Thr59Ile mutant and the wild-type enzyme in Km value and in the thermostability profile, we conclude that p.Thr59Ile might represent a mutation with a weak effect rather than a mere polymorphism. In contrast to the above-mentioned statements, there was some evidence supporting the association between the p.Thr59Ile mutation and the AIP phenotype, such as successful treatment of the clinical symptoms of AIP with glucose and Normosang. Moreover, the patient's erythrocyte HMBS activity was reduced to half-normal value, and considering the age of the patient (that is, under the age of 18), normal values of ALA and PBG may be observed during symptomatic periods. It may very well be that in this compound case, there is more than one causative factor with clinical relevance, which remains undetected.

In summary, two novel mutations c.176C>T (p.Thr59Ile) and c.643G>A (p.Val215Met) within the HMBS gene were identified. Despite not having full AIP-affirming biochemical evidence in one case, both mutations were associated with AIP. The study demonstrates that *in vitro* characterisation of mutations in the HMBS gene can add valuable information to the interpretation of clinical, biochemical and genetic data in establishing a diagnosis of AIP. It also highlights the fact that there are still many causative aspects to be investigated in AIP.

In this study, we cooperated with Central Laboratory, Triemli Hospital, Zurich, Switzerland; National Laboratory for the Biochemical Diagnoses of Porphyrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel; Department of Biochemistry, The University of Texas Health Science Center at San Antonio, USA; Department of Pediatrics, Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, Israel; Department of

Human Molecular Genetics and Biochemistry, The Sackler Faculty of Medicine, Tel Aviv University, Israel. I contributed to this study by the identification of the proband´s mutation c.176C>T (p.Thr59Ile) in the HMBS gene using direct sequencing and by expression, purification, protein analyses and enzyme activity measurements of all of the proteins.

Publication D

Correlation between biochemical findings, structural and enzymatic abnormalities in mutated HMBS identified in six Israeli families with acute intermittent porphyria *(Ulbrichova D et al., 2008)*

In this study, a total of 26 individuals from six unrelated Israeli AIP families of Caucasian origin underwent biochemical and mutational analysis in order to establish an AIP diagnosis. Variability with respect to the ALA/PBG levels and erythrocytic HMBS activity was found among the index patients. Each family carried a unique mutation in the HMBS gene.

Following the molecular screening of the HMBS gene, one novel heterozygous missense mutation c.95G>C (p.Arg32Pro) was shown to exist *de novo* in one family, along with five known mutations c.176C>T (p.Thr59Ile), c.532G>A (p.Asp178Asn), c.643G>A (p.Val215Met), c.730_731delCT and c.982_983delCA identified separately in each family.

We expressed p.Arg32Pro and p.Asp178Asn mutant proteins in the prokaryotic system, since the other missense mutations have been expressed and characterised in our previous study. We performed biochemical testing of the purified mutant enzymes. The structure-function consequences of all mutations were studied at the protein level. We analysed the correlation between biochemical findings, the impact of the mutation on the protein function, and the structural predictions of the affected individuals as part of the study.

Of the four missense mutations, p.Arg32Pro and p.Val215Met had not only detrimental effects on the enzyme *in vitro,* with residual activities of 1% and 19% respectively, but these mutations were also associated with high levels of ALA/PBG comparable with that of frameshift mutations c.730_731delCT and c.982_983delCA in patients. In addition, p.Val215Met was shown to be extremely thermo labile. Therefore we labelled them “strong” mutations. In contrast, the *in vitro* effect of both of the “weak” p.Thr59Ile and p.Asp178Asn mutations was much lower, as demonstrated by the relatively high residual activity of 81%. In accordance with this analysis, a common feature shared by these two patients with “weak”

mutations was their normal or borderline levels of ALA/PBG although they presented characteristic clinical symptoms.

All six HMBS mutations were evaluated at the structural level based on the 3D structure of the *E. coli* enzyme. Based on the 3D structure, the two “strong” missense mutations as well as the two frameshift mutations were all predicted to have detrimental effects on the structure and function of the enzyme either due to their location or due to the nature of the substitution. The two “weak” mutations on the other hand were located at less critical positions and therefore exerted limited impact on the structure and function of the enzyme.

In summary, one novel heterozygous mutation c.95G>C (p.Arg32Pro), along with five known mutations c.176C>T (p.Thr59Ile), c.532G>A (p.Asp178Asn), c.643G>A (p.Val215Met), c.730_731delCT and c.982_983delCA, were identified separately in each family. We performed an extensive *in vitro* characterisation of the proteins with the introduced mutations, including residual activity analysis and kinetic activity assays. The results of the *in vitro* study broaden our understanding of the impact of individual mutations on enzyme activity and consequently, our understanding of AIP disease.

In this study, we cooperated with Central Laboratory, Triemli Hospital, Zurich, Switzerland; National Laboratory for the Biochemical Diagnoses of Porphyrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel; Laboratory of Molecular Pathology, Institute of Inherited Metabolic Disorders, 1st School of Medicine, Charles University, Prague, Czech Republic; Department of Human Molecular Genetics and Biochemistry, The Sackler Faculty of Medicine, Tel Aviv University, Israel. I contributed to this study by the identification of the proband´s mutation c.95G>C (p.Arg32Pro) in the HMBS gene using direct sequencing as well as by the DNA screening of his family members. I performed expression, purification, protein analyses and enzyme activity measurements of all of the proteins.

Publication E

Acute intermittent porphyria - impact of mutations found in the hydroxymethylbilane synthase gene on biochemical and enzymatic protein properties *(Ulbrichova D et al., 2009)*

In the present study, six patients who were newly diagnosed with AIP were studied. Five of them were women. The diagnosis of AIP was made on the basis of clinical features typical for AIP and the excretion pattern of porphyrin precursors. The most prominent symptom in all patients was severe abdominal pain.

HMBS genes of all probands were screened for DNA variations by denaturing gradient gel electrophoresis (DGGE). Six samples with abnormal patterns suggesting mutations were detected. Direct DNA sequencing revealed seven mutations in these samples. Of the identified mutations, three were novel, c.610C>A (p.Gln204Lys), c.750A>T (p.Glu250Asp) and c.675delA (p.Ala226ProfsX28); and four mutations were previously reported c.76C>T (p.Arg26Cys), c.77G>A (p.Arg26His), c.518G>A (p.Arg173Gln) and c.771+1G>T (r.spl?). One patient had two mutations, c.[518G>A; 610C>A], located in the same allele, which is a rare molecular defect in the HMBS gene. Overall, 33 individuals from proband´s families were screened and nine carriers of an affected HMBS gene were identified.

To study the impact of the various mutations on the protein structure and subsequent functional consequences, mutated proteins were expressed in *E. coli* and the enzymatic properties were characterised. All the recombinant expressed and purified proteins displayed homogeneous bands when inspected by SDS-PAGE, except for two mutants, one with the small deletion mutation p.Ala226ProfsX28 as expected, and surprisingly with the mutation p.Glu250Asp. The residual enzymatic activity measurement of the HMBS proteins with mutant alleles revealed that, with the exception of the p.Gln204Lys mutation (which exhibited ~ 46% of wild-type activity), all mutations lead to little, if any, enzymatic activity. The observation of low residual activity for most mutations is consistent with the expected 50% decrease in final HMBS activity, which is observed in individuals with acute intermittent porphyria. These findings further support the AIP-causality of these mutations in the HMBS gene. In the case of the patient with two combined mutations, both located on the same allele, mutation p.Arg173Gln has a severe effect on enzyme function. From the additional testing of the protein properties, we concluded that the p.Gln204Lys mutation has a milder impact on protein function and structure, but can still be associated with AIP.

To further determine the structure–function relationships for these mutations, the 3D structure of the *E. coli* and newly-determined human proteins, as well as the sequence alignment of prokaryotic and eukaryotic HMBS nonredundant sequences was used. From the structure and sequence information, it can be inferred that the patient's mutations of Arg26 to Cys or His may lead to the loss of interactions with the cofactor, which explains our observation of the near complete loss of enzyme activity. In the case of the Arg173 to Gln mutation, the change results in an apo form of the enzyme that is incapable of catalysis. Most likely, the mutant is unable to interact properly with the cofactor. In the case of the p.Gln204Lys mutant, the mutant enzyme exhibited ~ 46% of wild-type activity. This can be explained by the fact that the Gln204 residue is exposed on the surface of the central domain, remote from the active site of the protein. It is likely that the introduction of the positive charge of the lysine amino group changes the configuration of the two surface loops, which may destabilise the enzyme. The small deletion p.Ala226ProfsX28 causes a truncation which leads to an unstable and inactive protein, which is likely to be rapidly degraded by the proteosome. In mutant p.Glu250Asp, the Glu250 residue is conserved in all sequences with no exception. It creates an interaction that fixes the C-terminal domain to the interdomain hinge whose mobility is important for access of the substrate to the active site. Nevertheless, the effect of the new mutation p.Glu250Asp is unexpected because the change from Glu to Asp results only in a subtle change of the molecule structure: The abolition of the activity demonstrates the importance of exact geometry in the interior of the enzyme. In the case of such mutations as c.771+1G>T, the deletion of the entire exon 12 is expected, and the function of this mutant is expected to be completely abolished.

In summary, we identified four previously reported mutations c.76C>T (p.Arg26Cys), c.77G>A (p.Arg26His), c.518G>A (p.Arg173Gln), c.771+1G>T (r.spl?); and three novel ones c.610C>A (p.Gln204Lys), c.675delA (p.Ala226ProfsX28), c.750A>T (p.Glu250Asp) in Czech AIP patients. Of particular interest, one patient had two mutations (c.518G>A; c.610C>A), both located in the same allele. We performed an extensive *in vitro* characterisation of the mutant proteins by introducing the mutation of interest and performing residual activity measurements, kinetic studies on recombinant enzymes, and structure-function correlations. These findings provided further insights into the causal relationship between HMBS mutations and AIP.

In this study, we cooperated with Laboratory of Molecular Pathology, Institute of Inherited Metabolic Disorders, 1st School of Medicine, Charles University, Prague, Czech

Republic. I contributed to this study by the identification of the mutations in the HMBS gene of AIP probands using DGGE analysis and direct DNA sequencing as well as by the DNA screening of all AIP families. I performed expression, purification, protein analyses and enzyme activity measurements of all of the proteins.

Publication F

Detection of DNA Variations in the Polymorphic Hydroxymethylbilane Synthase Gene by High-Resolution Melting Analysis *(Ulbrichova Douderova D et al., in press in Anal Biochem 2009)*

In this report, we tested the high-resolution melting (HRM) procedure on the LightScanner instrument as a method of screening DNA variations in the polymorphic HMBS gene.

In the selected subjects tested, the diagnosis of porphyria was made based on the clinical features typical for each porphyria type and the related specific porphyrin excretion pattern. The samples were amplified in the presence of a 1x concentration of the saturating DNA dye LC-Green PLUS dye. Straight afterwards, the PCR plate was transferred to the LightScanner in which the HRM analyses were performed. Collected data were analysed with the commercial LightScanner software 1.5 with the Call-IT function. Identified DNA variations were confirmed by independent sequencing of the second PCR product.

To start off, we determined whether the presence of previously detected polymorphisms would adversely interfere with further testing of the gene using this method. In all four cases of polymorphisms consistently detected in our patients, we detected three discrete groups of genotypes (two homozygous and one heterozygous).

The ability of the HRM method to detect DNA variations in the HMBS gene was tested on DNA samples with ten known mutations using a curve shape scan generated with the LightScanner instrument. Each of the ten mutations tested had an altered melting profile compared to the melting profile of the controls. Even if the mutation was localised in the amplicon together with the other known polymorphisms, the mutation was identified correctly.

Finally, we evaluated the HRM method on the group of 97 subjects with suspected acute hepatic porphyria. From the DNA variations identified, three were previously described mutations: c.70G>A (p.Gly24Ser), c.87+5G>T (r.(spl?), c.[518G>A; 610C>A] (p.[Arg173Gln; Gln204Lys]), one was a novel mutation c.899_900delins TGCCTGCATCTG

(p.His300LeuFsX10), two were previously described polymorphisms g.3119T/G (rs1006195) and g.7998G/A (rs1799997), and three were novel, rare DNA variations g.2922T>G, g.3059G>A and g.7175A>G (found in one subject). The finding of these three novel rare DNA variations requires further investigation, since these variations are localised in the intronic part of the HMBS gene, but were only present in one allele out of 200. We found the small, indel mutation interesting because there are only four such cases (out of 307 mutations in the HMBS gene) registered in the mutation database. At the protein level, the mutation p.His300LeuFsX10 would cause a frame shift leading to a premature stop codon after ten completely different amino acids. A rare, novel DNA variation, g.2922T>G, found in one subject with porphyria variegata suggests the possibility of a rare case of dual porphyria. This finding requires further investigation, since this variation is localised in the regulatory segment of the erythroid promoter of HMBS.

In summary, screening the group of subjects with suspected porphyria revealed nine different DNA variations, four of which were novel. HRM is a fast, cost-effective pre-screening method for detecting DNA variations in the HMBS gene. Moreover, the screening can be extrapolated to an entire family in the event of possible misdiagnosis or rare dual porphyria. We showed that the HRM method can serve as a useful screening tool to identify DNA variations, even in amplicons with other polymorphisms.

I contributed to this study by the introduction of new prescreening method of HRM using LightScanner instrument into the molecular diagnostic of AIP. I identified the mutations in the HMBS gene of AIP subjects using HRM analysis and direct DNA sequencing.

Publication G

Lichen sclerosus et atrophicus in a patient carrying a novel hydroxymethylbilane synthase mutation *(Ulbrichova Douderova D et al., prior to submission)*

In this case report, we present a latent AIP patient incidentally identified during clarification of a skin disorder that was eventually diagnosed as lichen sclerosus et atrophicus (LS). A cutaneous porphyria disorder was suspected based on skin problems of a 48-year-old female Swiss patient. Biochemical analyses revealed normal fecal porphyrin content, a normal protoporphyrin concentration in erythrocytes and a negative plasma fluorescence

scan, typical features of cutaneous porphyria. However, repeated measurement of erythrocytic HMBS activity revealed a ~ 50% reduction in enzyme activity and slightly increased urinary ALA and PBG, biochemical findings that are compatible with the latent status of AIP. The skin condition in this patient was subsequently diagnosed as LS by biopsy.

After molecular screening of the HMBS gene, the novel heterozygous substitution mutation c.601C>G in exon 10 was identified and the status of latent AIP was confirmed, since the patient had never experienced an acute attack. At the protein level, this missense mutation leads to a change of Arg201 amino acid residue to glycine residue (p.Arg201Gly).

The mutation p.Arg201Gly was subsequently expressed and the mutant protein was characterised *in vitro*. A residual enzymatic activity of 5.9% of wild-type was measured in the mutant enzyme. Moreover, compared to the wild-type enzyme, the mutant was extremely unstable when faced with heat treatment and exhibited a shift in the optimal pH.

In the 3D structure of human HMBS published recently, two lobes of the central domain are joined by ion-pairing between Arg201 and Asp178. Both of these amino acid residues are highly conserved among prokaryotic as well as eukaryotic HMBS enzyme sequences. Such high degree of conservation suggests that the ion pair plays an important role in the enzyme structure.

In summary, the novel mutation c.601C>G (p.Arg201Gly) was found in a Swiss patient with AIP. The result of the *in vitro* characterisation of the mutant suggested that the p.Arg201Gly mutation has a deleterious effect on the HMBS protein. This result is in accordance with the *in vivo* measurement of decreased erythrocytic HMBS activity. Thus, a latent status, so typical in this disorder, but hardly identifiable without family history, was granted in this patient, suffering simultaneously with lichen sclerosus et atrophicus, taking into account that she has so far not shown any symptoms of AIP.

In this study, we cooperated with Central Laboratory, Triemli Hospital, Zurich, Switzerland; Laboratory of Molecular Pathology, Institute of Inherited Metabolic Disorders, 1st School of Medicine, Charles University, Prague, Czech Republic; Outpatient Clinic of Dermatology, Triemli Hospital, Zurich, Switzerland; and Department of Dermatology, University Hospital, Zürich, Switzerland. I contributed to this study by protein expression, purification, protein analyses and enzyme activity measurements.

Report of a novel mutation identification

Publication H

Gene symbol: HMBS. Disease: Porphyria, acute intermittent *(Ulbrichova D et al., 2008)*

From our cooperative laboratory we received whole blood of a Turkish AIP patient. His diagnosis was made based on clinical findings typical for AIP and almost half-normal (57.8%) erythrocytic HMBS activity. After gDNA isolation, we performed molecular screening in the HMBS gene by direct DNA sequencing. The DNA screening revealed a novel heterozygous mutation in exon 4, c.89T>G. At the protein level, this mutation results in the amino acid substitution of leucine to arginine, p.Leu30Arg.

We expressed the mutated gene in the prokaryotic system and performed biochemical testing of the purified enzyme. The purified mutant enzyme had a relative activity 0.03% of the average wild-type level (*unpublished data*). This is in good agreement with the results measurement of half-normal erythrocytic HMBS activity as the patient was heterozygous for the mutation. The detrimental effect of this amino acid substitution on the protein structure is further emphasised by the fact that the Leu30 residue is highly conserved in both the prokaryotic and eukaryotic HMBS sequences and therefore likely of special importance to the enzyme structure.

In summary, the novel mutation c.89T>G (p.Leu30Arg) was found in a Turkish patient with AIP. This finding further confirmed the diagnosis at the molecular level.

In this report, we cooperated with Department of Clinical Biochemistry in Gulhane School of Medicine, Ankara, Turkey. I contributed to this report by the identification of the proband´s mutation in the HMBS gene using direct sequencing. I performed protein expression, purification, protein analyses and enzyme activity measurements (*unpublished data*).

Publication I

Gene symbol: HMBS. Disease: Porphyria, acute intermittent *(Ulbrichova Douderova D et al., sent to Hum Genet 2009)*

The patient, female, was hospitalised while having her first acute attack characterised by severe abdominal pain, hyponatraemia and behavioural disturbances. From the biochemical measurements, the highly elevated level of porphyrin precursors was detected, a distinct peak in the fluorimetric plasma scan at 404/622 nm was identified and her erythrocytic HMBS activity was 71% of the normal value.

DNA sequence analyses revealed a small heterozygous insertion c.184_185insT within the HMBS gene and confirmed the diagnosis of AIP.

At the protein level, this mutation p.Lys62IlefsX3 causes a frameshift and creates a stop codon after three completely different amino acids, resulting in a truncated protein of 64 amino acids. The effect of such a truncation is expected to be detrimental.

In summary, the novel mutation c.184_185insT (p.Lys62IlefsX3) was found in a patient with AIP. This finding further confirmed the diagnosis at the molecular level.

In this report, we cooperated with National Laboratory for the Biochemical Diagnoses of Porphyrias, Rabin Medical Center, Beilinson Hospital, Petah-Tikva, Israel and Department of Internal Medicine A, Shaare Zedek Medical Center, Jerusalem, Israel. I contributed to this report by the identification of the proband's mutation in the HMBS gene using direct DNA sequencing.

Publication J

Gene symbol: HMBS. Disease: Porphyria, acute intermittent *(Ulbrichova Douderova D et al., sent to Hum Genet 2009)*

The patient, a Ukrainian female, was diagnosed with AIP by a clinician while having clinical manifestations typical for acute hepatic porphyria- abdominal pain, dark urine and muscle weakness. During an acute attack, the level of porphyrin precursors PBG and ALA were elevated (16.6 mg/100ml and 18.2 mg/100ml, respectively).

DNA sequence analysis revealed a novel heterozygous small insertion c.384_385insT in exon 8 within the HMBS gene and confirmed the diagnosis of AIP.

At the protein level, this mutation p.Val130CysfsX80 causes a frameshift and creates a stop codon after eighty completely different amino acids resulting in a truncated protein. The effect of such truncation is expected to be detrimental.

In summary, the novel heterozygous mutation c.384_385insT (p.Val130CysfsX80) was found in a patient with AIP. This finding further confirmed the diagnosis at the molecular level.

I contributed to this report by the identification of the proband´s mutation in the HMBS gene using direct DNA sequencing.

CONCLUSIONS

In the present study, the patients carrying mutation in the HMBS gene were characterised at the molecular level. The major achievements of the work in this thesis are the following:

- Twenty-eight DNA variations were identified in patients with AIP. Out of them, thirteen were novel mutations, ten were previously reported mutations, two were previously reported polymorphisms, and three were novel rare DNA variations, which require further investigation. Moreover, out of the novel mutations identified, two were *de novo* mutations, which are rare events in this disorder. To the six mutations known to exist in the Slavic population to date, another nine mutations were identified, broadening the molecular heterogeneity of the HMBS gene in our population.

- The comparison of the clinical manifestation of AIP patients disclosed the evidence of the variability with respect to the ALA/PBG levels and erythrocytic HMBS activity among the index patients. This clearly demonstrates that although biochemical measurements should be included as a first diagnostic step, the detection of the causal mutation in the HMBS gene is the ultimate diagnostic criteria for AIP.

- In order to improve molecular testing of the HMBS gene, a fast, cost-effective pre-screening method of high-resolution melting using the LightScanner instrument was established.

- Fourteen different proteins with introduced mutations were expressed in the prokaryotic system. These mutants were characterised at the biochemical level. Even though most of them exhibited a residual activity close to zero, some of them exhibited as high a residual activity as 81% that of wild-type. Only further characterisation allowed the association of these mutations with AIP. This demonstrates that *in vitro* expression of HMBS mutant genes and characterisation of their structure-function consequences can improve the interpretation of clinical, biochemical and genetic data and the diagnosis of AIP. Moreover, the results suggest

that there is more than one causative factor with clinical relevance, which remains undiscovered.

- Based on the identification of the causal mutation in the HMBS gene of the index patient, appropriate genetic counselling based on the DNA diagnostics was applied within the AIP affected families.

Future challenges

One of the unknown aspects in the pathogenesis of AIP is the mechanism of incomplete penetrance and high variability of the clinical manifestation among the porphyria patients. According to the literature, only 10-20% of the mutation carriers will ever develop clinical symptoms. This suggests that the genetic background of individuals may explain differences in susceptibility to an acute attack, though the genes involved have not yet been identified. Future studies may be focused on the identification of such modifier genes which influence the susceptibility of patients with acute hepatic porphyrias to acute attack.

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SUPPLEMENTS: ORIGINAL PUBLICATIONS

PUBLICATION A

De Novo mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study *(Ulbrichova D et al., 2006)*

Physiol. Res. 55 (Suppl. 2): S145-S154, 2006

De Novo Mutation Found in the Porphobilinogen Deaminase Gene in Slovak Acute Intermittent Porphyria Patient: Molecular Biochemical Study

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Received November 3, 2006
Accepted December 13, 2006

Summary

The porphyrias are group of mostly inherited disorders in which a specific spectrum of accumulated and excreted porphyrins and heme precursors are associated with characteristic clinical features. There are eight enzymes involved in the heme synthesis and defects in seven of them cause porphyria. Four of them are described as acute hepatic porphyrias, which share possible precipitation of acute attacks with symptoms engaging the nervous system. Acute intermittent porphyria (AIP), caused by partial deficiency of the porphobilinogen deaminase (PBGD), is the most frequent among hepatic porphyrias. Clinical expression is highly variable and ~ 90 % of AIP heterozygotes remain asymptomatic throughout life. During systematic genetic analysis of the AIP patients diagnosed in the Czech and Slovak Republics, we found a special case of AIP. In a 15-year-old boy with abdominal and subsequent neurological symptomatology, we identified *de novo* mutation 966insA within the PBGD gene leading to a stop codon after 36 completely different amino acids compared to the wt-sequence. To establish the effects of this mutation on the protein structure, we expressed mutant constructs with described mutation in *E. coli* and analyzed their biochemical and enzymatic properties. Moreover, computer-assisted protein structure prediction was performed.

Key words

Heme • Acute intermittent porphyria • Porphobilinogen deaminase • Porphyria

Introduction

Heme biosynthesis is one of the most conserved pathways in all living cells through many species (Mauzerall 1998). There are eight enzymes involved and defects in seven of them lead to porphyria.

The porphyrias are group of mostly inherited disorders in which specific spectrum of accumulated and excreted porphyrins and heme precursors are associated with characteristic clinical features. Porphyrias can be divided into either hepatic or erythropoietic forms with respect to their anatomical origin and into acute or cutaneus forms with respect to their clinical presentation. Four of them are described as acute hepatic porphyrias, which share possible precipitation of the acute attacks. Acute attacks are manifested by a wide variety of clinical

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features, including autonomous, central, motor or sensory symptoms, but the most common clinical presentation is the abdominal pain caused by the neurovisceral crises. An acute attack can be provoked by porphyrinogenic drugs such as barbiturates and sulfonamides, determining acute porphyria to be pharmacogenetic (Tschudy *et al.* 1975). Other known precipitants are various factors, including hormonal changes, alcohol, stress, infection and caloric deprivation; attacks are more common in women. Acute attacks can be potentially life-threatening and death may result from the respiratory paralysis (Goldberg 1959).

Acute intermittent porphyria (AIP) is an autosomal dominant inborn disorder (Meyer and Schmid 1978) and is the most frequent among hepatic porphyrias (Grandchamp 1998). The prevalence of symptomatic disease is only 1-2 per 100 000 (Badminton and Elder 2002). Clinical expression is highly variable and ~90 % of AIP heterozygotes remain asymptomatic throughout life (Petrides 1998). Individual gene carriers differ from each other in biochemical and clinical manner. Despite increased understanding of AIP, the mechanism causing symptoms of this disease remains unclear. There are many theories; two of them are on the hot spot of the current investigation – porphyrin precursor neurotoxicity (Brennan *et al.* *1980,* Solis *et al.* 2004) and heme deficiency (Yeung Laiwah *et al.* 1987, Kupferschmidt *et al.* 1995, Litman and Correia 1985). Existence of the PBGD knock-out mouse, exhibiting many of the AIP symptoms (Lindberg *et al.* 1996), can greatly improve understanding of the mechanism of the pathophysiology of this disorder.

AIP results from the half-normal activity of the porphobilinogen deaminase enzyme (PBGD, EC 4.3.1.8) (Strand 1970, Meyer *et al.* 1972, Strand *et al.* 1972). PBGD is determined by a single gene located on the chromosome 11 (Meisler *et al.* 1980); assigned the locus to the long arm in the segment 11q23-qter (Wang *et al.* 1981). PBGD gene contains 15 exons and 14 introns and spans approximately 10 kb of DNA (Chretien *et al.* 1988). It has been shown that two distinct promoters generate the housekeeping and erythroid-specific transcripts by alternative splicing (Grandchamp *et al.* 1987, Chretien *et al.* 1988). The housekeeping HMBS transcript contains exons 1 and 3-15, the erythroid HMBS transcript is encoded by exons 2-15 (Chen *et al.* 1994).
PBGD is the third enzyme of the heme biosynthetic pathway that catalyzes the step-wise head to tail condensation of four porphobilinogen molecules into the linear tetrapyrrole hydroxymethylbilane. Two PBGD protein isoforms differ approximately by 2 kDa (40 and 42 kDa) (Grandchamp *et al.* 1987), the housekeeping form consists of 361 amino acids and the erythroid variant of 344 amino acids. The housekeeping isoform contains additional 17 amino acid residues at the N-terminus, but the function of these extra amino acids is not yet known (Grandchamp *et al.* 1987). The three-dimensional structure of PBGD has been defined in *E. coli* by X-ray analysis (Louie *et al.* 1992). The monomeric protein is organized in three domains, approximately equal in size (Louie *et al.* 1996). In the active site, there is dipyrromethane co-factor (Jordan and Warren 1987). The three-dimensional structure of the PBGD from *E. coli* indicates strong conservation of the protein and many amino acid similarities with the human PBGD (Brownlie *et al.* 1994).

To date, around 300 mutations in the PBGD gene have been identified. In this study, we report *de novo* mutation 966insA found within the PBGD gene in a Slovak AIP patient. To establish the effect of this mutation on the protein structure and its function, we expressed mutant protein with the desired mutation in *E. coli* and analyzed its biochemical and enzymatic properties. Moreover, computer-assisted protein structure prediction was performed.

Methods

Patient

A 15-year-old boy was hospitalized and diagnosed as having AIP after his first attack. The diagnosis of AIP was made on the basis of clinical features typical for AIP, including acute abdominal pain, cognition failure, hypertension, tachycardia and subsequent neurological symptomatology. Symptoms were in combination with highly increased urinary ALA and PBG and other porphyrin precursors and hyponatremia (coproporphyrins 1240.5 μmol/l (N: 38-153), uroporphyrins 3298,3 μmol/l (N: 6-24), ALA 1178.2 μmol/l (N :< 42), PBG 331.2 μmol/l (N :< 9). Five other family members out of two generations were without any clinical symptoms. Urinary porphyrin measurements were performed in the family, all subjects except the proband were within the normal concentration range.

Isolation and amplification of DNA

Genomic DNA was extracted from the peripheral blood leucocytes anticoagulated with EDTA

according to the standard protocol. Coding sequences of all exons 1-15 with flanking exon/intron boundaries were amplified. The PCR/DGGE primers were designed as previously reported (Puy *et al.* 1997). The PCR reactions of exon 1-15 were amplified in the total volume of 50 µl including 1x Plain PP Master Mix (150mM Tris-HCl, pH 8.8, 40mM $(NH_4)_2SO_4$, 0.02 % Tween 20, 5mM $MgCl_2$, 400µM dATP, 400µM dCTP, 400µM dGTP, 400µM dTTP, 100 U/ml Taq DNA polymerase; Top-Bio, Ltd.) and 0.4mM of each primer. Thermal cycling conditions: initial denaturation was performed at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55.8 or 59,3 or 62.9 °C for 30 sec (62.9 °C for exons 1 and 11, 59.3 °C for exons 3 and 5/6, 55.8 °C for the rest of the exons) and elongation at 72 °C for 40 sec, followed by final step 72 °C for 5 min, 95 °C for 5 min and 72 °C for 5 min.

DGGE analysis

Fourteen different PCR products were designed to cover the whole encoding sequence, including ~ 50 bp upstream and downstream of each exon/intron boundary of the PBGD gene (Puy *et al.* 1997). Complete DGGE setup was optimized according to previously reported method (Myers *et al.* 1987). DGGE was performed on linearly increasing denaturing gradient polyacrylamide gels of 35 – 90 % of denaturant (7M urea and 40 % deionized formamide, Bio-Rad). PCR products were analyzed using DCode™ (Bio-Rad). Gels had been run at 60 °C, 150 V for 3.5 hours in 1 x TAE buffer (40mM Tris-HCl, 20mM acetic acid, 1mM EDTA).

DNA sequencing and RFLP

The PCR-amplified double-stranded DNA products were purified from agarose gel using a QIAquick gel extraction kit (Qiagen). Exon 15 was sequenced in both directions on automatic sequencer ABI PRISM 3100/3100-Avant Genetic analyzer (Applied Biosystems) using the ABI PRISM BigDye terminator v 3.1 (Applied Biosystems). Identified mutation in exon 15 caused loss of the restriction site, as confirmed by RFLP. Restriction enzyme digestion was performed using *Mae III* (Roche Diagnostic) according to the manufacturer's instructions.

Plasmid construction and mutagenesis

Total RNA was extracted from the peripheral leukocytes isolated from EDTA-anticoagulated whole venous blood. cDNA sequence was obtained by reverse transcription (RT-PCR) of the total RNA using oligo(dT) (GE Healthcare) as primer in the first step. Specific cDNA for PBGD with restriction sites *BamH I* and *Xho I* was amplified using specific primers in the second step: cDNA BamH I Fw: ata tgg atc cat gtc tgg taa cgg, cDNA *Xho I* Rev: tat act cga gtt aat ggg cat cgt taa. Human cDNA for PBGD was ligated into the pGEX-4T-1 expression vector (GE Healthcare) and transformed into *E. coli BL21 (DE3)* (Stratagene). Plasmid DNA was amplified and isolated using the QIAprep Spin Miniprep Kit (Qiagen). Site-directed mutagenesis of the mutation 966insA was performed with the following mutagenic primers: Fw: 5′gca tca ctg ctc gta aac att cca cga ggg c 3 and Rev: 5′gcc ctc gtg gaa tgt tta cga gca gtg atg c 3′, using QuikChange® Site-directed Mutagenesis Kit (Stratagene). The results of the mutagenesis were confirmed by sequencing.

Protein expression

All the proteins were expressed as GST-fusion proteins. *BL21* cells were grown at 37 °C in TB medium containing ampicillin (100 µg/ml, Sigma). 0.05 % of the volume of the overnight-shaked culture was used to inoculate the growth medium. The cells were induced by IPTG (the final concentration of 0.5mM, Sigma) when reached OD at 600 nm between 0.4-0.6. Bacterial culture was grown under aerobic conditions for 4 hours at 30 °C. Bacterial cells were harvested by centrifugation at 4 °C for 10 min at 6000g.

Protein purification

All purification steps were carried out at 4 °C. Washed cells were resuspended in the lysis buffer: PBS (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1,8mM KH_2PO_4, pH 7.3), protease inhibitor cocktail (Sigma) and Triton X-100 (0.5 % v/v, Sigma). The cells were lysed by lysozyme (1 mg/ml, ICN Biomedicals) on ice with gentle shaking for 1 hour. The lysate was sonicated 5 x 3 minutes with a 3 min pause in each cycle. Sonicated cells were centrifuged at 4 °C, 33.000g for 30 min. The supernatant was loaded onto the Glutathione Sepharose 4B column (GE Healthcare) and washed three times using the wash buffer (20mM Tris-HCl, 100mM NaCl, 1mM EDTA, 0.5 % Nonidet P-40, pH 8.2). Proteins were eluted in the freshly prepared 50mM Tris-HCl, pH 8.5 buffer with 20 mM gluthatione (Sigma). Thrombin digest was performed by gentle shaking of protein mixed with thrombin (ICN Biomedicals) in concentration of 20 U thrombin / 1mg of the protein overnight at 20 °C.

Glycerol was added to the final concentration of 20 % and the aliquots were frozen and stored at – 80 °C. All results of the protein purification and digestion were confirmed by the SDS-PAGE (Laemmli 1970).

PBGD enzymatic assay

The PBGD activity assay was optimized according to the previously described methods (Erlandsen *et al.* 2000, Brons-Poulsen *et al.* 2005). 1 and 2.5 µg of the protein were diluted by the incubation buffer (50mmol/l Tris, 0.1 % BSA, 0.1 % Triton, pH 8.2) to the final volume of 360 µl. After pre-incubation at 37 °C for 3 min, 40 µi of 1mM PBG (ICN Biomedicals) was added and samples were incubated in the dark at 37 °C for exactly 1 hour. The reaction was stopped by adding 400 µi of 25 % TCA, (Fluka). Samples were exposed to fotooxidation for 30 min under UV light (350 nm) and then centrifuged for 10 min at 1.500g. To determine enzymatic activity, the fluorescence intensity was measured using Perkin Elmer LS 55 spectrofluorometer immediately thereafter. Uroporphyrin I (URO I, ICN Biomedicals) was used as the standard and 12.5 % TCA as a blank. The exact concentration was determined at 22 °C by measuring absorbance at 405 nm and calculated as A_{405}/ε (ε – 505 x 10^3 l cm^{-1} $mol^{-1)}$. Standard curve in the linear range of fluorescence emission intensity and the concentration of URO I in 12.5 % TCA were created. All measurements were performed in triplicates and the negative control was included. The spectrofluorometer wavelength settings were set up at the maximum excitation of 405 nm and the maximum emission of 599 nm for URO I.

Computer-based structural modeling

For the prediction of the possible impact of the mutation on the protein structure and function, we used the correlations between a three-dimensional structure of human and bacterial *E. coli* (Brownlie *et al.* 1994, Louie *et al.* 1996) and human protein. The amino acid sequence of PBGD corresponding to proband was used to construct a homology model with *E. coli* PBGD as template. We also constructed a homology model with wild-type human PBGD. The resulting models were refined using CNS program to remove bad contacts and inspected using the program O.

Results

The proband was diagnosed at the age of nearly 15 after he was hospitalized with an acute attack (abdominal and severe neurological symptomatology) after surgery for appendicitis. The patient was the only member of the family known to have AIP symptoms (Fig. 1). No clinical and molecular patterns were found after biochemical and molecular screening of his family. Nonpaternity was excluded by DNA microsatellite analysis. Based on these results, we suggested that 966insA was a *de novo* mutation.

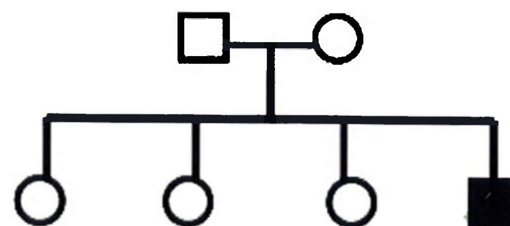

Fig. 1. Pedigree of the Slovak acute intermittent porphyria patient. Affected individual is indicated by solid symbol.

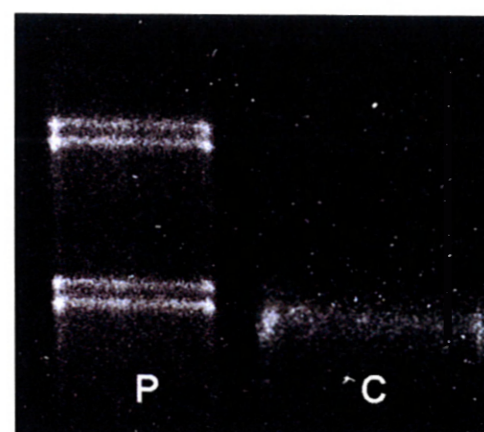

Fig. 2. Denaturing gradient gel electrophoresis (DGGE) based mutation screening of the porphobilinogen deaminase gene. Lane **P** – patient, lane **C** – control. The 35 – 90 % DGGE gels had been run at 60 °C, 150V for 3.5 hours in 1 x TAE buffer. In case of the heterozygous mutated carrier, there occurs a specific exhibition of three- or four-band pattern. Two lower bands represent the normal and mutated homoduplexes and, the upper ones corresponding to the two types of the normal/mutated heteroduplexes. Only in one fragment of exon 15 did we detect an abnormal four-band pattern suggesting a DNA variation.

All encoding sequences and exon/intron boundaries of the proband PBGD gene were screened for mutations. The PCR products were subjected to DGGE analysis. The sample with abnormal pattern suggesting certain sequence variation was detected (Fig. 2). This sequence variation was subsequently confirmed by direct sequencing and was further characterized as an insertional point mutation (Fig. 3). Mutation 966insA was located in the exon 15 and leading to formation of STOP

codon after 36 completely different amino acids compared to the original sequence. To further confirm the mutation, we proceeded to RFLP. Pursuant to which the identified mutation caused loss of the restriction endonuclease recognition site. The 317-kb fragment of genomic DNA containing exon 15 and flanking sequence was digested with the restriction endonuclease *Mae III*. In case of the normal homozygous allele control, there were two bands of 80 bp and 197 bp. In case of the mutant allele, three bands of 80 bp and 197 bp and, further, undigested 317 bp fragments appeared thus confirming the existence of the mutation.

To investigate the impact of the mutation on the protein structure and to study further functional consequences, we decided to purify protein expressed from mutated gene in the procaryotic system. We isolated mRNA from the peripheral leukocytes, produced cDNA of PBGD by reverse transcription and amplified a fragment containing the complete encoding region. This fragment was inserted into prokaryotic expression vector. The cells of recombinant strain *E. coli* BL21 were transformed by this construct. By side-directed mutagenesis we introduced desired mutation into the construct. The accuracy and absence of additional base changes were confirmed by direct DNA sequencing.

To determine optimal growth conditions for high-level production of mutant PBGD, we proposed three different temperatures levels and expression durations. The decrease of the temperature during protein expression did not show any impact on the protein stability or solubility. As expected, in the case of the normal enzyme the purified protein product showed single homogeneous band on SDS-PAGE. In the case of mutant protein several bands appeared on SDS-PAGE suggesting low protein stability (Fig. 4). The amounts of proteins displayed in each lane of the SDS-PAGE gel were adjusted to be identical (the same volume of affinity beads as well as elution buffer) for the normal and mutant enzyme to reflect the potential of mutant protein expression and its solubility. PBGD enzymes of the normal as well as of the mutant were similar in size having approximately Mr 68 kDa with GST-tag and Mr 42 kDa without GST-tag.

Specific activities were estimated to be 1677.9 ± 75 U/mg for the normal PBGD (U/mg = nmol URO I/hr/mg of protein), 1320.1 ± 55 U/mg for the normal PBGD expressed in fusion with GST, 2.763 ± 0.136 U/mg for the mutant PBGD and 2.334 ± 0.129 U/mg for the mutant PBGD expressed in fusion with GST, when

Fig. 3. Mutation detected in the porphobilinogen deaminase gene of a Slovak acute intermittent porphyria patient by sequencing analysis. The mutation 966insA was localized in the exon 15 and this single-base insertion was further identified as the causative mutation.

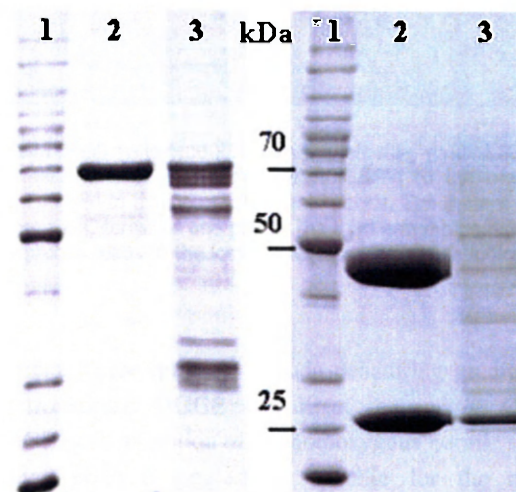

Fig. 4. SDS/PAGE analysis of the porphobilinogen deaminase. Left: GST-fusion purified enzymes. Right: Thrombin digested purified enzymes. Lane 1 – Marker, lane 2 – wild-type protein, lane 3 – mutated protein. PBGD enzymes of the normal as well as of the mutant were similar in size having approximately Mr 68 kDa with GST-tag and Mr 42 kDa without GST-tag. The amount of protein displayed in each lane of the SDS-PAGE gel was adjusted to be identical (the same volume of affinity beads as well as elution buffer) for the normal and mutant enzyme to reflect the potential of mutant protein expression and its solubility.

measured at pH 8.2. The purified mutant enzyme had a relative activity 0.18 % of the average level expressed by the normal allele. We concluded that there was a significant difference in the specific and relative activity between the thrombin digested protein and the fusion protein. Fusion protein exhibited approximately 22 % higher activity than the thrombin digested enzyme.

Using the computer-assisted structure prediction, we designed the mutant protein structure. Due to the truction, two helixes $\alpha 2_3$ and $\alpha 3_3$ of the domain 3 were missing at the mutant protein in comparison with the normal protein (Fig. 5).

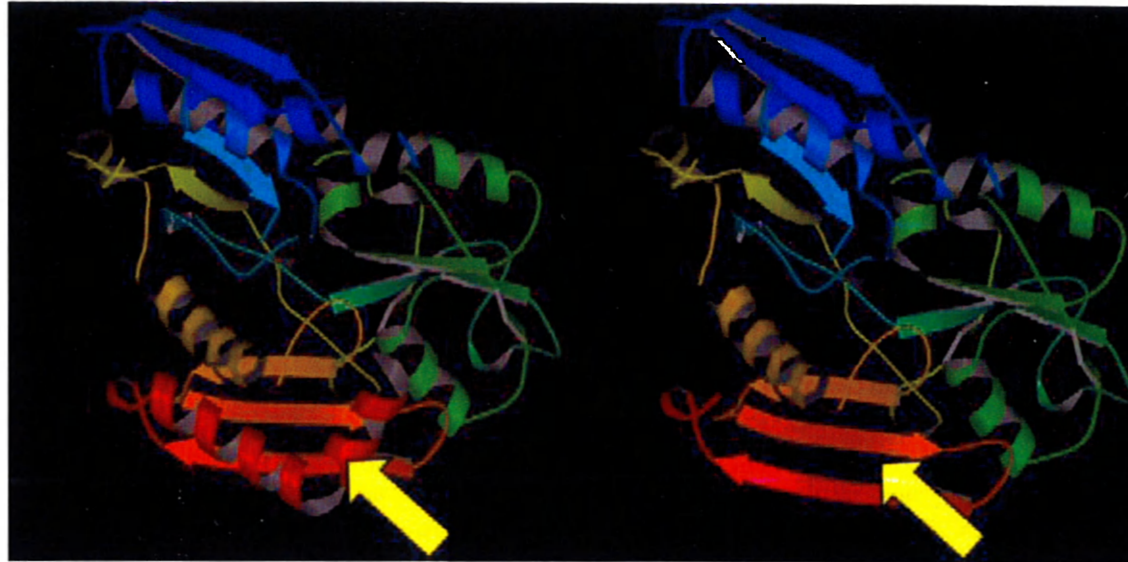

Fig. 5. Computer-based con-structed homology model with wild-type human PBGD (wild-type PBGD on the left side, mutant PBGD on the right side). This structural model shows small insertion 966insA, which resulted in the frameshift leading to the premature truncation. The trun-cated mutant protein consists of 357 amino acids as opposed to the normal 361 amino acids. The mutant protein includes 36 amino acids different from the normal, lacks 4 amino acids from the C-terminus and part of the third enzyme domain takes up a different shape formation. The premature termination of the mutant protein leads to the loss of protective helixes as indicated by yellow arrows.

Discussion

The identification and characterization of the novel mutations within PBGD gene of the newly diagnosed AIP patients emphasize insight into the molecular heterogeneity of AIP. Accurate molecular diagnosis and detection of asymptomatic carriers in affected families helps them prevent potential precipitating factors. Investigation of the effects of the present mutation on the protein structure and its function provide further understanding of the molecular basis and cooperation of the molecules in the system.

PBGD is the third enzyme of the heme biosynthetic pathway and catalyzes the step-wise head to tail condensation of four porphobilinogen molecules into the linear tetrapyrrole hydroxymethylbilane. In AIP patients, the half-normal activity of PBGD results in the accumulation of high amount of porphyrin precursors as ALA and PBG (Strand 1970, Meyer *et al.* 1972, Strand *et al.* 1972). The accumulation leads to the potentially life-threatening state of acute attacks.

A great step forward was the isolation and characterization of the human PBGD cDNA and genomic sequence (Grandchamp 1987, Yoo *et al.* 1993). This resulted in a rapid progress in molecular diagnosis enabling us to employ several DNA variation screening methods for the accurate identification of mutation AIP carriers. These methods include denaturing gradient gel electrophoresis DGGE and direct sequencing. On the DGGE gels, migration of the homozygous normal subject alleles gives a single-band specific for the normal homoduplex. In case of the heterozygous mutated carrier, there occurs a specific exhibition of three- or four-band pattern. DGGE detected sequence variations might be either disease causing the mutation or a neutral polymorphism. DGGE of the entire coding sequence including exons and exon/intron boundaries was performed. Only in one fragment of exon 15, we detected an abnormal four-band pattern suggesting a DNA variation. We proceeded to sequencing this fragment in both directions and we identified a single-base insertion 966insA as the causative mutation. In exon 15, together with exons 10 and 12, high mutation frequency within PBGD gene in AIP patients was detected (Gu *et al.* 1992, Gu *et al.* 1993a, Mgone *et al.* 1993, Grandchamp *et al.* 1996).

The deciphering of the structure of the PBGD enzyme had the same impact on the protein level. The three-dimensional structure of PBGD has been defined in *E. coli* by X-ray analysis (Louie *et al.* 1992). It has been shown that this monomeric protein is organized in three domains, approximately equal in size (Louie *et al.* 1996). The x-ray structure and results from the site-directed mutagenesis provided evidence for a single catalytic site

(Louie *et al.* 1992). In the active site, there is a dipyrromethane co-factor (Jordan and Warren 1987). The active site is located between domains 1 and 2 and, the dipyrromethane cofactor is covalently linked to a flexible loop of side-chain of cysteine 242 residue (Hart *et al* 1988, Jordan and Warren 1987). Other important interactions in the process of tetrapyrol formation are hydrogen-bonds and salt-bridges that are formed between its acetate and propionate side-groups and the polypeptide chain, which are supposed to anchor the cofactor within the active site cleft (Louie *et al.* 1996). Secondary structure of domains 1 and 2 is a modified doubly wound parallel beta-sheet which has a similar overall topology. Domain 3 is an open-faced three-stranded antiparallel beta-sheet, with one face covered by three alpha-helices (Louie *et al.* 1996, Lambert *et al.* 1994). The three-dimensional structure of the PBGD from *E. coli* shows approximately 45 % amino acid sequence identity and nearly 80 % of the amino acids are structurally related to human PBGD (Brownlie *et al.* 1994). Small insertion 966insA resulted in the frameshift leading to the incorporation of 36 completely different residues and to premature truncation. Usually, the truncation would lead to an unstable and inactive protein. Truncated proteins are likely to be rapidly degradated by proteosome. The truncated mutant protein consists of 357 amino acids as opposed to the normal 361 amino acids. The mutant protein includes 36 amino acids different from the normal, lacks 4 amino acids from the C-terminus and, part of the third enzyme domain takes up a different shape formation. The mutation is located in the $\beta 3_3$ sheet; two helixes $\alpha 2_3$ and $\alpha 3_3$ of the domain 3 are completely missing compared to the normal protein. In the wild-type protein, the C-terminal helixes protect the beta-strands from being exposed to solvent. This would be expected to expose the beta-strand core that could make the mutant protein prone to aggregation. In agreement with this prediction, stability of the mutant PBGD was severely impaired as we detected from SDS-PAGE results. During protein expression, high precipitation occurred, which decreased the amount of the soluble recombinant protein.

We presumed that the premature truncation would cause a loss of enzymatic activity. We found that there was a significant difference in the specific and relative activity between the thrombin digested and the fusion protein. Fusion protein exhibited approximately 22 % higher activity than the thrombin digested enzyme. This observation was in agreement with another study (Brons-Poulsen *et al* 2005) and suggests that due to GST tag presence in this case, there is no correlation between steric barrier and lower substrate accessibility. The residual activity was extremely low, about 0.18 % of the normal protein activity, corresponding to the massive breakdown of the protein; therefore, kinetic studies of this mutant were not suitable.

Currently, around 300 mutations in the PBGD gene are known. Such known mutations include one promoter region mutation (Whatley *et al.* 2000), missense, nonsense, splicing and frame-shift mutations and in-frame deletion and insertion as well. Mutations are equally distributed among the PBGD gene and no prevalent site for mutation has been identified. In Czech and Slovak patients, 7 different mutations: G24S, R26C, R26H, 158insA, G111R, IVS 12 771+1 and V267M has been found (Rosipal *et al.* 1997, Kauppinen *et al.* 1995, Gu *et al.* 1993b, Llewellyn *et al.* 1993, Puy *et al.* 1997). 966insA is the first mutation found in the exon 15 in this population and indicates that AIP is a heterogeneous disease at the molecular level.

In summary, one *de novo* mutation was found in the Slavic population. Due to truncated protein sequence with an abnormal C-terminus, this small insertion mutation leads to an almost complete loss of the enzymatic function and decreases the stability of the protein structure. The probability of the life-threatening porphyric attack in AIP is a significant personal burden and poses a challenge for counseling and medical management. Introduction of molecular biology techniques to diagnostics increases our chances of more effective treatment of AIP.

Acknowledgements

This research was supported by a grant GA UK 10/2004 from the Grant Agency of the Charles University, GA CR 303/03/H065 from the Grant Agency of the Czech Republic, and grant MSM 0021620806.

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PUBLICATION B

A new mutation within the porphobilinogen deaminase gene leading to a truncated protein as a cause of acute intermittent porphyria in an extended Indian family *(Flachsová E et al., 2007)*

Original Article

A New Mutation within the Porphobilinogen Deaminase Gene Leading to a Truncated Protein as a Cause of Acute Intermittent Porphyria in an Extended Indian Family

(acute intermittent porphyria / OMIM 176000 / porphobilinogen deaminase / exon 15 / 973insG mutation)

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Abstract. Based on Internet search, we were contacted by a 50-year-old man suffering from severe abdominal pain. Acute hepatic porphyria was considered from positive Watson-Schwartz test. He, not being a health professional, searched for centres with ability to do molecular diagnosis and for information about therapeutic possibilities. He asked his physician for haem-arginate (Normosang, Orphan Europe, Paris) treatment, arranged sending his blood to our laboratory and mediated genetic counselling for him and his family. Molecular analyses of the *PBGD* gene revealed a novel mutation in exon 15, the 973insG. Subsequently, genetic analysis was performed in 18 members of the proband's extensive family. In 12 members of the family, the same mutation was found. The mutation, which consisted of one nucleotide insertion, resulted in addition of four different amino acids leading to a protein that is prematurely truncated by the stop codon. The effect of this mutation was investigated by expression of the wild-type and mutated PBGD in a prokaryotic expression system. The mutation resulted in instability of the protein and loss of enzymatic function. The increasing access to a number of disease- and symptom-oriented web pages presents a new and unusual venue for gaining knowledge and enabling self-diagnosis and self-help. It is, therefore, important that disease-oriented Internet pages for public use should be designed with clarity and accurate current knowledge-based background.

Received September 7, 2007. Accepted September 21, 2007.

E. F., D. U., J. Z., and P. M., are supported by grants MSM 0021620806 and 1M6837805002 from the Ministry of Education, Youth and Sports of the Czech Republic. D. U. is also supported by the Grant Agency of Charles University, GAUK 257540 54007.

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Abbreviations: AHP – acute hepatic porphyria, AIP – acute intermittent porphyria, ALA – δ-aminolevulinic acid, DP – Doss porphyria, GST – glutathione synthetase, HC – hereditary coproporphyria, PBG – porphobilinogen, PBGD – porphobilinogen deaminase, VP – variegate porphyria.

Introduction

Haem, an iron-containing tetrapyrrole, is critical for life. It is utilized by numerous proteins involved in basic cellular processes such as respiration (cytochrome oxidase), oxygen transport (haemoglobin), detoxification of foreign compounds (cytochromes P450), vascular homeostasis (nitric oxide synthases), cellular signalling (guanylate cyclase), cell death (cytochrome *c*), and a whole host of other functions (Granick and Beale, 1978; Ponka, 1999; Tang et al., 2003). Haem synthesis is a well-coupled complex of cellular machinery comprising eight enzymes (Meyer and Schmidt, 1978; Anderson et al., 2001; Kauppinen, 2005). The pathway is evolutionarily conserved from bacteria to humans. In mammals, the pathway is localized in cytosolic as well as in mitochondrial compartments (Fig. 1). Haem and porphyrins in general have unique structural features (Kral et al., 2006). The biological functions of haem are ensured by its metallocomplex with iron. It is, therefore, evident that a pathway of such biological significance will be regulated at many levels.

Porphyrias are caused by decreased activities of the enzymes in the haem biosynthetic pathway, with the exception of the first one in which a defect causes anaemia (Meyer and Schmidt, 1978; Anderson et al., 2001)

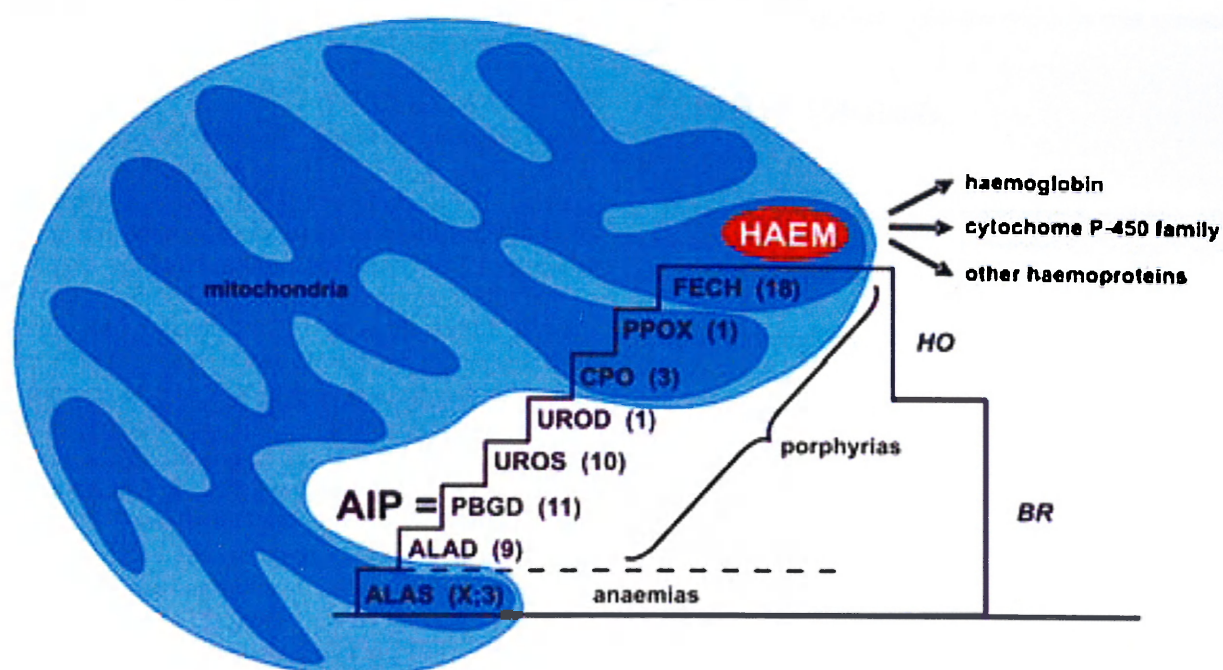

Fig. 1. Haem synthetic and degradation metabolic pathway. Enzymes involved are in Abbreviations and parenthetical numbers indicate chromosomal localization of the gene involved. ALAS: δ-aminolevulinic acid synthase; ALAD: δ-aminolevulinic acid dehydratase; PBGD: porphobilinogen deaminase; UROS: uroporphyrinogen III cosynthetase; UROD: uroporphyrinogen decarboxylase; CPO: coproporphyrinogen oxidase; PPOX: protoporphyrinogen oxidase; FECH: ferrochelatase; *Haem degradation enzymes:* HO – haem oxygenase; BR – biliverdin reductase. AIP: acute intermittent porphyria (defect in PBGD).

(Fig. 1). Each porphyria is characterized by a typical spectrum of accumulated and excreted porphyrins and their precursors, porphobilinogen (PBG) and δ-aminolevulinic acid (ALA). Acute porphyrias (acute intermittent porphyria, AIP; variegate porphyria, VP; hereditary coproporphyria, HC; Doss porphyria, DP) are all considered to be hepatic (Grandchamp, 1998; Martasek, 1998; Kauppinen, 2005). They all share possible precipitation of acute attacks. This might be a life-threatening medical emergency with neurovisceral symptomatology, believed to be caused by the administration of drugs (archetypal – barbiturates), which are metabolized by cytochrome P450 monooxygenases (Meyer and Schmidt, 1978; Yeung et al., 1987; Anderson et al., 2001; Kauppinen, 2005). This increased need in haem production may trigger a porphyric attack by a mechanism which is not yet understood. Other triggers for porphyric attack include a profound decrease in caloric intake, infections, and hormonal influences. The main clinical symptoms of an acute porphyric attack are (from the more frequent to the less frequent): abdominal pain, tachycardia, peripheral motor neuropathy, constipation, nausea, vomiting, mental changes, hypertension, sensory neuropathy, convulsion (Meyer and Schmidt, 1978; Yeung et al., 1987; Anderson et al., 2001). In case of VP and HC, skin symptoms may be present as well. Diagnosis in clinically manifested cases is based on typical biochemical findings (dark urine in all acute attacks, especially if exposed to light; AIP: increased urinary PBG and ALA, no elevation of faecal porphyrins; VP: increased faecal proto- and coproporphyrins, fluorescence plasma emission maximum 627 nm (exc. 400 nm); HC: high increase of faecal and urinary coproporphyrin, red fluorescence of faeces in UV light; DP: extremely rare, increased ALA urinary excretion) (Meyer and Schmidt, 1978; Grandchamp et al., 1996; Kauppinen, 2005). Once the mutation is found in responsible genes, a genetic-based diagnosis can be offered to all family members. Detection of the mutation will identify members with latent porphyria, and unaffected family members can be distinguished with assurance. An acute attack of porphyria in most cases requires hospital admission. High glucose intake (oral/intravenous) and haem-arginate are the principal therapeutic remedies (Kauppinen, 2005). An important aspect of therapy of acute hepatic porphyria (AHP) is prevention. Drugs known to provoke acute attacks should be avoided (for a complete list see web sites: http://www.porphyria-europe.com/03-drugs/how-to-use-info.asp) (Deybach et al., 2006)

The study of porphyria, as with other metabolic disorders, deals with gaining a deeper understanding of how defects in the enzymes that generate porphyrins

cause disease. Acute intermittent porphyria (AIP; OMIM 176000) is a low-penetrant autosomal dominant disorder caused by reduced activity (~50%) of porphobilinogen deaminase (PBGD; EC 4.3.1.8), the third enzyme of the haem synthetic pathway (Goldberg, 1959; Strand et al., 1970; Meyer et al., 1972; Meyer and Schmidt, 1978; Puy et al., 1997). This enzyme, also known as hydroxymethylbilane synthase, catalyses the head-to-tail condensation of four molecules of porphobilinogen to form hydroxymethylbilane (Jordan and Warren, 1987; Hart et al., 1988). The human *PBGD* gene has been cloned and his organization characterized. The *PBGD* gene is localized in 11q23.3 region (Wang et al., 1981; Namba, et al., 1991). It is split into 15 exons spread over 10 kb with a single open reading frame of 1038 bp (Raich et al., 1986; Yoo, et al., 1993). Two distinct promoters are located in the 5' flanking region and in intron 1, respectively, and generate housekeeping (exon 1 and 3–15) and erythroid-specific (exon 2–15) transcripts by alternative splicing of exon 1 and 2 (Grandchamp et al., 1987; Chretien et al., 1988; Chen et al., 1994).

AIP is the most frequent acute hepatic porphyria (Puy et al., 1997; Grandchamp, 1998). The disease is characterized by intermittent acute porphyric attacks with abdominal pain, hypertension, tachycardia, neurologic and psychiatric manifestation as previously mentioned. In difference to other hepatic porphyrias, skin photosensitivity is not present (Grandchamp, 1998; Kauppinen, 2005). To date, more than 300 mutations within the *PBGD* gene have been identified (Hrdinka et al., 2006).

In this study, we identify a new, previously non-characterized mutation, the 973insG, found in exon 15 of the *PBGD* gene in an extended Indian family. To establish the effect of this mutation on the protein function, we expressed the mutant protein in the prokaryotic expression system and analysed its biochemical and enzymatic properties.

Material and Methods

Patients

Based on Internet search, we were contacted by a 50-year-old proband suffering from severe abdominal pain accompanied with dark urine; acute hepatic porphyria was considered from positive Watson-Schwartz test. He, not being a health professional, searched for more information about porphyrias and centres with ability to do molecular diagnosis and give him information about therapeutic possibilities. He asked his physician for haem-arginate (Normosang, Orphan Europe, Paris), a treatment which achieved excellent clinical effects with prompt relief of abdominal pain. He subsequently arranged sending blood or genomic DNA via clinical geneticist (I.V., co-author of this paper, he also provided genetic counselling) from 18 members of his family to our laboratory (Fig. 2). We, therefore, analysed DNA from this extensive Indian family from Nepal.

DNA analysis

Genomic DNA was extracted from peripheral blood samples anticoagulated with EDTA according to a standard protocol. All 15 exons of the *PBGD* gene with surrounding exon/intron boundaries (more than 60 bp of their flanking regions) were amplified by PCR using specific primers (Puy et al., 1997; Genbank accession numbers, *HMBS* gene, M95623; HMBS cDNA, NM000190). Consequently, fragments of amplified DNA were analysed by denaturing gradient gel electrophoresis (Myers et al., 1987). Sequence analysis was performed in automatic sequencer ABI PRISM 3100/3100-Avant Genetic analyser (Applied Biosystems, Foster City, CA).

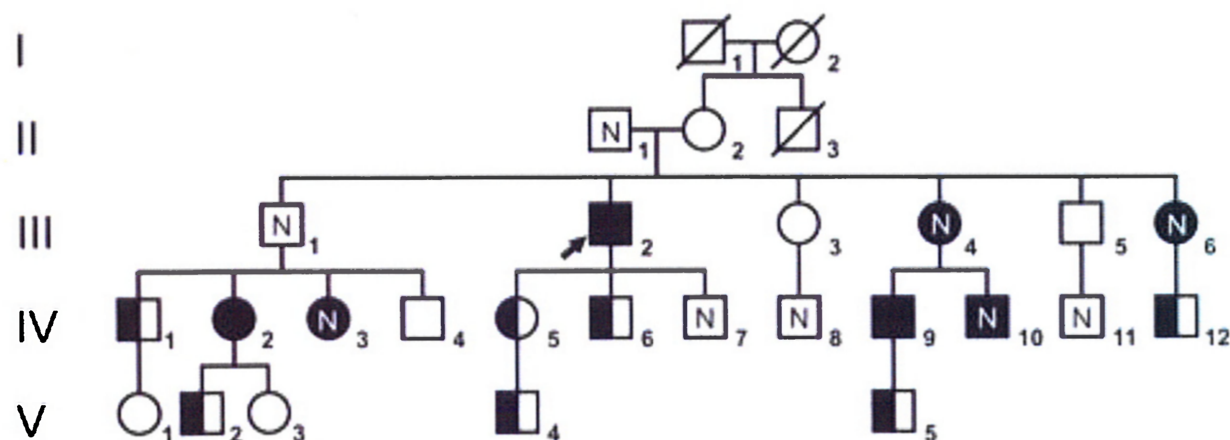

Fig. 2. Pedigree of the Indian family with acute intermittent porphyria (AIP). Proband is indicated by an arrow. Solid symbol represents a patient with clinically manifested disease. Half-solid symbols represent members of the family who carry the mutation but are asymptomatic. N – not tested

Plasmid construction and mutagenesis

Total RNA was extracted from fresh leukocytes isolated from EDTA-anticoagulated whole venous blood. One microgram of total RNA was reverse transcribed using 200 U M-MLV SuperScript III (Invitrogen, Carlsbad, CA) in the presence of oligo(dT) (Invitrogen) as a primer in the first step. The complementary cDNA spanning exons 1 and 3–15 was PCR-amplified using primers with specific restriction sites in the second step (cDNA *Bam*HI Fw: 5´ata tgg atc cat gtc tgg taa cgg 3´, cDNA *Xho*I Rev: 5´tat act cga gtt aat ggg cat cgt taa 3´). The human PBGD cDNA was ligated into the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech, Piscataway, NJ) and then transformed into *E. coli* BL21 (DE3) (Stratagene). Plasmid DNA was amplified and isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Site-directed mutagenesis of the 973insG mutation was performed with the following mutagenic primers: Fw: 5´cac tgc tcg taa cat tcc agc gag ggc ccc 3´, Rev 5´ggg gcc ctc gct gga atg tta cga gca gtg 3´ using QuikChange® Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The PCR products were subsequently submitted to direct sequencing to confirm the result of mutagenesis.

Protein expression and purification

Mutated PBGD and wild-type PBGD were expressed as glutathione synthase (GST)-fusion proteins in BL21 cells using the optimal growing conditions (TB medium with ampicillin (100 μg/ml), IPTG (0.5 mM), 4 h at 30°C, aerobic conditions). All purification steps were carried out at 4°C. Bacterial cells were lysed using lysis buffer (PBS, pH 7.3) containing lysozyme (1 mg/ml) and Triton X-100 (0.5% v/v, Sigma, St. Louis, MO). The lysate was subjected to sonication thereafter. Cell debris was centrifuged at 4°C, 33,000 *g* for 30 min. Proteins were purified using affinity chromatography on the Glutathione Sepharose 4B column (Amersham Biosciences). GST-tag was cleaved by thrombin (20 U thrombin/1 mg of the protein, ICN Biomedicals, Irvine, CA) overnight at 20°C. All steps of the protein purification and digestion were confirmed by the SDS-PAGE (Laemmli et al., 1970).

PBGD enzymatic assay

The PBGD activity assay was carried out according to previously described methods (Erlandsen et al., 2000; Brons-Poulsen et al., 2005; Ulbrichova et al., 2006). The standard incubation system contained 1 μg of PBGD enzyme, incubation buffer (50 mM Tris-HCl, pH 8.2), and substrate PBG (100 μM, ICN Biomedicals) in a final volume of 400 μl. After pre-incubation at 37°C for 3 min, the reaction was started by substrate addition and incubation was carried out in the dark at 37°C for exactly 1 h. The reaction was stopped by adding TCA (trichloracetic acid, final concentration 12.5%). The uroporphyrinogens formed were photooxidized by exposing them to daylight for 60 min. The precipitated protein was discarded by centrifugation and total fluorescence intensity was measured using a Perkin Elmer LS 55 spectrofluorometer immediately thereafter. Uroporphyrin I (URO I, ICN Biomedicals) was used as the standard and 12.5% TCA as a blank. Protein concentration was determined by the method of Lowry (Lowry et al., 1951) employing bovine serum albumin (BSA) as standard.

Sequence and structure analysis

Sequence alignment was produced with T-coffee software on the server tcoffee@igs (Poirot et al., 2003). The structure was displayed and examined with The Molecular Biology Toolkit, Moreland et al. (2005).

Results and Discussion

The fragments of amplified DNA were analysed in the proband's DNA by denaturing gradient gel electrophoresis. An abnormal pattern in exon 15 was found. Subsequent sequencing analysis showed the insertion of one extra G in position 9205 on genomic DNA, resulting in a shift of the reading frame (Fig. 3). Analysis of the protein sequence indicated that following the mutation, four amino acids were different, leading to a prematurely truncated protein due to a stop codon in which 44 amino acids of the C-terminal of PBGD were missing. This mutation was subsequently found in 12 members of the family in addition to the proband. It is known that clinical expression of AIP is highly variable and a high percentage of AIP heterozygotes remain asymptomatic throughout life (Petrides, 1998). However, knowledge of their heteroallelic nature of gene abnormality might be an extremely important way to prevent devas-

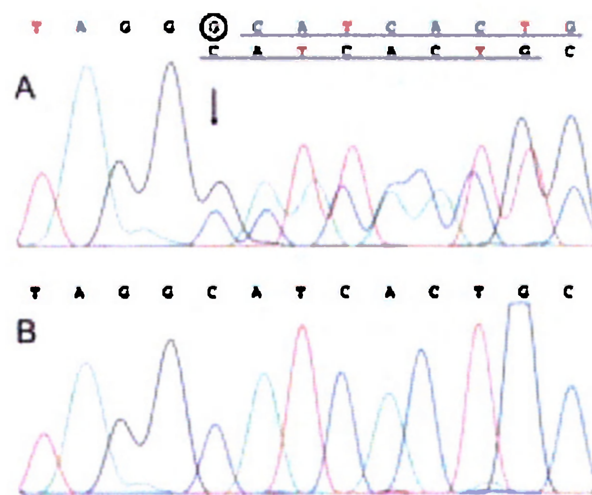

Fig. 3. DNA sequencing analysis in the *PBGD* gene of the proband (A) and (B) control. The mutation 973insG was localized in exon 15. This single-base insertion resulted in a shift in the open reading frame leading to a truncated and inactive protein.

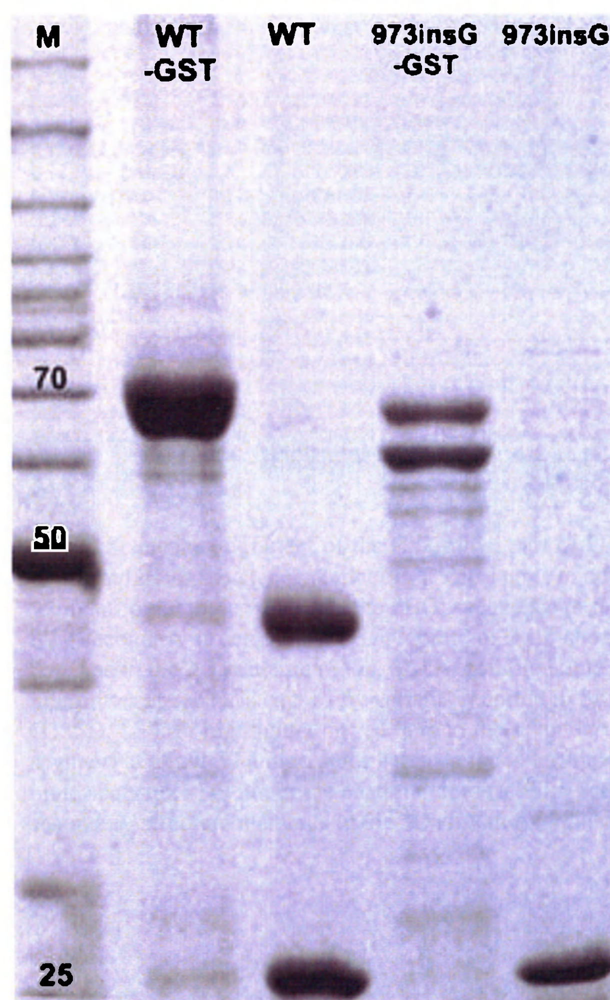

Fig. 4. SDS/PAGE analysis of wild-type and mutated porphobilinogen deaminase (PBGD). From the left: Marker; GST-fusion purified wild-type protein; thrombin-digested purified wild-type protein; GST-fusion purified mutated protein; Thrombin-digested mutated GST-fusion mutated products. Wild-type protein showed approximately Mr 68 kDa with GST-tag and Mr 42 kDa without GST-tag. The amount of protein loaded in each lane of the SDS-PAGE gel was adjusted to be identical (the same volume of affinity beads as well as elution buffer) for wild-type and mutant enzymes.

tating acute porphyria attack by carefully avoiding its precipitating factors. Additionally, two known polymorphisms within the *PBGD* gene were found in the proband: 3581A/G heteroallelic in ivs3 (RFLP *Bsm*AI) and 7064C/A heteroallelic in ivs10 (RFLP *HinfI*).

In vitro expression remains a very useful tool in studying mutations in the *PBGD* gene (Delfau et al., 1990; Chen, et al., 1994; De Siervi, et al., 1999 & 2000; Solis, et al., 1999; Ulbrichova et al., 2006). The wild-type PBGD and the truncated mutated PBGD were simultaneously expressed as GST-fusion proteins (Fig. 4). The resulting SDS-PAGE gel showed distinct band(s) corresponding to the GST-PBGD of wild type and its cleaved products, PBGD and GST with the appropriate size. However, expression of the mutated protein was only possible, to a certain extent, as a GST-fusion protein. Attempts to purify the mutated PBGD, free of the GST, failed. We speculated at this point that this is due to instability of the truncated mutated protein. Results of the corresponding activity measurements are depicted in Table 1. Due to extreme instability of the mutated protein as described above, measurement of the activity was performed in GST-PBGD fusion protein of the mutated form and compared with the GST-PBGD fusion protein of the wild-type form expressed simultaneously under identical conditions. The activity of the mutated form was very low and amounted to only 0.5% of the activity of the control wild-type protein.

Table 1. Measurement of the activity was performed in GST-PBGD fusion protein of the mutated form and compared with the GST-PBGD fusion protein of the wild-type form expressed simultaneously under identical conditions

Activity	WT	973insG
Specific Activity (nmol URO I / h / μg of protein)	**615**	**32**
Relative Activity (%)	**100**	**0.52**

The availability of recent powerful sequence alignment algorithms and the increase of the number of PBGD homologues in the databases allowed us to improve the original (Louie et al., 1992; Lambert et al., 1994) *E. coli* and human PBGD sequence alignment. The sequences share 41% identity and 57% similarity in our alignment. The crystal structure of *E. coli* PBGD (Brownlie et al., 1994; Lambert et al., 1994; Louie et al., 1998) can thus be used for assessing the molecular and functional consequences of the missense mutation in the human enzyme.

Figure 5 shows the amino acid alignment of the N-terminal sequence for several bacterial and vertebrate PBGDs. It can be seen that the regular secondary structure elements are conserved among bacteria and vertebrates (β-sheet and two helices). Higher eukaryotes contain an extra segment (29 amino acids in humans) inserted between the β-sheet and the penultimate helix forming a large surface linker.

The effect of the deletion and few mutations is visualized in Fig. 6. The enzyme is formed by three domains in close interaction. Their interface forms the active site (Fig. 6a). The truncation described in the present report (2007) is located in the N-terminal domain (Fig. 6b). The domain consists of a three-stranded antiparallel β-sheet meander and three α-helices. Whereas the first two helices form a tightly packed unity with the β-sheet, the terminal helix is much more independent of the rest

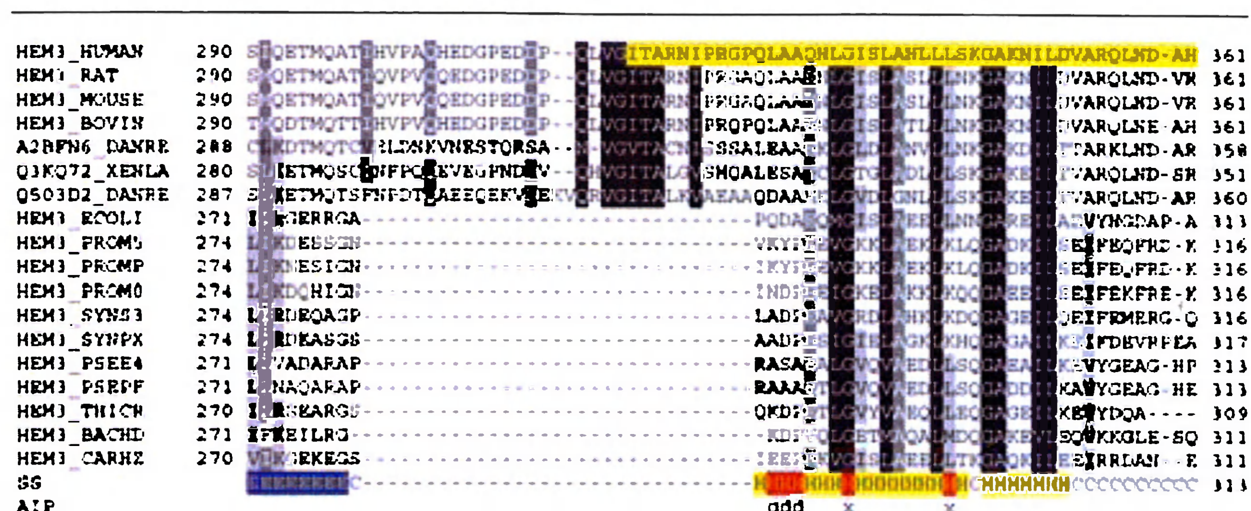

Fig. 5. Sequence alignment of the C-terminal part of PBGD. Selected vertebrate and bacterial sequences are compared (UniProt IDs and sequence numbering). The degree of conservation is expressed in three grades of background shade. The segment found to be deleted in the PBGD sequence of the AIP patient is highlighted in yellow in the human sequence.
SS – secondary structure in the *E.coli* protein: E – β-sheet, H – α-helix (highlighted in deep blue and yellow, respectively, here and in the 3D structure in Fig. 6), C – coil (invisible from position 306 to the end in the crystal structure). AIP – acute intermittent porphyria caused by variants reported in Swiss-Prot database: d – deletion, m – missense mutations G335S, G335D, L343P (highlighted in red here in the secondary structure and 3D structure in Fig. 6b). Note a large, conserved segment of about 30 amino acids between the C-terminal β-sheet strand and C-terminal helix that is present in the vertebrate sequences but not in the bacterial ones (cf. Fig. 6, the segment would be inserted between the deep blue and yellow secondary structure elements in the 3D structure of the vertebrate proteins).

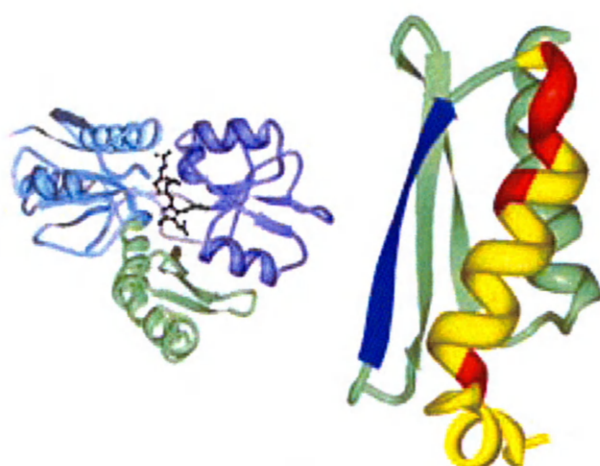

Fig. 6. Three-dimensional structure of *E. coli* porphobilinogen deaminase (PDB ID 1pda).
a) Left panel: Overall structure with N-terminal, central and C-terminal domains coloured in light blue, medium blue and green, respectively, connecting loops in grey and the dipyrromethane cofactor in the active site in black, b) Right panel: C-terminal domain, colour-coding as in Fig. 5.

of the structure and points towards the solvent. In agreement, the mutations in the penultimate helix or its removal destabilize the whole C-terminal domain. If the penultimate helix is missing, the N-terminal domain cannot fold in a stable unity. This will destabilize the whole protein. Even point mutations in the penultimate helix lead to AIP (Fig. 6b), putting in evidence the importance of this helix for the enzyme stability. On the other hand, no mutations have been reported in the C-terminal helix or in the vertebrate-only insert. Not surprisingly, the protein without the penultimate helix is unstable and if the GST in the fusion protein is removed, the protein probably precipitates and cannot be isolated.

The approach taken in this study is novel at several levels. First, it was initiated by a concerned patient who searched for knowledge on web pages. Second, it involved the full scale of finding the relationship between sequence and function. Hence, sequencing of genomic DNA was followed by protein analysis, expression of the mutated protein and measurement of functional activity. This full-scale analysis brought a yet unidentified mutation in the *PBGD* gene. This mutation yields a truncated and inactive protein and provides a mechanistic explanation for the symptoms this patient had. Third, the discovery of this mutation allowed genetic screening of the patient's family, 12 members of which had this mutation and thereby are at risk for AIP acute attack.

In summary, the increasing access to a number of disease- and symptom-oriented web pages presents a new and unusual venue for gaining knowledge and enabling self-diagnosis and self-help. It is, therefore, important that disease-oriented Internet pages for public use should

be designed with clarity and accurate current knowledge-based background. The current study together with the increasing global access to information over the Internet formed the impetus to our initiative of establishing a web page in conjunction with the activity of the European Porphyria Initiative – http://www.porphyria-europe.com (Deybach et al., 2006). We do believe it will add to the knowledge of the professional health-care providers as well as patients.

Acknowledgements

Authors thank Dr. Michal Laniado Schwartzman, Department of Pharmacology, New York Medical College for critical reading of the manuscript and Lubomir Kralik, MSc. for his help during manuscript preparation.

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PUBLICATION C

Characterization of two missense variants in the hydroxymethylbilane synthase gene in the Israeli population, which differ in their associations with acute intermittent porphyria *(Xiaoye Schneider-Yin et al., 2008)*

Molecular Genetics and Metabolism 94 (2008) 343–346

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Characterization of two missense variants in the hydroxymethylbilane synthase gene in the Israeli population, which differ in their associations with acute intermittent porphyria

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ARTICLE INFO

Article history:
Received 6 March 2008
Accepted 6 March 2008
Available online 11 April 2008

Keywords:
Acute intermittent porphyria
Hydroxymethylbilane synthase
Missense mutation
In vitro expression

ABSTRACT

Acute intermittent porphyria (AIP) is an autosomal dominant disorder of heme biosynthesis caused by molecular defects in the hydroxymethylbilane synthase (HMBS) gene. In this study, we report two novel missense sequence variations in the HMBS gene, T59I (C176T) and V215M (G643A), in two patients with clinical symptoms compatible with acute attacks of porphyria. However, only the patient who carried V215M presented with full AIP-affirming biochemical evidence. Both variant proteins were expressed in a prokaryotic system and characterized in vitro. Recombinant T59I and V215M had residual activity of 80.6% and 19.4%, respectively, of that of the wild type enzyme. Moreover, changes in K_m, V_{max} and thermostability observed in the recombinant V215M suggest a causal relationship between V215M and AIP. The association between the T59I substitution and AIP is less obvious. Based on our investigation, substitution T59I is more likely to be a mutation with a weak effect than a rare form of polymorphism. This study demonstrates that in vitro characterization of missense variations in the HMBS gene can provide valuable information for the interpretation of clinical, biochemical and genetic data, for establishing a diagnosis of AIP. It also highlights the fact that there are still many aspects to be investigated concerning AIP and corroborates the need to report new data that can help to clarify the genotype–phenotype relationship.

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Introduction

Acute intermittent porphyria (AIP, MIM #176000) is an autosomal dominantly inherited disorder of heme biosynthesis resulting from an ~50% deficiency of hydroxymethylbilane synthase activity (EC 4.3.1.8; HMBS). This enzyme, also known as porphobilinogen deaminase, catalyzes the head-to-tail condensation of four molecules of porphobilinogen (PBG) to form hydroxymethylbilane. Clinically, AIP is characterized by acute intermittent neurovisceral attacks that can be provoked by various factors such as drugs, hormones and alcohol [1]. Biochemical diagnosis of AIP is based on measurement of the urinary porphyrin precursors, δ-aminolevulinic acid (ALA) and PBG, in combination with the determination of erythrocyte HMBS activity. Molecular analysis of the HMBS gene has been shown to be more efficient than enzymatic analysis in detecting latent AIP patients who do not excrete excess amounts of ALA and PBG in the urine [2,3].

The locus for this disorder has been mapped on chromosome 11q24.1–q24.2 [4]. The length of the HMBS gene is ~10 kb, and the cDNA, encoded by 15 exons, is 1.4 kb, with a single open reading frame of 1038 bp [5,6]. Two distinct promoters, located in the 5′ flanking region and in intron 1, respectively, generate housekeeping (contains exon 1 and 3–15) and erythroid-specific (contains exon 2–15) transcripts by alternative splicing of exon 1 and 2 [7].

To date, a total of 301 different mutations have been identified in the HMBS gene [8]. Among them, 97 mutations (32%), were missense. Most of the missense mutations were documented only at the DNA level, while only a small fraction was expressed and characterized in vitro [9–12]. The availability of the crystal structure of *Escherichia coli* HMBS made it possible to postulate the molecular

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1096-7192/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2008.03.001

and functional consequences of missense mutations in the human enzyme because the *E. coli* and human HMBS amino acid sequences have 35% homology and more than 70% similarity [13]. Nevertheless, in vitro expression remains a powerful tool in studying HMBS gene mutations in the absence of information on the tertiary structure of the human enzyme.

In this study, we report on mutation analysis and in vitro characterization of HMBS variants identified in two individuals who were suspected of having AIP. One of the two subjects, however, presented with biochemical features that were not fully compatible with the AIP diagnosis.

Materials and methods

Subjects and clinical presentations

Two Ashkenazi Jewish females were studied. The first subject aged 17, has been experiencing menstruation-related recurrent episodes of severe abdominal pains accompanied by vomiting, tachycardia and hypertension, for the last three years. Biochemical analyses failed to show elevations in urinary PBG (Table 1) and ALA (not shown) levels during three latent and four acute phases.

Normal fecal porphyrin profile and fluorometric plasma scan were recorded (not shown). However, erythrocyte HMBS activity, measured in three independent occasions, showed an average activity of 60% of that of normal (the enzyme activity among our AIP patients ranged from 38% to 61% of that of normal). Since no other diagnosis besides AIP was established, the patient was treated with either glucose or heme-arginate (Normosang®) for acute attacks. Both substances were proven to be effective. Genetic testing was performed on the patient in order to clarify the conflicting situation between clinical symptoms and biochemical analyses.

The second subject aged 30, was suffering from recurrent acute attacks of abdominal pain, hyponatremia and urinary retention for 12 years. She was diagnosed as having AIP during an acute attack three years ago, treated with glucose, and has remained symptom-free since then. The diagnosis was established on the basis of increased urinary ALA and PBG, and reduced erythrocyte HMBS activities to 61% and 50% of normal, measured two months and three years after her last acute attack, respectively (Table 1).

Peripheral blood samples were collected from both subjects and their family members, as well as from 50 non-porphyric subjects of Jewish origin (Ashkenazi) with informed consent. This study was approved by the Helsinki committee of the Israeli Ministry of Health.

Mutation analysis

Genomic DNA was isolated from peripheral blood by using the QIAamp Blood Kit (Qiagen). The 15 coding exons of the HMBS gene, along with more than 60 bp of their flanking regions, were amplified by PCR using specific primers (Genbank Accession Numbers, HMBS gene, M95623; HMBS cDNA, NM000190) as we published previously [14,15]. Sequence analysis was performed on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Plasmid construction and mutagenesis

An EDTA-anticoagulated blood sample from a healthy volunteer was used for RNA extraction. Total RNA was reverse transcribed into cDNA by RT-PCR using an oligo(dT) primer. cDNA of the HMBS gene was obtained by PCR using sequence-specific primers with restriction sites BamHI and XhoI attached to the 5′- and 3′-end, respectively (forward: 5′ata tgg atc cat gtc tgg taa cgg 3′ and reverse: 5′tat act cga gtt aat ggg cat cgt taa 3′. The HMBS cDNA was ligated into an expression vector, pGEX-4T-1 (Amersham Pharmacia Biotech) and then transformed into *E. coli BL21* (*DE3*) (Stratagene). Plasmid DNA containing the HMBS sequence was purified by using the QIAprep Spin Miniprep Kit (Qiagen). Site-directed mutagenesis was performed by using the QuikChange® Site-directed Mutagenesis Kit (Stratagene). Primers 5′agt ttg aaa tca ttg cta tgt cca cca **t**ag ggg aca aga ttc 3′ (forward) and 5′gaa tct tgt ccc ct**a** tgg tgg aca tag caa tga ttt caa act 3′ (reverse) were used to generate variant T59I; primers 5′cct gag aaa tgc atg tat gct **a**tg ggc cag ggg 3′ (forward) and 5′ccc ctg gcc ca**t** agc ata cat gca ttt ctc agg 3′ (reverse) were used to generate variant V215M (the sites of mutagenesis are *in bold* and *underlined*). The results of the mutagenesis were confirmed by sequencing.

In vitro expression and purification of variants and wild type enzyme

Variants and wild type enzyme were expressed as GST-fusion proteins in *BL21* cells using a standard technique. Bacterial cells were subjected to protein purification using a Glutathione Sepharose 4B column (Amersham Biosciences). The purity of the proteins was confirmed on SDS–PAGE.

Enzymatic assay in recombinant enzymes

The HMBS activity assay was carried out according to a previously published method [16]. One microgram of purified enzyme was diluted with an incubation buffer containing 50 mM Tris, 0.1% BSA, 0.1% Triton, pH 8.2, to a final volume of 360 μl. After pre-incubation of the sample at 37 °C for 3 min, 40 μl of 1 mM PBG (ICN Biomedicals) was added. The sample tube was further incubated in the dark for 1 h before 400 μl of 25% TCA were added to terminate the reaction. The samples were subjected to photooxidation under daylight for 60 min and were then centrifuged for 10 min at 1500*g*. Fluorescence intensity was measured in the supernatant immediately after centrifugation using a Perkin-Elmer LS 55 spectrofluorometer. The fluorescence wavelengths were set at 405 nm for excitation and 599 nm for emission. Uroporphyrin I (URO I) purchased from ICN Biomedicals, was used as a standard for calculation of enzyme activity. Protein concentration was determined by the Lowry method. One unit of HMBS activity was defined as 1 nmol URO/h/μg protein.

To determine K_m values of the various recombinant enzymes, a series of substrate concentrations was used, i.e., 1, 2.5, 5, 10, 20, 25, 50, 75, 100 and 150 μM of PBG in the final reaction mixture. The time of incubation varied from 1 to 9 min. To determine optimal pH, HMBS activity was measured within a range from pH 7.0 to pH 9.0. To study thermostability, enzymes were pre-incubated at 65 °C at pH 8.2 for 0, 30, 60, 90, 120, 180 and 240 min, respectively, before activity measurements. K_m and V_{max} values were calculated by Sigma Plot using a single rectangular hyperbolic curve (Michaelis–Menten curve).

Results

Direct sequencing of PCR-amplified HMBS gene fragments from the first subject unveiled C to T transition at nucleotide position 176 in exon 5 resulting in the substitution of Thr59 by an isoleucine residue (T59I). This missense variation was absent in the HMBS gene of her mother, the only family member available for testing. A different missense variation G643A (V215M) was identified in the second subject, as well as in members of her family, including her father, her 3-year-old son, and two of her paternal cousins. Both subjects were heterozygous for the respective sequence variations. Both T59I and V215M were absent in the HMBS gene of 50 non-porphyric Ashkenazi Jewish subjects.

To examine the effect of these missense variations on the HMBS enzyme, T59I and V215M were expressed in a prokaryotic system and purified to near homogeneity based on SDS–PAGE analysis (data not shown). A series of experiments were then carried out using purified enzymes in order to further characterize the two variants.

HMBS activity was measured in triplicate in all three recombinants enzymes, T59I, V215M and wild type. The mean activity of

Table 1
Biochemical, enzymatic and genetic data

	Biochemical analyses			Genetic analysis	In vitro enzymatic studies		
	Urinary PBG (μmol/24 h)[a]		Erythrocyte HMBS activity (% normal)[b]	Sequence variation in HMBS gene	K_m (μM)	V_{max} (nmol/min)	Optimal pH
	Latent	Acute					
Subject 1	6.2 ± 1.7 (*n* = 3)	8.4 ± 1.4 (*n* = 4)	60[c]	C176T, T59I	6.6 ± 0.3	1.3 ± 0.1	7.8
Subject 2	92.8	287.3	50[c]	G643A, V215M	70.0 ± 16.3	0.4 ± 0.1	8.0
				Wild type	4.2 ± 0.5	2.1 ± 0.1	8.2

[a] Normal value: <8.8 μmol/24 h.
[b] Normal value: >70%.
[c] Activity measured in the latent phase.

Table 2
Residual activities in the recombinant HMBS enzymes

Nucleotide change	Amino acid substitution	Residual activity (% of normal activity)	Reference
A100C	Q34P	1.3	De Siervi et al. [11]
C104T	T35M	4.0	De Siervi et al. [20]
G277T	V93F	1.1	Chen et al. [10]
C346T	R116W	1.4	Chen et al. [10]
G500A	R167Q	0.7	Delfau et al. [9]
G518A	R173Q	0.6	Delfau et al. [9]
C601T	R201W	41.6	Chen et al. [10]
A634G	M212V	1.7	Solis et al. [12]
G740T	C247F	11.0	Chen et al. [10]
G1003A	G335S	2.5	De Siervi et al. [11]
C176T	T59I	80.6	This study
G643A	V215M	19.4	This study

each variant was expressed as a percentage of that of the wild type enzyme. T59I exhibited a slight reduction in HMBS activity with a residual activity of 80.6%, whereas the residual activity of V215M was shown to be 19.4% (Table 2).

Kinetic studies on the recombinant enzymes involved measurements of both K_m and V_{max}. A slightly increased K_m value (6.63 ± 0.33 μM) compared to that of the wild type (4.24 ± 0.47 μM), was observed in T59I, as opposed to a 16-fold difference in K_m values between V215M (70.0 ± 16.3 μM) and wild type. The V_{max} value of T59I was reduced to 1.3 ± 0.1 nmol/min and that of V215M to 0.4 ± 0.1 nmol/min, compared to a V_{max} of 2.1 ± 0.1 nmol/min determined in the wild type enzyme (Table 1 and Fig. 1a).

The two variants also exhibited different stabilities at 65 °C. Thermostability of T59I was similar to that of the wild type enzyme. As shown in Fig. 1b, T59I lost approximaltely10% of its activity after a pre-incubation period of 240 min at 65 °C. However, the HMBS activity of V215M fell below 10% of that of the wild type enzyme after a short pre-incubation period of 30 min. An inverse situation was observed in the optimal pH of the variants; the optimal pH of V215M (pH 8.0) was close to that of the wild type (pH 8.2). Yet, T59I (pH 7.8) and wild type differed in optimal pH by 0.4 (Table 1 and Fig. 1c).

Discussion

Porphyria experts concur that acute attacks of neurovisceral symptoms are attributed to porphyria only if an elevation in urinary PBG concentration is observed. According to our newly published study, the PBG level was increased during the acute phase at least 2.3-fold of that during the latent phase [17]. Indeed, a 3.1-fold increase in the PBG level was observed in subject 2 who carried the V215M mutation. The 19.4% residual activity measured in the recombinant V215M was consistent with the 50% reduction in erythrocyte HMBS activity in this subject, since AIP is an autosomal dominantly inherited disorder. These findings suggest a causal relationship between mutation V215M in the HMBS gene and the disease AIP in subject 2.

The association between the T59I substitution and AIP is less obvious. The biochemical finding in subject 1, i.e., normal urinary excretion of ALA and PBG during acute attacks was not in accordance with the current accepted knowledge on AIP. In addition, the results from in vitro expression study demonstrated similarities between mutant T59I and the wild type enzyme in K_m value and in the thermostability profile. Moreover, mutant T59I displayed the highest residual activity (80.6%) among all recombinant enzymes that have been characterized so far. As shown in Table 2, missense R201W has a residual activity of 41.6%, the second highest next to T59I. However, unlike T59I, recombinant R201W was proven to be extremely thermolabile at 65 °C comparing to the wild type enzyme. Indeed, R201W was associated with AIP [10].

In contrast to the above-mentioned evidences, a few of the additional observations support the scenario of T59I having a functional role in the AIP phenotype. Subject 1 had typical clinical symptoms of AIP which were successfully treated with glucose and Normosang, and her erythrocyte HMBS activity was reduced, features compatible with AIP. Moreover, the biochemical finding of normal urinary ALA and PBG during acute attacks do not necessarily contradict a diagnosis of AIP, once the young age of the patient is taken into account. According to Hultdin et al., in children under the age of 18, normal values of ALA and PBG may be observed during symptoms [18]. In addition, the substitution was absent in 100 chromosomes from Ashkenazi control subjects, as well as in the mother, the only family member available for testing. In view of the above, we have to con-

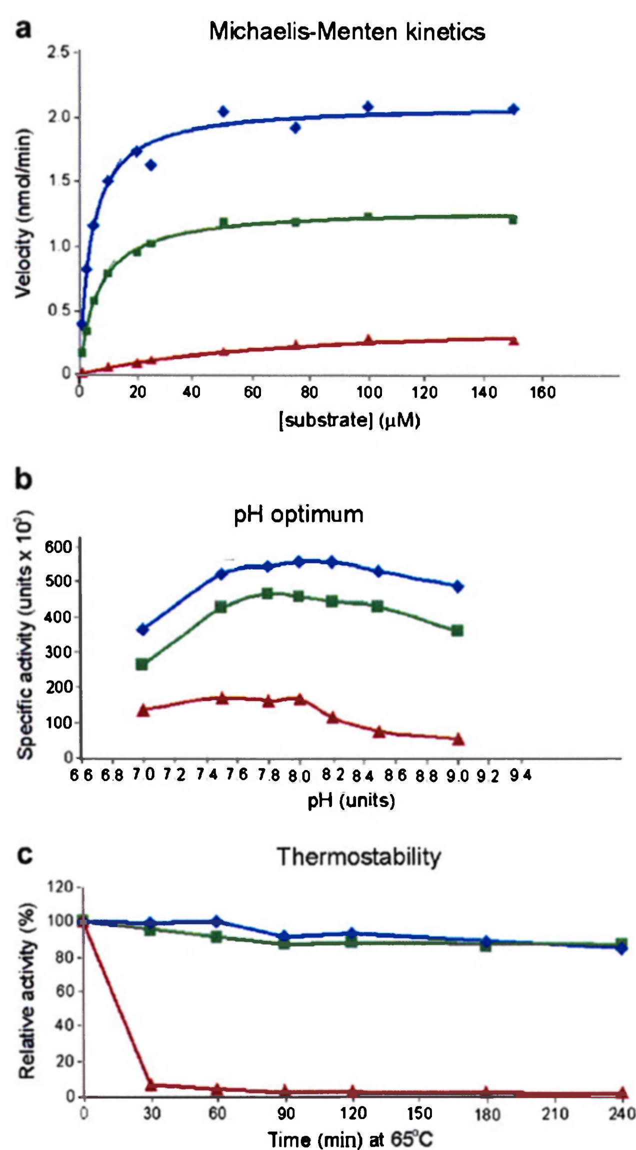

Fig. 1. In vitro enzymatic studies of two sequence variations in the HMBS gene, C176T (T59I) (■) and G634A (V215 M) (▲) compared to wild type (◆).

clude that T59I might represent a mutation with a weak effect rather than a mere polymorphism.

In accordance with the "synergistic heterozygosity" hypothesis [19], it may very well be that in this compound case, there is more than one causative factor. A few partial defects in multiple steps of the heme biosynthetic pathway or even in an additional pathway may have a great clinical relevance.

The present study demonstrates that in vitro expression of mutations in the HMBS gene can add valuable information to the interpretation of clinical, biochemical and genetic data in establishing a diagnosis of AIP. It also highlights the fact that there are still many aspects to be investigated concerning AIP, and corroborates the need to report new data that can help to clarify the genotype–phenotype relationship.

Acknowledgments

We appreciate the participation of the patients and their relatives in the study. We thank Sulejman Ahmetovic, Zürich, Switzerland, for his excellent technical assistance.

This work was supported by Ministry of Education, Sport and Youth of Czech Republic, The Granting Agency of Charles University; Contract Grant Number: MSM0021620806, 1M6837805002, GAUK 257540 54007, to D.U. and P.M.

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PUBLICATION D

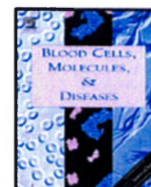
Correlation between biochemical findings, structural and enzymatic abnormalities in mutated HMBS identified in six Israeli families with acute intermittent porphyria *(Ulbrichova D et al., 2008)*

Blood Cells, Molecules, and Diseases 42 (2009) 167–173

Contents lists available at ScienceDirect

Blood Cells, Molecules, and Diseases

journal homepage: www.elsevier.com/locate/ybcmd



Correlation between biochemical findings, structural and enzymatic abnormalities in mutated HMBS identified in six Israeli families with acute intermittent porphyria

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ARTICLE INFO

Article history:
Submitted 29 October 2008
Revised 10 November 2008
Available online 12 January 2009

(Communicated by M. Lichtman, M.D., 19 November 2008)

Keywords:
Acute intermittent porphyria
HMBS mutation
In vitro expression
Structure-function correlation

ABSTRACT

Mutations in the hydroxymethylbilane synthase (HMBS) gene are responsible for the inherited disorder of acute intermittent porphyria (AIP). AIP is diagnosed on the basis of characteristic clinical symptoms, elevated levels of urinary porphyrin precursors aminolevulinic acid (ALA) and porphobilinogen (PBG) and a decreased erythrocytic HMBS activity, although an identifiable HMBS mutation provides the ultimate proof for AIP. Six Israeli AIP families underwent biochemical and mutation analysis in order to establish an AIP diagnosis. Variability with respect to the ALA/PBG levels and HBMS activity was found among the index patients. Indeed, each family carried a unique mutation in the *HMBS* gene. A novel missense c.95G>C (p.R32P) was shown to be a *de novo* mutation in one family, along with five known mutations p.T59I, p.D178N, p.V215M, c.730_731delCT and c.982_983delCA identified in the rest of the families. Both R32P and D178N were expressed in a prokaryotic system. Recombinant p.R32P was enzymatically inactive as demonstrated by a <1% residual activity, whereas p.D178N possessed 81% of the activity of the wild type enzyme. However, the p.D178N mutant did display a shift in optimal pH and was thermo labile compared to the wild type. Among the four missense mutations, p.R32P and p.V215M had not only harmful effects on the enzyme *in vitro* but also were associated with high levels of ALA/PBG in patients. On the other hand, the *in vitro* effect of both p.T59I and p.D178N, and the impact of these mutations on the enzyme structure and function as interpreted by the 3-D structure of the *Escherichia coli* enzyme, were weaker than that of p.R32P and p.V215M. Concomitantly, patients carrying the p.T59I or p.D178N had normal or borderline increases in ALA/PBG concentrations although they presented characteristic clinical symptoms. These findings provided further insights into the causal relationship between HMBS mutations and AIP.

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Introduction

Acute intermittent porphyria (AIP, OMIM 176000) is an autosomal dominantly inherited disorder of heme biosynthesis resulting from a ~50% deficiency of hydroxymethylbilane synthase activity (EC 2.5.1.61; HMBS). This enzyme, also known as porphobilinogen deaminase, catalyzes the head-to-tail condensation of four molecules of porphobilinogen (PBG) to form hydroxymethylbilane. Clinically, AIP is characterized by acute intermittent neurovisceral attacks that can be provoked by various factors such as drugs, hormones and alcohol [1]. Biochemical diagnosis of AIP is based on measurement of the urinary porphyrin precursors, δ-aminolevulinic acid (ALA) and PBG, in combination with the determination of erythrocytic HMBS activity.

The *HMBS* gene is located on chromosome 11q24.1–q24.2. It is approximately 10 kb long and contains 15 exons [2]. Two distinct promoters, located in the 5′ flanking region and in intron 1, respectively, generate housekeeping (contains exon 1 and 3–15) and erythroid-specific (contains exon 2–15) transcripts by alternative splicing [3,4]. Since the genetic information on the *HMBS* gene became available, DNA-analysis has been established as an important diagnostic tool for AIP [5]. Worldwide, more than 300 different mutations have been identified in the *HMBS* gene of AIP patients [6].

Recently, we published a study on two Israeli AIP patients, both of whom carried a unique missense mutation in the *HMBS* gene [7]. We performed an extensive *in vitro* characterization of the mutant proteins including residual activity and kinetic study on recombinant

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doi:10.1016/j.bcmd.2008.11.001

enzymes. The results of *in vitro* study helped to better understand the clinical situation and biochemical findings in these two patients. In this study, we report a systematic mutation analysis among six Israeli AIP families including family members of the two previously published cases.

Patients, material and methods

Biochemical and enzymatic analyses

A total of 26 individual from six unrelated Israeli AIP families of Caucasian origin were studied. Biochemical diagnosis of AIP was performed in the Israeli National Laboratory for the Diagnoses of Porphyrias. The diagnosis of AIP was established on the basis of reduced HMBS activity and/or increased urinary ALA and PBG. Detailed information in each family is given in Table 1. In addition, fluorescence plasma scan revealed a peak at 622 nm in a number of patients, especially during acute attacks. In all patients, the ratio of fecal coproporphyrin III/I was less than one, and total fecal coproporphyrin was normal (data not shown). The methods employed for biochemical analysis were previously described [7].

Family A

The index patient suffered from symptoms typical of acute porphyria. A massive increase in urinary porphyrin precursors i.e., 19-fold above the upper normal limit for ALA and 50-fold above the normal limit for PBG, was measured during an acute attack. HMBS activity was however within the normal range in the index patient during the latent phase. None of her family members including her parents and her younger sister, had symptoms and biochemical findings compatible with AIP. However, the father showed slightly increased urinary ALA/PBG concentrations (Table 1).

Family B and D

The clinical and biochemical characterization of AIP in the index patient from both families have been described in a previous publication [7]. In this study, we present results of biochemical analyses of family members of the two index patients. In Family B, the mother of the index did not present any symptoms of acute porphyria and had a normal HMBS activity (Table 1). Among eight family members/relatives of index D who were tested, four individuals had a decreased HMBS activity i.e., below 70% of normal. Two of these four individuals, the father and the grandfather, also showed typical symptoms of acute porphyria. The results of urinary ALA/PBG concentrations seemed to be less discriminative. In two out of the three non-affected subjects studied, urinary ALA/PBG levels were slightly increased (less than 2-fold).

Family C

Both the symptomatic patient (15 years old) and her asymptomatic mother had reduced activity of HMBS, 36% and 44% of normal, respectively. They both exhibited borderline levels of urinary ALA and PBG. No further increase in those values was noted during clinical exacerbation in the patient.

Family E

None of the tested individuals except for the index, had any symptoms compatible with AIP. The index patient and her daughter showed a clearly decreased HMBS activity and increased urinary ALA/

Table 1
Clinical, biochemical and genetic data on six Israeli AIP families

Family	Ethnical background	Age	Acute attacks	Erythrocyte HMBS activity* (>70% of normal)	Urinary ALA§ (< 38 μmol/24 h)	Urinary PBG§ (< 8.8 μmol/24 h)	Mutation status
Family A							c.G95 > C, p.R32P
1. Index (f)		21	Yes	85	282.2 (716.8)	238.7 (442.3)	+/-
2. Father	Ashkenazi	61	No	100	49.1	15.2	-/-
3. Mother	Non Ashkenazi	55	No	105	35.8	7.0	-/-
4. Sister		18	No	98	25.1	6.8	-/-
Family B	Ashkenazi						c.C176 > T, p.T59I
1. Index		15	Yes	50, 63, 68	25.1 (41.2)	6.2 (8.4)	+/-
2. Mother		40	No	100	ND	ND	-/-
Family C	Jewish from						c.532G > A, p.D178N
1. Index	Cochin, India	16	Yes	36	32.7 (35.8)	7.5 (11.0)	+/-
2. Mother		45	No	44	41.2	13.3	+/-
3. Father		50	No	90	ND	ND	-/-
Family D	Ashkenazi						c.643G > A, p.V215M
1. Index (f)		30	Yes	61, 50	114.4 (205.9)	97.2 (287.3)	+/-
2. Son		3	No	ND	ND	ND	+/-
3. Father		57	Yes	61	30.5	26.5	+/-
4. Grandfather		85	Yes	61	45.0	24.0	ND
5. Brother		29	No	100	64.0	17.2	-/-
6. Sister		23	No	87	62.5	14.6	-/-
7. Cousin (m)		27	No	69	54.9	23.6	+/-
8. Cousin (m)		25	No	63	62.5	30.5	+/-
9. Aunt		52	No	110	29.0	11.5	-/-
Family E	Ashkenazi						c.730_731delCT, FS244
1. Index		45	Yes	42	56.4 (267.2)	42.0 (490.5)	+/-
2. Daughter		15	No	46	54.9	15.5	+/-
3. Son		16	No	92	54.0	13.0	-/-
4. Brother		50	No	ND	ND	ND	-/-
5. Niece		25	No	69	ND	ND	-/-
6. Niece		26	No	70	ND	ND	-/-
Family F	Ashkenazi						c.982_983delCA, FS 328#
1. Index (m)		30	Yes	55	76.2 (266.0)	57.0 (185.6)	+/-
2. Father		60	No	66	78	33	ND

* values measured in the latent phase; § values measured in the latent phase, those measured during acute attacks are given in the *parentheses*; #identified in Hôpital Louis Mourier, Colombes, France by the group of professor JC Deybach; Symptomatic patients are highlighted in *dark gray*, asymptomatic carriers in *light gray*. ***Bold***: new mutation; ND: not determined; +/−: a particular HMBS mutation is present in one allele; −/−: a particular HMBS mutation is absent in both alleles.

PBG levels. In addition, two other relatives (nieces) of the index had a borderline HMBS activity.

Family F

HMBS activity in the index patient was decreased to 55% of the normal, and urinary ALA/PBG concentrations were 7-fold/21-fold above the upper normal limit during acute attacks and 2-fold/6-fold above the upper normal limit in the latent phase. His father, although non-symptomatic, also showed decreased HMBS activity and increased ALA/PBG levels.

Mutation analysis

Genomic DNA was isolated from peripheral blood by using the QIAamp Blood Kit (Qiagen, Hilden, Germany). The 15 coding exons of the *HMBS* gene, along with more than 60 bp of their flanking regions, were amplified by PCR using specific primers (GenBank accession numbers, *HMBS* gene, M95623; HMBS cDNA, NM000190). Sequence analysis was performed on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster city, USA).

Plasmid construction and mutagenesis

Total RNA was extracted from leukocytes isolated from EDTA-anticoagulated whole venous blood (Qiagen, Hilden, Germany). cDNA sequences were obtained by reverse transcription (RT-PCR, SuperScript III, Invitrogen, Carlsbad, USA) of total RNA using oligo$(dT)_{20}$ (Invitrogen, Carlsbad, USA) as primer. cDNA of the *HMBS* gene was amplified by PCR using specific primers, ligated into the *p*GEX-4T-1 expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden) and transformed into *Escherichia coli* BL21 (DE3) (Stratagene, La Jolla, USA). Site-directed mutagenesis was performed with specific mutagenic primers using the QuikChange2009/01/02 Site-directed Mutagenesis Kit (Stratagene, La Jolla, USA). Successful mutagenesis was confirmed by sequencing.

In vitro characterization of mutant enzymes

In vitro expression, purification and enzymatic assay in mutant and wild type enzymes were performed as previously described [7].

Analysis of structure–function correlation

337 UniProt (www.uniprot.org) and 32 Ensembl (www.ensembl.org) HBMS protein sequences were aligned with MUSCLE software [8]. The dataset contained 46 eukaryotic and 323 prokaryotic diverse sequences; a subset of 216 sequences showed amino acid identity smaller then 90%, and a subset of 59 sequences identity smaller then 60%. The three-dimensional structure was displayed and examined with MTB platform using the coordinates of the PDB structure ID 1pda [9,10].

Results

Molecular diagnosis of AIP

The coding exons and intron–exon boundaries of the *HMBS* gene were sequenced in the index patient of each family. In family A, a G to C transversion at nucleotide position 95 in exon 4 resulting in substitution of Arg32 by a proline residue (p.R32P) was identified in the index patient. This novel missense was however absent in the *HMBS* gene of both parents (Fig. 1). Since nonpaternity was excluded in this family, p.R32P therefore presents a *de novo* mutation.

Each of the remaining five families carried a unique mutation which had been known to the *HMBS* gene p.T59I, p.D178N, p.V215M, c.730_731delCT and c.982_983delCA [5,7,11,12]. Seven of symptomatic patients who underwent DNA-testing, were all carriers of a specific mutation (Table 1). In addition, family screening allowed identification of a total of five asymptomatic carriers including one (D7) with a borderline HMBS activity of 69% (normal value >70%). Moreover, mutation analysis was able to exclude mutation c.730_731delCT in two members of family E who also had borderline HMBS activities (69% in E5, 70% in E6).

In vitro characterization of mutant enzymes

Missense mutations p.R32P and p.D178N were expressed in *E. coli*. Recombinant as well as wild type enzymes were purified to homogeneity as judged by SDS-PAGE (Fig. 2). HMBS activity in recombinant enzymes was measured in three independent experiments and expressed as percentage of that of the wild type.

The deleterious effect of mutation p.R32P on the enzyme was clearly demonstrated by a <1% residual activity (Table 2). No further characterisation was performed in this respect. The p.D178N mutant on the other hand, exhibited a residual activity of 81% of the wild type (Table 2). To further study this missense mutation, optimal pH and thermo stability were determined in the recombinant enzyme. The optimal pH of mutant p.D178N was reduced to pH7.5 as compared to pH8.2 in the wild type enzyme. Thermo stability study was performed by pre-incubating the enzymes at 65 °C for various periods of time before activity measurement. While wild type enzyme retained ~80% of its activity after a pre-incubation period of 240 min, the p.D178N mutant lost ~70% of its activity under the same condition. The half-life of the mutant enzyme was estimated 60 min.

Structure–function correlation

All six HMBS mutations described in this study were subjected to structural analysis based on the available 3-D structure of the *E. coli* enzyme [10]. The alignment of all available HMBS protein sequences allowed the identification of corresponding amino acid residues between human and *E. coli* sequences. As observed previously, a segment of approximately 30 amino acids between the C-terminal β-sheet strand and the C-terminal helix is present only in the eukaryotic, but not in the prokaryotic enzymes. This segment therefore, cannot be modeled on the *E. coli* structure [13]. However, since none of the six mutants of this study are located within this C-terminal segment, their counterparts in the *E. coli* enzyme were unambiguously identified (Fig. 3 and Table 2).

p.R32P Although conserved in eukaryotes, Arg32 varies substantially in prokaryotes. However, proline is never present at this position. The corresponding residue of Arg32 in the *E. coli* enzyme, Leu17 is located in the N-terminal helix of which the apex participates in the forming of the active site [14,15]. The loss of activity of the p.R32P mutant to <1% is probably due to the helix-braking effect of proline residue in the apex and in turn, the distortion of the active site.

p.T59I Thr59 is located within a mobile surface loop connecting the $\beta 2_1$ and $\alpha 2_1$ segments [14,15]. It is one of the most variable positions in both eukaryotes and prokaryotes at which Ile appears more frequently than other residues. In the presence of substrate, the surface loop is mostly invisible in the X-ray structures even at very low temperatures [16]. For this reason, only the beginning and the end of the loop can be observed in the SD structure (Fig. 3). The relatively high residual activity of 81% measured in the p.T59I mutant suggests that the loop does not play an important role in the catalytic action of the enzyme, a scenario which is in agreement with the variability and mobility of this region.

p.D178N The crystal structure in the *E. coli* enzyme shows a salt bridge between Asp160 and Arg182 [14]. Both residues are

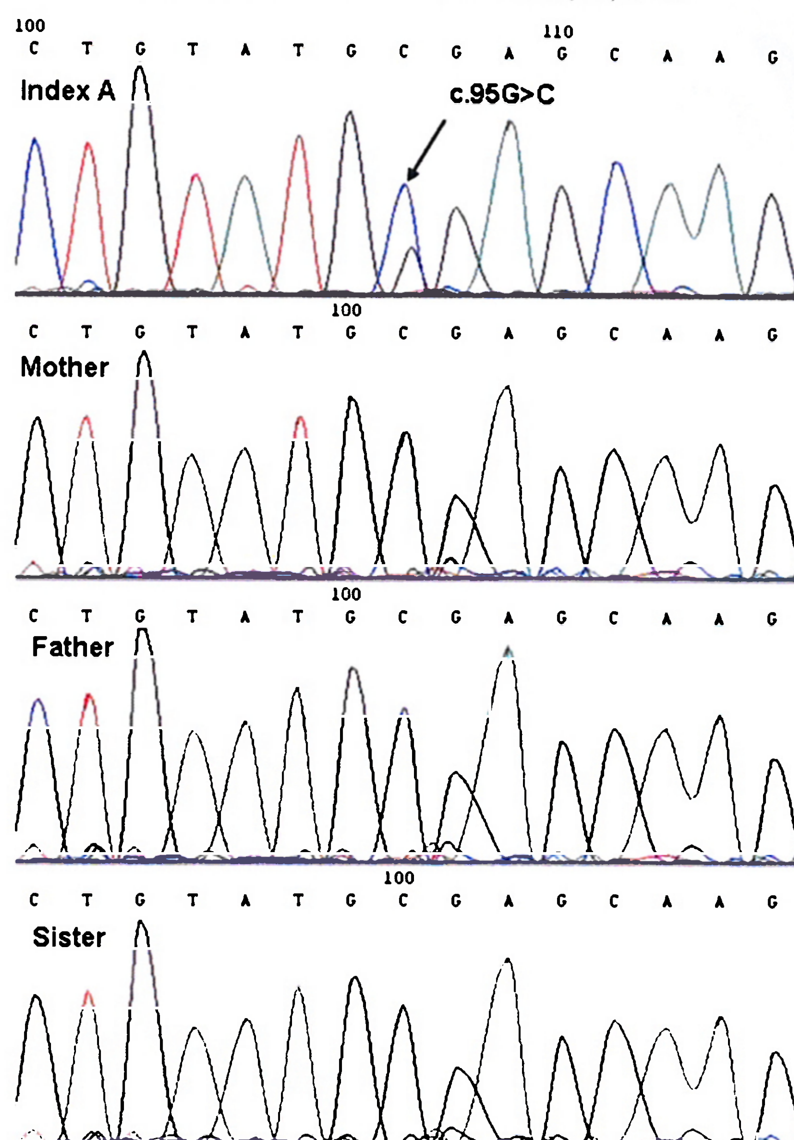

Fig. 1. Identification of the *de novo* c.95G > C (p.R32P) mutation in the *HMBS* gene of Family A. PCR was performed on genomic DNA using primers to specifically amplify the 15 coding exons of the *HMBS* gene. Sequencing detected a heterozygous G to C change at base 95 in the index patient as indicated by an *arrow* (the top panel). The p.R32P mutation was absent in three members of family A including the father, the mother and the younger sister of the index patient (the 2nd, 3rd and 4th panels). A segment of reversed sequence of the *HMBS* gene is shown in each panel.

conserved in the human sequence (Asp178/Arg201) as well as in other eukaryotic sequences. In very few prokaryotic enzymes in which the pair is not conserved, the basic residue can be replaced by an acidic residue, and vice versa, at these two positions. The conserved salt bridge is displayed in Fig. 3. The p.D178N mutation abolishes the negative charge of the salt bridge and suppresses the Coulombic interaction. Although these actions only resulted in a 19% reduction of activity, they did alter heat stability and pH optimum of the enzyme.

p.V215M Val215 is situated in the inner part of the active site cleft of the enzyme, where it forms one of two hinges between domains 1 and 2 [14,15]. The activity dropped to 19% in the p.V215M mutant protein as determined in our previous study [7]. Although it is difficult to predict the exact effect of the Val/Met substitution, it is clear that it occurs in the tightly packed core of the enzyme sensitive to subtle changes. Position 215 is conserved among eukaryotes and varies considerably among prokaryotes. Only in a few prokaryotic species can a methionine residue be found at this position which is otherwise always occupied by hydrophobic residues.

c.730_731delCT and c.982_983delCA Both deletions cause a frameshift prior to the premature termination, which results in the incorporation of 6 and 30 amino acid residues respectively, with sequences completely different from that of the original enzyme. The truncated protein generated from c.730_731delCT consists of 249 amino acids as opposed to 361 amino acids in the wild type. The deletion takes place at the beginning of the $\alpha 1_3$ helix which produces a protein lacking the entire domain 3 [14,15]. The truncated protein generated from c.982_983delCA contains 357 amino acids, only four amino acids shorter than the wild type enzyme. However, due to the change in amino acid sequence at the C-terminus, part of the third enzyme domain is most likely misfolded and may lead to aggregation. The deletion is situated in the $\beta 3_3$ sheet causing complete disappearances of both helixes $\alpha 2_3$ and $\alpha 3_3$ in domain 3. Given the fact that a truncation of the C-terminal helix leads to the abolition of the activity,

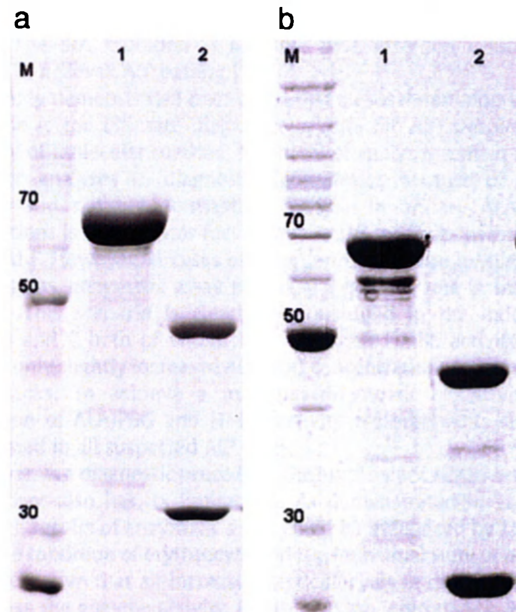

Fig. 2. Assessment of homogeneity in purified mutant enzymes by SDS-PAGE. (a) the p.R32P mutant; (b) the p.D178N mutant. M, molecular weight marker; lane 1, mutant enzyme (MW 68 kDa) before thrombin digestion of the GST-tag; lane 2, mutant enzyme (MW 42 kDa) after thrombin digestion of the GST-tag.

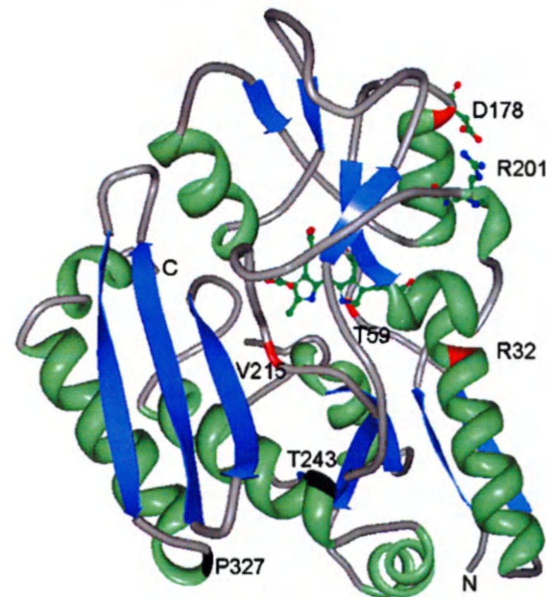

Fig. 3. Locations of human *HMBS* mutants in the 3D structure of *E. coli* enzyme. The helices are indicated in green, the beta-strands in blue, the irregular secondary structures in gray. The position of missense mutations are marked in red and the position of the last amino acid before frameshifts in black. The dipyrromethane cofactor in the active site and the salt-bridge forming residues Asp160-Arg201 are in ball and stick representations. The N- and C-termini are labeled N and C, respectively.

any larger truncations will most likely have a similar effect on the enzyme [13,17].

Discussion

All mutations described in this study occurred in exons common to both the housekeeping and erythroid-specific isoforms of HMBS. These mutations are therefore associated with the classical form of AIP. The so called "variant AIP" is caused by mutations in the first exon of the *HMBS* gene affecting only the liver-specific isozyme. The novel missense c.95G>C (p.R32P) is the first *de novo* mutation observed in the Israeli AIP population. According to an early estimation, *de novo* mutations occur in ~3% of AIP index cases [18]. However, the data that are available so far seem to indicate an even lower rate of *de novo* events in AIP since only four such cases have been described in the literature [5,17,18,19].

Most of the *de novo* events in human genes occur at the CpG dinucleotides which are known to be hypermutable due to oxidative deamination of methylated cytosines [20]. Indeed, four of the five known *de novo* mutations in the *HMBS* gene including p.R32P, are CpG mutations, CGC→CAC (p.R26H), CGC→CCC (p.R32P), CGG→TGG (p.

Table 2
In vitro characterization and structural analysis of HMBS mutants

Exon	Mutation in the DNA sequence	Amino acid change or other effect	*In vitro* enzymatic studies			Equivalent amino acid residue in *E. coli*	Location in the secondary structure of *E. coli* enzyme	Conservation among HMBS of different species	Effect	Reference
			Residual activity§	Optimal pH•	Thermo-stability•					
4	c.95G>C	p.R32P	<1%	ND	ND	Leu17	N-terminal helix	8/23	Breakdown of the N-terminal helix, distortion of the active site	This study
5	c.176C>T	p.T59I	81%*	7.8*	10%*	Arg44	Loop between $\beta2_1$ and $\alpha2_1$	10/23	Mild effect	Schneider-Yin et al., 2008 [7]
10	c.532G>A	p.D178N	81%	7.5	70%	Asp160	$\alpha2_2$	12/23	Salt bridge to Arg201, loss of stabilizing interaction	Puy et al., 1997 [5]
11	c.643G>A	p.V215M	19%*	8.0*	>99%*	Val196	Loop between $\beta4_2$ and $\beta5_1$	15/23	Packing changes in the protein core near the active site cleft	Schneider-Yin et al., 2008 [7]
12	c.730_731del CT	FS244 Δ Stop + 6	ND	ND	ND	FS224	Domain 3	-	Frame shift, truncated protein	Mgone et al., 1993 [11]
15	c.982_983del CA	FS328 Δ Stop + 30	ND	ND	ND	FS280	C-terminal helix	-	Frame shift, truncated protein	Maeda et al., 2000 [12]

§In percentage of the wild type enzyme; •reduction in enzyme activity (percentage of the wild type) after a pre-incubation period of 240 min at 65 °C; optimal pH of the wild type enzyme 8.2; *published in our previous work (reference No.7); ND: not determined.

R173W) and CGC→TGC (p.R195C). All four missenses affect arginine residues. The 5th mutation is a single base insertion c.966insA, identified in a Slovak AIP patient [17].

This study demonstrated once again that a causal mutation in the *HMBS* gene is the ultimate diagnostic criteria for AIP. Despite the availability of molecular method, biochemical analyses remain to be first-choice analyses in diagnostic laboratories because of their simplicity and rapidity. A massive increase in urinary ALA/PBG concentrations is an indicator for AIP, as seen in the index of families A, D, E and F. However, in cases of a borderline increase in ALA/PBG concentrations, enzymatic assay may play a decisive role in the AIP diagnosis. This scenario is clearly demonstrated in the index of families B and C both of whom had decreased HMBS activity and normal to only slightly increased ALA/PBG concentrations even during acute attacks. To achieve a maximal diagnostic sensitivity, a combination of ALA/PBG and HMBS activity measurements should be performed in all suspected AIP cases.

However, as a diagnostic procedure, the erythrocytic HMBS activity measurement also has its limitations. As demonstrated in various studies, the results of enzymatic assay could be influenced by factors such as the condition of erythrocyte and the individual state of health [1,21]. It is known that an increase in reticulocytes in the circulation can increase the enzyme activity. For this reason, erythrocytic HMBS activity can be normal during acute attacks and decrease to subnormal levels during remissions [22]. As a consequence of the interaction among various factors, certain overlaps in the values can be observed between healthy individuals and affected patients [5,23]. Such example can be found in the index of family A. The patient had frequent attacks, each of them was accompanied by immensely increased ALA/PBG concentrations. However, her erythrocytic HMBS activity was normal between attacks (Table 1) and could go up to as much as 144% of the normal during an acute attack (data not shown).

In vitro characterization of HMBS mutants provides another means to interpret the often heterogeneous biochemical findings in AIP patients. Among the four missense mutations identified in this study cohort, p.R32P and p.V215M were proven to be deleterious *in vitro* as seen by a residual activity of 1% and 19%, respectively. In addition, p.V215M was shown to be extremely thermo labile [7]. The *in vitro* effect of the other two missenses p.T59I and p.D178N was much weaker compared to p.R32P and p.V215M. Both p.T59I and p. D178N had a relatively high residual activity of 81% although D178N seemed to be more thermo labile than p.T59I. In all four missenses, the *in vitro* residual activity did not seem to correlate with the *in vivo* erythrocytic HMBS activity (Tables 1 and 2). Patients who carried the "weak" mutations had different levels of enzyme activity i.e., borderline values of 50–68% in patient B and a low value of 36% in patient C. However, a common feature shared by these two patients was the slightly increased levels ALA/PBG as discussed earlier. The "strong" missense mutations p.R32P and p.V215M on the other hand, were apparently associated with high levels of ALA/PBG comparable with that of frameshift mutations c.730_731delCT and c.982_983delCA.

To further explore the genotype-phenotype correlation in AIP, all six HMBS mutations were evaluated at the structural level based on the 3-D structure of the *E. coli* enzyme [14]. The two "strong" missense mutations as well as the two frameshifts, were all predicted to have detrimental effects on the structure and function of the enzyme either due to their location or due to the nature of the substitution. The weak mutations p.T59I and p.D178N on the other hand, besides the similarities between wild type and substituted amino acid residues, were located at less critical positions and therefore exerted limited impact on the structure and function of the enzyme.

Only 18 of the nearly 120 known missense mutations of the *HMBS* gene, including the four missenses of this study, have so far been characterized in a prokaryotic system *in vitro* [7,24,25]. Indeed, discrepancies seem to exist between *in vitro* and *in vivo* enzyme activities as discussed earlier. This problem could be solved in the future by expression of mutant enzymes in eukaryotic systems. Nevertheless, the current prokaryotic expression systems such as the one used in this study have proven to be effective tools in understanding the impact of individual mutations on enzyme activity and consequently, the AIP disease.

During the final preparation of this manuscript, the structure of human HMBS was published [26]. The superposition of the *E. coli* and human structures shows strong similarities. The structural interpretation of human mutants in the *E. coli* enzyme is fully compatible with that in the human enzyme in light of the new data.

Acknowledgments

We would like to thank the patients and their relatives for participation in the study and Mr. Sulejman Ahmetovic for his excellent technical assistance. This work was supported by the Ministry of Education, Sport and Youth of Czech Republic, the granting agency of Charles University; contract grant number: MSM0021620806, 1M6837805002 and GAUK 257540 54007 to D.U. and P.M. E.I.M. is a recipient of grants from the Hartmann-Muller Stiftung (No. 1187) and from the Velux Stiftung (Project No. 480).

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PUBLICATION E

Acute intermittent porphyria - impact of mutations found in the hydroxymethylbilane synthase gene on biochemical and enzymatic protein properties *(Ulbrichova D et al., 2009)*

the FEBS Journal

Acute intermittent porphyria – impact of mutations found in the hydroxymethylbilane synthase gene on biochemical and enzymatic protein properties

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Keywords
acute intermittent porphyria; heme; hydroxymethylbilane synthase; porphobilinogen deaminase; porphyria

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(Received 25 November 2008, revised 28 January 2009, accepted 2 February 2009)

doi:10.1111/j.1742-4658.2009.06946.x

Acute intermittent porphyria is an autosomal dominantly inherited disorder, classified as acute hepatic porphyria, caused by a deficiency of hydroxymethylbilane synthase (EC 2.5.1.61, EC 4.3.1.8, also known as porphobilinogen deaminase, uroporphyrinogen I synthase), the third enzyme in heme biosynthesis. Clinical features include autonomous, central, motor or sensory symptoms, but the most common clinical presentation is abdominal pain caused by neurovisceral crises. A diagnosis of acute intermittent porphyria is crucial to prevent life-threatening acute attacks. Detection of DNA variations by molecular techniques allows a diagnosis of acute intermittent porphyria in situations where the measurement of porphyrins and precursors in urine and faeces and erythrocyte hydroxymethylbilane synthase activity is inconclusive. In the present study, we identified gene defects in six Czech patients with acute intermittent porphyria, as diagnosed based on biochemical findings, and members of their families to confirm the diagnosis at the molecular level and/or to provide genetic counselling. Molecular analyses of the hydroxymethylbilane synthase gene revealed seven mutations. Four were previously reported: c.76C > T, c.77G > A, c.518G > A, c.771 + 1G > T (p.Arg26Cys, p.Arg26His, p.Arg173Gln). Three were novel mutations: c.610C > A, c.675delA, c.750A > T (p.Gln204Lys, p.Ala226ProfsX28, p.Glu250Asp). Of particular interest, one patient had two mutations (c.518G > A; c.610C > A), both located in exon 10 of the same allele. To establish the effects of the mutations on enzyme function, biochemical characterization of the expressed normal recombinant and mutated proteins was performed. Prokaryotic expression of the mutant alleles of the hydroxymethylbilane synthase gene revealed that, with the exception of the p.Gln204Lys mutation, all mutations resulted in little, if any, enzymatic activity. Moreover, the 3D structure of the *Escherichia coli* and human protein was used to interpret structure–function relationships for the mutations in the human isoform.

Acute intermittent porphyria (AIP; Online Mendelian Inheritance in Man database®: 176000) represents the most frequent type of acute porphyria throughout the world, with the exception of South Africa and Chile, where variegate porphyria is prevalent [1]. This autosomal dominantly inherited disorder, classified as acute

Abbreviations
AIP, acute intermittent porphyria; DGGE, denaturing gradient gel electrophoresis; GST, glutathione *S*-transferase; HMBS, hydroxymethylbilane synthase; PBG, porphobilinogen; TAE, Tris–acetic acid-EDTA buffer; TCA, trichloroacetic acid; URO I, uroporphyrin I.

hepatic porphyria, is characterized by a deficiency of hydroxymethylbilane synthase, the third enzyme in heme biosynthesis [2]. Inheritance of one copy of a mutated allele decreases enzyme activity by approximately 50%.

Expression of the disease is highly variable, determined in part by environmental, metabolic and hormonal factors that induce the first and rate-limiting enzyme of heme biosynthesis in the liver, δ-aminolevulinic acid synthase. The upregulated activity of this enzyme increases the production of the potentially toxic porphyrin precursors, δ-aminolevulinic acid and porphobilinogen (PBG) [3]. Clinical expression of the disease is associated with an acute neurological syndrome accompanied by acute attacks. These are manifested by a wide variety of clinical features, including autonomous, central, motor or sensory symptoms. However, the most common clinical presentation is abdominal pain caused by neurovisceral crises [4]. Individuals differ from each other with respect to their biochemical and clinical manifestations, and approximately 90% of AIP carriers remain asymptomatic throughout life [5].

Human hydroxymethylbilane synthase (HMBS; EC 2.5.1.61, EC 4.3.1.8, also known as porphobilinogen deaminase, uroporphyrinogen I synthase) is encoded by a single gene located on chromosome 11 [6], assigned to the segment 11q24.1-q24.2 of the long arm [7]. The *HMBS* gene is divided into 15 exons of 39–438 bp in length and 14 introns of 87–2913 bp in length and spans approximately 10 kb of DNA [8]. HMBS is the third enzyme of the heme biosynthetic pathway. Two isoenzymes, 42 kDa housekeeping and 40 kDa erythroid-specific, are independently expressed [9–11]. The housekeeping isoform consists of 361 amino acids, containing an additional 17 amino acid residues at the N-terminus compared to the erythroid variant, which consists of 344 amino acids. [10,11]. HMBS isoforms from several different species have been studied and their enzymatic and kinetic properties have been described [12,13]. The crystallographic structure of HMBS from *Escherichia coli* [14,15] and human [16] has been determined.

The diagnosis of AIP is crucial for the prevention of life-threatening acute attacks among both symptomatic and asymptomatic carriers. In the majority of acute attacks, the concentration of urinary PBG is dramatically increased (20- to 50-fold compared to normal values) [17], but biochemical diagnosis is not reliable in all cases. Therefore, molecular screening techniques have become established as the ultimate diagnostic tool.

The prevalence of symptomatic disease varies in the range 1–10 per 100 000 but, due to frequent misdiagnosis and incomplete penetrance, it may be much higher. No statistical data exist for the prevalence of AIP in the Czech Republic. Establishing the diagnosis of porphyria can be difficult because different types of porphyria often reveal uncharacteristic clinical symptoms, leading to misdiagnosis. Additionally, patients with acute attack symptoms and asymptomatic carriers or asymptomatic carriers and healthy individuals can have similar measured values of porphyrins and their precursors [18]. Together with biochemical diagnoses, much effort is dedicated to the identification of clinically asymptomatic mutation carriers, particularly in families with AIP-affected individuals. The most powerful and coveted diagnostic tool in recent years comprises the detection of DNA sequence variation by molecular techniques. The search for the disease-causing mutation in each affected family is an important tool for individualized medicine, allowing for careful drug prescription and acute attack prevention. Currently, more than 300 mutations in the *HMBS* gene leading to AIP are known [19]. Mutations are equally distributed throughout the *HMBS* gene, and no prevalent site for mutation has been identified. In Czech and Slovak patients, nine different mutations have been described to date.

The present study aimed to identify gene defects in newly-diagnosed AIP patients and their families aiming to provide early genetic counselling. We report seven mutations: four previously described and three novel mutations. Prokaryotic expression of the *HMBS* mutant alleles revealed that, with the exception of one case, all mutations lead to little, if any, enzymatic activity. Moreover, the 3D structure of the *E. coli* and newly-determined human protein 3D structure was used to interpret structure–function relationships for the mutations in the human isoform.

Results and Discussion

In the present study, six patients who were newly diagnosed with AIP were studied. Overall, 33 individuals from their families were screened and nine carriers of an affected *HMBS* gene were identified. These results were used for genetic counselling within the families. *HMBS* genes of all probands, including all encoding sequences and exon/intron boundaries, were screened for DNA variations. In the first phase of the study, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified exonic and flanking intronic sequences was used as a pre-screening method. DGGE is an effective method that allows the screening of several

samples at one time. However, it is necessary to sequence the specific PCR product to pinpoint the DNA variation exactly. Six samples with abnormal patterns suggesting mutations were detected (Fig. 1). These mutations were subsequently confirmed by direct sequencing in both directions. Of the identified mutations, three were novel, including two missense mutations c.610C > A (p.Gln204Lys) and c.750A > T (p.Glu250Asp) and one small deletion c.675delA (p.Ala226ProfsX28), leading to the formation of a STOP codon after 28 completely different amino acids compared to the original sequence. Four of the identified mutations were previously reported (c.76C > T, c.77G > A, c.518G > A, c.771 + 1G > T) (p.Arg26Cys, p.Arg26His, p.Arg173Gln) [20–23]. One patient had two mutations, p.Arg173Gln and p.Gln204Lys (Fig. 2) and both were located in exon 10 of the same allele, which is a rare molecular defect of *HMBS* gene. All family members were offered screening for the individual mutation.

To study the impact of the various mutations on protein structure and functional consequences, mutated proteins were expressed in *E. coli* and enzymatic properties were characterized. Measurement of the activity of these mutant proteins helps to distinguish mutations from rare polymorphisms as well as to establish causality between the genetic defect and the disease.

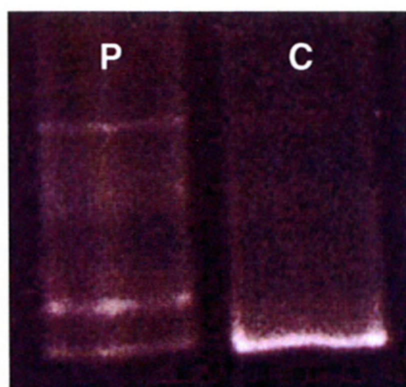

Fig. 1. Example of the abnormal pattern of DGGE-based mutation screening of the *HMBS* gene. Lane P, patient; lane C, negative control. DGGE of exon 10 was performed on a linearly increasing denaturing gradient polyacrylamide gel of 50–80% of denaturant (7 M urea and 40% deionized formamide). Electrophoresis was performed at 60 °C, 150 V for 3 h in 1× TAE buffer. In the case of the heterozygous mutated carrier, a specific exhibition of a four-band pattern was observed. The two lower bands represent the normal and mutated homoduplexes and, the upper bands correspond to the two types of the normal/mutated heteroduplexes. In this patient, an abnormal four-band pattern suggesting a DNA variation was detected only in one fragment of exon 10.

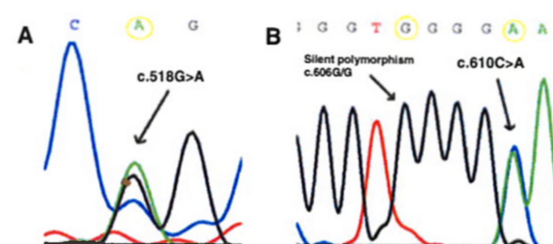

Fig. 2. Two mutations detected in the *HMBS* gene of an AIP patient detected by sequencing analysis. Two point mutations, a previously reported mutation c.518G>A (p.Arg173Gln) and the novel mutation c.610C>A (p.Gln204Lys), were identified in exon 10. After further investigation, both mutations were found to be located on the same allele of this exon.

This was especially interesting in the unique case where two mutations, p.Arg173Gln and p.Gln204Lys, were both located on the same allele. Because mutation c.771 + 1G > T, which causes a donor splice site defect, was not located in the coding sequence, it was not included in the expression and subsequent enzymatic analyses.

All the recombinant expressed and purified proteins were inspected by SDS/PAGE. Both the wild-type enzyme as well as those with introduced mutations, p.Arg26Cys, p.Arg26His, p.Arg173Gln and p.Gln204Lys, displayed homogeneous bands on SDS/PAGE before and after thrombin digest of the glutathione *S*-transferase (GST) tag (see Fig. S1). As expected for the enzyme with the small deletion mutation p.Ala226ProfsX28, but surprisingly for the enzyme with the missense mutation p.Glu250Asp, only a very light band was observed before GST cleavage. After GST cleavage, the band almost entirely disappeared, suggesting strong impairment of protein structure stability. Both wild-type and mutant recombinant HMBS enzymes, with the exception of the truncated protein (p.Ala226ProfsX28), were similar in size (approximately 68 kDa with the GST tag and 42 kDa after GST cleavage). The p.Ala226ProfsX28 mutant protein was approximately 53 kDa before cleavage and strongly degraded after thrombin digest.

HMBS enzymatic activity was measured for mutant and wild-type enzymatic and expressed as percentage of activity compared to that of wild-type enzyme. Five of the mutants, p.Arg26Cys, p.Arg26His, p.Arg173Gln, p.Ala226ProfsX28 and p.Glu250Asp, showed little, if any, enzymatic activity. By contrast, one mutant, p.Gln204Lys, exhibited approximately 46 ± 0.72% of wild-type activity (Table 1). The observation of low residual activity for most mutations was consistent with the expected approximately 50% decrease in final

Table 1. Mutations in the *HMBS* gene in Slavic AIP patients. Activity measurements were performed with HMBS GST-fusion protein of recombinant enzymes carrying selected mutations and compared with the HMBS GST-fusion protein of the wild-type form expressed simultaneously under identical optimal conditions (50 mM Tris–HCl, pH 8.2, 37 °C for 1 h). All measurements were performed in triplicate for all recombinant enzymes and wild-type. Values are expressed as the arithmetic mean.

Amino acid substitution	Nucleotide change	Location	Residual activity (% of wild-type)	Reference
p.Arg26Cys	c.76C>T	Exon 3	0.3	Kauppinen *et al.* [20]
p.Arg26His	c.77G>A	Exon 3	0.2	Llewellyn *et al.* [21]
p.Arg173Gln	c.518G>A	Exon 10	0.15	Delfau *et al.* [22]
p.Gln204Lys	c.610C>A	Exon 10	46	Present study
p.Ala226ProfsX28	c.675delA	Exon 12	0.05	Present study
p.Glu250Asp	c.750A>T	Exon 12	0.5	Present study
Deletion of exon 12	c.771 + 1G>T	Intron 12	–	Rosipal *et al.* [23]

activity of HMBS in cells when affected by acute intermittent porphyria. These findings further support the causality of those mutations in the *HMBS* gene and their association with the AIP disorder. However, even alleles with significant residual activity (11–42% of the normal mean) have been linked to the porphyria disorder [24]. In the observed rare case of two mutations located on the same allele (one having low residual activity and the second one having relatively high residual activity), further investigation of the contribution of the mutation p.Gln204Lys was required. Given the extremely low residual activity of most of the mutant proteins, further kinetic studies of those mutants were not performed.

Comparison of thermal protein stability, pH optimum and kinetic properties of the p.Gln204Lys mutant protein with wild-type HMBS aimed to confirm or negate the causality of the second mutation. As shown in (Fig. 3A), a slight decrease in K_m value in the mutant protein (3.42 μM) compared to that of the wild-type (4.45 μM) was observed; *V*max, however, was decreased three-fold to 0.66 nmol·min^{-1} compared to 2.14 nmol·min^{-1} in the wild-type enzyme. Heat inactivation studies indicated that the recombinant HMBS enzyme is very stable overall because the wild-type enzyme lost approximately 30% of its activity after a pre-incubation period of 240 min at 65 °C (Fig. 3B). This is in agreement with the structure possessing a large number of ion pairs that may contribute to the heat stability of the enzyme [15]. The half-life of the mutant enzyme was approximately 100 min (Fig. 3B), indicating that the protein had approximately one-third of the stability of the wild-type enzyme. The pH optimum for both the wild-type and mutant proteins was pH 8.2 (Fig. 3C), indicating that the pH sensitivity of the mutant was unchanged. From these findings, we concluded that the p.Gln204Lys mutation has an impact on protein function and structure, and therefore can be associated with AIP. In the case of two combined mutations, both located on the same allele, the mutation p.Arg173Gln has a much more severe effect on enzyme function, which is close to zero, but the p.Gln204Lys mutation increases the negative effect, particularly on the protein stability.

The human 3D structure of HMBS has been determined and the function of the important residues analyzed in detail [16]. The enzyme is monomeric in solution and organized into three domains. The catalytic active site cleft contains the dipyrromethane cofactor. The active site is located between the N-terminal and central domains and the dipyrromethane cofactor is covalently linked to Cys261. The interaction of the cofactor with the enzyme side chains is well understood. The position of the observed mutations in the 3D structure is shown in Fig. 4. The structure of the *E. coli* homolog [14] and the mode of interaction with the cofactor are almost identical. Three hundred and twenty prokaryotic and 46 eukaryotic HMBS nonredundant sequences were found (October 2008) in the UniProt and ENSEMBL databases. Owing to the availability of two 3D structures with diverse sequences (39% identity), a very precise sequence alignment can be achieved [25]. Thus, the effect of a mutation can be evaluated by observing the function of the residue conserved in the structure and by assessing its conservation in the sequence in relation to structure and evolution.

p.Arg26Cys, p.Arg26His

Analysis of the active site shows that Arg26 is close to the C2 ring of dipyrromethane (Fig. 4B) [14,16] and potentially is able to protonate the amine group

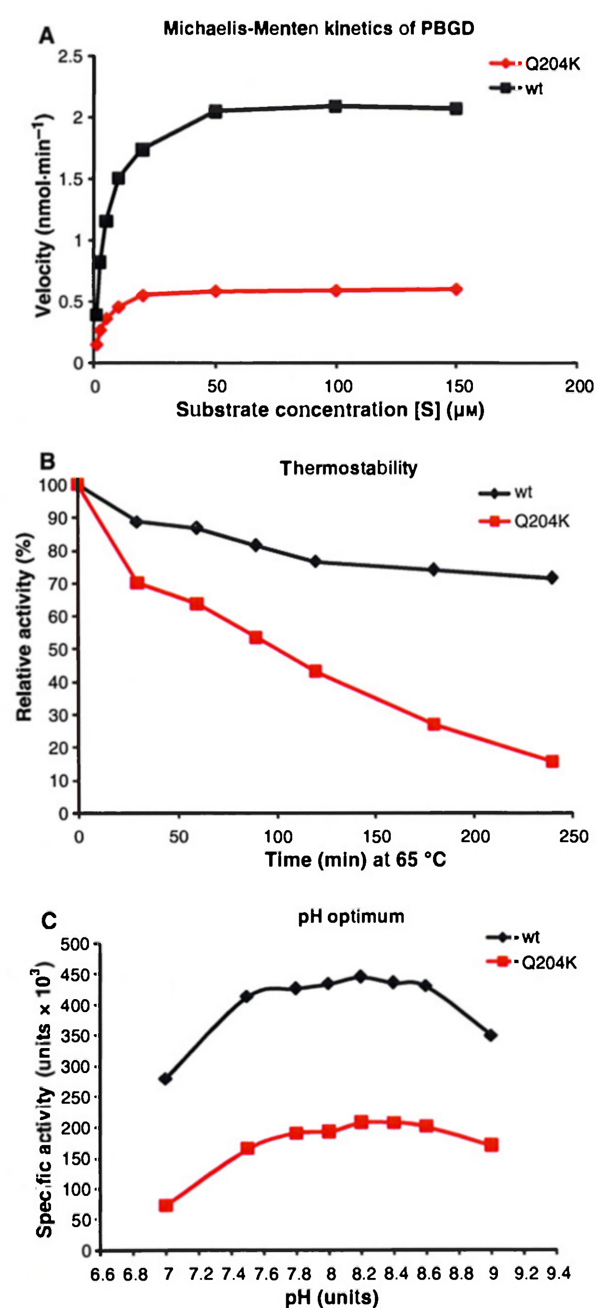

Fig. 3. *In vitro* enzymatic studies of wild-type (wt) HMBS and HMBS with mutation p.Gln204Lys. (A) Michealis–Menten kinetics of normal and mutated HMBS. Determination of kinetic constants K_m and V_{max} was performed under optimal conditions (50 mM Tris–HCl, pH 8.2). K_m of p.Gln204Lys mutant and wild-type HMBS was estimated to be 3.42 μM and 4.45 μM, respectively. V_{max} of p.Gln204Lys mutant (0.66 nmol·min^{-1}) was decreased by more than three-fold compared to that of wild-type HMBS (2.14 nmol·min^{-1}). The results were calculated as the arithmetic mean of two independent assays. (B) Thermostability of normal and mutated HMBS. Purified wild-type and mutant HMBS were incubated at 65 °C and pH 8.2. HMBS enzyme activities were measured at the indicated times. The wild-type enzyme lost approximately 30% of its activity after 240 min, whereas the half-life of the mutant enzyme was approximately 100 min. The results are expressed as the percentage of initial activity based on mean of two independent assays. In the graph, each point represents the mean of two measurements. (C) The pH optimum of normal and mutated HMBS. The pH optimum was measured in 50 mM Tris–HCl. We obtained corresponding values for both the wild-type and mutant protein at pH 8.2. The results were calculated as the mean of two independent assays. In the graph, each point represents the mean of two measurements.

of the incoming porphobilinogen. Arg26 is conserved absolutely in all available sequences. Its site-directed mutation to alanine leads to inactivation of HMBS [16]. Therefore, it can be inferred that the patient's mutations of Arg26 to Cys or His may lead to the loss of interactions with the cofactor, which explains well our observation of the almost entire loss of enzyme activity. Although the imidazole group in the p.Arg26His mutation could potentially interact with the porphobilinogen in a similar manner to the Arg guanidino group, the new side chain might be too short to do so.

p.Arg173Gln

The amide nitrogen of Arg173 forms a 2.7 Å hydrogen bond with the carboxyl oxygen of the propionic acid side chain of the C1 ring of the cofactor (Fig. 4B) [14,16]. Arg173 is an invariant residue in all known sequences. The mutation of Arg173 to Gln results in an apo form of the enzyme that is incapable of catalysis [26]. The missense mutant p.Arg173Trp in AIP patients has also been found to be inactive [27]. The residual activity of the patient's p.Arg173Gln mutant in our measurements (< 1% of wild-type activity) is consistent with a previous study [22]. Most likely, the mutant is unable to interact properly with the cofactor.

p.Gln204Lys

Gln204 is exposed on the surface of the central domain, remote from the active site. The only residue side chain in its close proximity is Glu135 (Fig. 4D). Both residues are only moderately conserved in the eukaryotic sequences (approximately 85%) and are broadly variable in the prokaryotic ones, although the position of Glu204 is never occu-

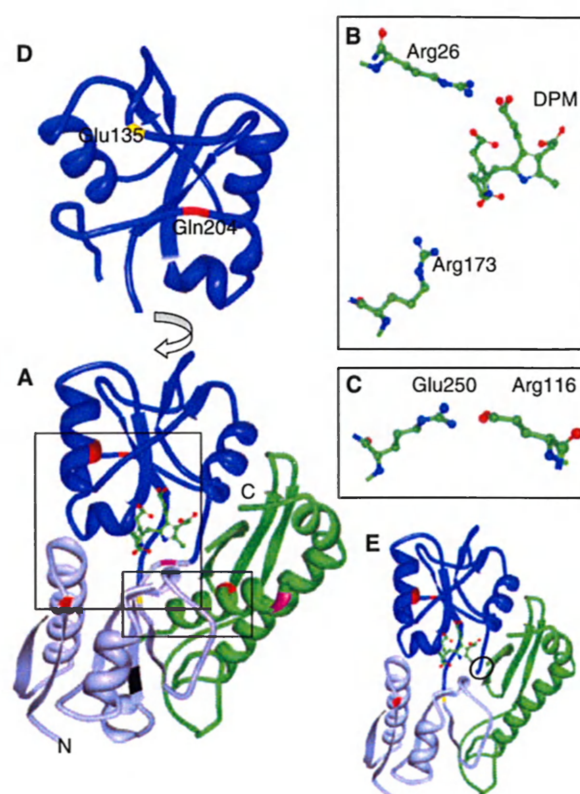

Fig. 4. 3D structure of human HMBS indicating the positions of the mutations observed in the patients. The N-, central and C-domains are shown in silver, blue and green, respectively; the positions of the mutated amino acids are indicated in red; and the position of the last amino acid before the frameshift is shown in black. The interacting partners of the mutated residues are shown in yellow and the dipyrromethane (DPM) cofactor is shown in the ball and stick representation. Magenta indicates the beginning and the end of the region coded by exon 12. The N- and C-termini are labeled N and C, respectively. (A) Global view in ribbon representation. The side chain interactions of the mutated residues from the boxed regions are expanded in (B) and (C). (D) Central domain rotated by approximately 180° with respect to (A). (E) The structure with the excised region coded by exon 12. The black oval indicates where the chains were able to connect after the deletion.

pied by a positively charged residue. Both residues lie in loops of the structure, which is different in human and *E. coli* enzymes. All these observation indicate that the mutation is in a quite variable region. Nevertheless, it leads to a substantial reduction of activity (46 ± 0.72% compared to the wild-type) and to a reduction of thermal stability. It is likely that the introduction of the positive charge of the lysine amino group attracts the carboxyl of Glu135 and brings the two loops into close proximity, which may destabilize the enzyme.

p.Ala226ProfsX28

The observed single base deletion in the present study causes a frameshift resulting in the incorporation of 28 completely different residues and premature termination. The mutated protein consists of 253 amino acids (361 in wild-type). The abrogated mutant lost the end of one β-sheet, one helix at the end of the central domain and the entire C-terminal (Fig. 4A). In general, such truncation leads to an unstable and inactive protein, which is likely to be rapidly degraded by the proteosome. As expected, the stability of the expressed truncated HMBS was devoid of any enzymatic function and its folding was severely impaired, as determined from results obtained by SDS/PAGE.

p.Glu250Asp

Glu250 forms an ion pair with Arg116 (Fig. 4B). The same pair is also found in the *E. coli* enzyme. Both residues are conserved in all sequences with no exception. The interaction fixes the C-terminal domain to the interdomain hinge whose mobility is important for access of the substrate to the active site [16]. The novel mutation p.Glu250Asp found in our patient was completely inactive. Mutations p.Arg116Trp and p.Arg116Gln in the acceptor residue in the ion pair, reducing their ability to form ionic interaction, have previously been found in AIP patients [28,29]. The effect of the new mutation p.Glu250Asp is unexpected because the change from Glu to Asp results only in a subtle change: the shortening of the bridge length by one methylene group. The abolition of the activity demonstrates the importance of an exact geometry in the interior of the enzyme.

c.771 + 1G>T

Several different single base changes at position 771 + 1 have been reported in AIP patients. The mutations c.771 + 1G>A and c.771 + 1G>C were responsible for the deletion of the entire exon 12, although, surprisingly, a protein product was still obtained [30–32]. Exon 12 codes for amino acids 218–257 and its deletion results in the excision of one β-sheet and two α-helixes. Cys261, to which the dipyrromethane cofactor is covalently attached, remains preserved (Fig. 4E). In agreement with the previous studies [30–32] on mutants without exon 12, the function of our mutant c.771 + 1G>T is expected to be completely abolished. Figure 4A shows that the lost

Table 2. Biochemical data of the Czech patients. ALA, 5'-aminolevulinic acid; m.i., markedly increased.

Patient	ALA ($mg{\cdot}100\ mL^{-1}$)[a]	PBG ($mg{\cdot}100\ mL^{-1}$)[a]	Total porphyrins	
			$\mu g{\cdot}L^{-1}$	$\mu g\ day^{-1}$
1	m.i.[b]	m.i.[b]	m.i.[b]	m.i.[b]
2	0.7	1.89	206	494
3	10.76	10.7	835	534
4	6.68	25.17	906	3488
5	3.82	7.79	919	1050
6	2.64	6.72	2505	1754
Normal values	< 0.45	< 0.25	< 80	< 200

[a] Maximal values measured in urine. [b] Data collected in local county hospital (values not available).

segment is connected to the rest of the protein by two irregular loops (the C-terminal one is mobile and invisible in the crystal structure). Figure 4C indicates that, despite the large excision, the chains could reconnect without major distortions. This may explain the stability of the expressed proteins.

In summary, the identification of three new and four previously reported mutations in the *HMBS* gene has increased our understanding of the molecular basis and heterogeneity of AIP. The present study demonstrates that *in vitro* expression of mutations in the *HMBS* gene can provide valuable information with respect to the interpretation of clinical, biochemical and genetic data and establishing a diagnosis of AIP. The use of the crystal structure of HMBS for structure–function correlations of real mutations in the human enzyme helps our understanding of the molecular basis of enzymatic defects. Moreover, the detection of causal mutations within affected lineages is very important for asymptomatic carriers, who can steer clear of precipitating factors, thus avoiding life-threatening acute attacks.

Experimental procedures

Subjects

The diagnosis of AIP, which lead to patients' DNA being brought to our laboratory for molecular diagnosis, was made on the basis of clinical features typical for AIP and the excretion pattern of porphyrin precursors. Out of six index patients studied, five were women. The most prominent symptom in all patients was severe abdominal pain. Table 2 shows the highest values for porphyrin excretion for each patient in the present study, although these data are not necessarily correlated with the stage of the disease. Fecal porphyrins were not increased.

The study was performed according to guidelines approved by the General Faculty Hospital Ethics Committee in Prague (approved 2003). Informed consent was obtained from each patient, and the study was carried out in accordance with the principals of the Declaration of Helsinki.

Isolation and amplification of DNA

Genomic DNA was extracted from peripheral blood leukocytes anticoagulated with EDTA according to a standard protocol. Coding sequences of all exons 1–15 with flanking exon/intron boundaries were amplified. The PCR/DGGE primers were designed as described previously [33]. The PCR reactions of exon 1–15 were amplified in a total volume of 50 μL that included 1× Plain PP Master Mix (Top-Bio Ltd, Prague, Czech Republic) and 0.4 mM of each primer. Thermal cycling conditions (DNA Engine Dyad Cycler, MJ Research, Waltham, MA, USA): initial denaturation was performed at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55.8, 59.3 or 62.9 °C for 30 s (62.9 °C for exons 1 and 11, 59.3 °C for exons 3 and 5/6; 55.8 °C for the remaining exons) and elongation at 72 °C for 40 s, followed by a final step at 72 °C for 5 min, 95 °C for 5 min and 72 °C for 5 min.

DGGE analysis

Fourteen different PCR products were designed to cover the entire coding sequence, including approximately 50 bp upstream and downstream of each exon/intron boundary of the *HMBS* gene. The complete DGGE setup was optimized as described previously [34]. DGGE was performed on linearly increasing denaturing gradient polyacrylamide gels (35–90%; denaturant was 7 M urea and 40% deionized formamide). PCR products were analyzed using DCode™ (Bio-Rad, Hercules, CA, USA). Gels were run at 60 °C, 150 V for 3–6 h in 1× TAE buffer.

DNA sequencing

The PCR-amplified double-stranded DNA products were purified from an agarose gel using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). Exons were sequenced in both directions on the automatic sequencer ABI PRISM 3100/3100-Avant Genetic analyzer (Applied Biosystems, Foster City, CA, USA) using the ABI PRISM BigDye terminator, version 3.1 (Applied Biosystems).

Allelic mutation localization

To identify allelic localization of two mutations found in exon 10, used molecular cloning techniques were employed. After PCR of exon 10, the insert was ligated into the

pCR®4-TOPO vector from the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) and then transformed into *E. coli* TOP10 competent cells (Invitrogen). Plasmid DNA was amplified and DNA from ten different colonies was isolated using the QIAprep Spin Miniprep Kit (Qiagen). DNA was sequenced with primer T7 using the TOPO TA Cloning Kit for Sequencing (Invitrogen).

Plasmid construction and mutagenesis

Total RNA was extracted from peripheral leukocytes isolated from EDTA-anticoagulated whole venous blood (Qiagen). cDNA sequences were obtained by RT-PCR (SuperScript III; Invitrogen) of total RNA using oligo(dT)20 (Invitrogen) as the primer in the first step. The cDNA for HMBS, with restriction sites *Bam*HI and *Xho*I, was amplified using specific primers in the second step: cDNA *Bam*HI forward, 5′-ATA TGG ATC CAT GTC TGG TAA CGG-3′, cDNA *Xho*I reverse, 5′-TAT ACT CGA GTT AAT GGG CAT CGT TAA-3′. Human cDNA for HMBS was ligated into the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden) and transformed into *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA, USA). Plasmid DNA was amplified and isolated using the QIAprep Spin Miniprep Kit (Qiagen). Site-directed mutagenesis to generate the mutations was performed with the mutagenic primers (see Table S1) using the QuikChange® Site-directed Mutagenesis Kit (Stratagene). Successful mutagenesis was confirmed by sequencing.

Protein expression

All the proteins were expressed as GST-fusion proteins. BL21 cells were grown at 37 °C in TB medium containing ampicillin (100 μg·mL^{-1}). An overnight culture was used to inoculate the growth medium. The cells were induced by isopropyl thio-β-D-galactoside (final concentration of 0.5 mM) at D_{600} in the range 0.4–0.6. The bacterial culture was grown under aerobic conditions for 4 h at 30 °C. Bacterial cells were harvested by centrifugation at 4 °C for 10 min at 6000 *g*.

Protein purification

All purification steps were carried out at 4 °C. Washed cells were resuspended in the lysis buffer: NaCl/P_i (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) and Triton X-100 (0.5% v/v; Sigma-Aldrich). The cells were lysed by lysozyme (1 mg·mL^{-1}) on ice with gentle shaking for 1 h. The lysate was sonicated five times for 3 min with a 3-min pause in each cycle. Sonicated cells were centrifuged at 4 °C for 30 min at 33 000 *g*. The supernatant was loaded onto the glutathione sepharose 4B column (Amersham Biosciences, Piscataway, NJ, USA) and washed three times using wash buffer [20 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40+ (Sigma-Aldrich), pH 8.2]. Proteins were eluted in freshly prepared 50 mM Tris–HCl (pH 8.3) buffer with 20 mM gluthatione (Sigma-Aldrich). Thrombin digest was performed by gentle shaking of protein mixed with thrombin (ICN Biomedicals, Costa Mesa, CA, USA), at a concentration of 20 U·mg^{-1} protein, overnight at 20 °C. Glycerol was added to a final concentration of 20%, and the aliquots were frozen and stored at −80 °C. All the results obtained from the protein purification and digestion were confirmed by SDS/PAGE.

HMBS enzymatic assay

The HMBS activity assay was optimized as described previously [35,36]. The protein (1 and 2.5 μg) was diluted with the incubation buffer (50 mmol·L^{-1} Tris, 0.1% BSA, 0.1% Triton, pH 8.2) to a final volume of 360 μL. After pre-incubation at 37 °C for 3 min, 40 μL of 1 mM PBG (ICN Biomedicals) was added, and samples were incubated in dark at 37 °C. The reaction was stopped by adding 400 μL of 25% trichloroacetic acid (TCA). Samples were exposed to photooxidation for 60 min under daylight and then centrifuged for 10 min at 1500 *g*. For determination of pH optima, HMBS activity was measured throughout the pH range 7.0–9.0. For determination of temperature stability, the relative stabilities of recombinant proteins were compared when incubated at 65 °C (pH 8.2). For determination of K_m, concentrations of PBG in the range 1–150 μM were used in the final reaction mixture. The incubation was carried out for different times (0–8 min). The reaction proceeded linearly with time under all kinetic experimental conditions. To determine enzymatic activity, the fluorescence intensity was measured using a Perkin Elmer LS 55 spectrofluorometer (Perkin Elmer Instruments LLC, Shelton, CT, USA) immediately thereafter. Uroporphyrin I (URO I; ICN Biomedicals) was used as the standard and 12.5% TCA as a blank. The exact concentration was determined at room temperature by measuring A_{405} and calculated as A_{405}/ε (ε − 505 × 10^3 L·cm^{-1}·mol^{-1}). A standard curve in the linear range of fluorescence emission intensity and the concentration of URO I in 12.5% TCA was created. Activity measurements were performed in triplicate; K_m, pH optimum and temperature stability determinations were performed in duplicate. The negative control was included. The spectrofluorometer wavelength settings were excitation at 405 nm and the emission at 599 nm for URO I.

Sequences and structure–function correlation

HMBS sequences were extracted from UniProt (http://www.uniprot.org) and Ensembl (http://www.ensembl.org) databases. They were identified using PSI-BLAST [37] with

the inclusion threshold $E < 0.001$ run to equilibrium and the query sequences of the human and *E. coli* proteins (UniProt IDs: HEM3_HUMAN and HEM3_ECOLI). The extracted sequences were aligned with 3D T-COFFEE software [25] using the 3D structures of *E. coli* [14] and human [16] HMBS as templates (Protein Data Bank code: 3ecr and 1pda). The 3D structures were displayed and examined with the Molecular Biology Toolkit platform [38], using the above Protein Data Bank coordinates.

Acknowledgements

We appreciate the participation of all patients and their relatives in the study. We would like to thank to Dr Linda J. Roman (Department of Biochemistry, UTHSC at San Antonio, TX, USA) for her critical review of the manuscript. This research was supported by the Ministry of Education, Sport and Youth of Czech Republic, The Granting Agency of Charles University; Contract grant number: MSM0021620806, 1M6837805002, GAUK 257540 54007.

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Supporting information

The following supplementary material is available:

Fig. S1. SDS/PAGE analysis of wild-type enzyme and HMBS carrying the p.Arg173Gln and p.Gln204Lys mutations.

Table S1. Primers for mutagenesis.

This supplementary material can be found in the online version of this article.

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PUBLICATION F

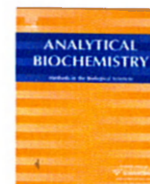
Detection of DNA Variations in the Polymorphic Hydroxymethylbilane Synthase Gene by High-Resolution Melting Analysis *(Ulbrichova Douderova D et al., in press in Anal Biochem 2009)*

Analytical Biochemistry xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio



Detection of DNA variations in the polymorphic hydroxymethylbilane synthase gene by high-resolution melting analysis

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ARTICLE INFO

Article history:
Received 1 June 2009
Available online xxxx

Keywords:
Acute intermittent porphyria
Hydroxymethylbilane synthase
Porphobilinogen deaminase
Porphyria
High-resolution melting
LightScanner

ABSTRACT

Acute intermittent porphyria (AIP) represents the most frequent type of acute porphyria. The underlying cause is a defect in the hydroxymethylbilane synthase (HMBS) gene. Diagnosis of AIP is crucial for preventing life-threatening, acute attacks among both symptomatic and asymptomatic carriers. We established the diagnostic tool, high-resolution melting (HRM), for diagnosing AIP. Of 13 amplicons amplified by PCR in the presence of the LCGreen Plus dye, 4 showed polymorphic backgrounds. The ability of the HRM method to detect DNA variations in the HMBS gene was tested on a DNA sample with 10 known mutations by a curve shape scan using the LightScanner instrument. Furthermore, genomic DNA (gDNA) samples from 97 individuals with suspected hepatic porphyria were tested. All possible genotypes from each of four polymorphic amplicons were detected. Each of the 10 mutations tested had an altered melting profile compared with the melting profile of the controls. Screening the group of subjects with suspected hepatic porphyria revealed nine different DNA variations, four of which were novel. In conclusion, HRM is a fast, cost-effective prescreening method for detecting DNA variations in the HMBS gene. Therefore, the screening can be easily applied to a porphyria family if misdiagnosis or rare dual porphyria is suspected.

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Porphyrias are mostly inherited disorders where accumulation and excretion of specific porphyrin and heme precursors lead to clinical features characteristic of these disorders. With the exception of the first enzymatic step, seven partial deficiencies linked to the seven enzymes of the heme biosynthetic pathway have been described. Most of the porphyrias are low-penetrance, autosomal dominant conditions in which clinical manifestation is the result of certain additional factors such as use of some common drugs, nutrition, alcohol abuse, stress, and hormones (e.g., progesterone). Porphyrias can be categorized as hepatic or erythropoietic, depending on the anatomical origin, and acute or cutaneous, depending on the clinical presentation. Four porphyrias are described as acute hepatic porphyries, with the liver being the major site of expression of the defective enzyme.

Acute intermittent porphyria (AIP,[1] OMIM 176000) represents the most frequent type of acute hepatic porphyria worldwide [1]. This disorder is characterized by a partial deficiency in hydroxymethylbilane synthase (HMBS, EC 2.5.1.61), the third enzyme in heme biosynthesis. The inheritance of one copy of the mutated allele decreases enzyme activity by approximately 50% [2]. Human HMBS is expressed from a single gene located on chromosome 11 [3] on the long arm of the segment 11q24.1–q24.2 [4]. HMBS has 15 exons and spans approximately 10 kb of genomic DNA (gDNA) [5].

The prevalence of AIP varies from 1 to 10 per 100,000. However, due to frequent misdiagnosis and incomplete penetrance, it may be much higher [2]. Individual carriers differ biochemically and clinically. It is well known that in approximately 90% of the carriers, the disease remains latent throughout their lives [1]. However, when a carrier enters acute porphyria following exposure to triggering factors, this condition is potentially fatal.

More than 300 mutations in HMBS have been described [6]. Mutations are equally distributed throughout the gene, and no predominant mutational hot spot has been identified. To date, 12 different mutations have been identified in the Czech and Slovak population: c.70G>A (p.Gly24Ser) [7,8], c.76C>T (p.Arg26Cys) [9], c.77G>A (p.Arg26His) [10], c.158_159insA (p.Ile54HisfsX12) [7,8], c.331G>A (p.Gly111Arg) [11], c.518G>A (p.Arg173Gln) [12], c.610C>A (p.Gln204Lys) [13], c.675delA (p.Ala226ProfsX28) [13], c.750A>T (p.Glu250Asp) [13], c.771+1G>T (r.spl?) [7], c.799G>A (p.Val267Met) [7,8], and c.965_966insA (p.Asn322LysfsX36) [14,15].

Identification of AIP heterozygotes is crucial to preventing life-threatening acute attacks among both symptomatic and asymp-

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[1] *Abbreviations used:* AIP, acute intermittent porphyria; HMBS, hydroxymethylbilane synthase; gDNA, genomic DNA; DGGE, denaturing gradient gel electrophoresis; PCR, polymerase chain reaction; HRM, high-resolution melting; PV, porphyria variegata; HC, hereditary coproporphyria; PCT, porphyria cutanea tarda; RFLP, restriction fragment length polymorphism; EDTA, ethylenediaminetetraacetic acid; cDNA, complementary DNA; HPLC, high-performance liquid chromatography.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.07.050

tomatic carriers. The diagnosis of porphyria is based on clinical symptoms, characteristic biochemical findings, and enzyme assays. Due to possible inaccuracies, these tools have recently been implemented as the first step in disease confirmation, whereas molecular techniques are used for the final verification of the gene carrier status [15,16]. In clinical diagnostics, several DNA-based screening methods have been employed to screen gene variations. In our previous study, we used the conventional methods of denaturing gradient gel electrophoresis (DGGE) and direct DNA sequencing for detecting HMBS gene variations [13]. In the current work, we tested a new generation method of amplicon melting using the high-resolution LightScanner. This post-polymerase chain reaction (PCR) method was performed in the presence of a saturating, double-stranded, DNA-binding dye. In the instrument, amplicons were slowly heated until fully denatured while the fluorescence was monitored. Amplicons with a sequence variation resulted in an altered melting curve profile compared with normal control samples [17]. This method has recently undergone significant improvements with respect to reagents, software, and instrumentation. In this regard, the detection of previously known HMBS mutations on a polymorphic background was evaluated. Furthermore, screening of HMBS in 97 subjects diagnosed with acute porphyria was performed. We show here that the high-resolution melting (HRM) method can serve as a useful screening tool of DNA variations, even in amplicons with other polymorphisms.

Materials and methods

Subjects

The diagnosis of porphyria was made based on clinical features typical for each porphyria type and the related specific porphyrin excretion pattern. Of 97 subjects of mostly Slavic origin, 44 were diagnosed with AIP (20 were family members), 17 were diagnosed with porphyria variegata (PV) (3 diagnoses were previously confirmed by sequencing), 5 were diagnosed with hereditary coproporphyria (HC) (2 diagnoses were previously confirmed by sequencing), 5 were diagnosed with porphyria cutanea tarda (PCT) (none was previously diagnosed), and the remaining 26 were included based on nonspecific porphyria diagnoses.gDNA of all subjects was identified, and the study was performed according to the guidelines approved by the General University Hospital ethics committee (Prague, Czech Republic). Informed consent was obtained from each patient, and the study was carried out in accordance with the principles of the Declaration of Helsinki.

Restriction fragment length polymorphism

To determine the polymorphic categories of previously detected polymorphic DNA variations in our patients—g.-64T/C (rs589925), g.3581A/G (rs17075), g.6479T/G (rs1131488), and g.7064C/A (rs1784304)—the genotypes of all DNA samples of the subjects diagnosed with AIP were confirmed by restriction enzyme digestion of appropriate PCR fragment using ApaI, BsmAI, MscI, and HinfI, respectively (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. To introduce a restriction site for the detection of g.6479T/G, we designed a specific mismatch primer: Rev: 5′ aat acg act cac tat agg cag gcc cta cct ggc c 3′. In addition, representative samples of each genotype were sequenced for confirmation. Moreover, in an attempt to define the allelic frequency of such polymorphisms in the general Slavic population, we screened 100 healthy donors using the restriction fragment length polymorphism (RFLP) assay.

PCR and HRM assay conditions

gDNA was extracted from peripheral blood, and then ethylenediaminetetraacetic acid (EDTA) was added, according to the standard protocol, to prevent coagulation. Amplicons were designed to cover the 10-kb fragment of the HMBS gene, encompassing all coding and flanking intronic regions. Primers were designed to avoid the known polymorphisms and minimize undesirable base-pairing interactions. Resulting amplicons, ranging from 219 to 634 bp in length, were generated using primers listed in Supplemental Table S1 (see supplementary material).

The samples were amplified in the presence of a 1 × concentration of the saturating DNA dye LCGreen Plus (Idaho Technology, Salt Lake City, UT, USA), 1 × Plain PP Master Mix (Top-Bio, Prague, Czech Republic), 0.5 μM forward and 0.5 μM reverse primers, 50 ng of DNA template, and PCR-grade water adjusted to a final volume of 10 μl. When preparing the amplicon samples of known polymorphisms, all genotypes were equally included in one run. A similar approach was applied to unknown samples. In addition, where possible, four doublets of each genotype were included as positive controls.

The thermal cycling profile (DNA Engine Dyad Cycler, Bio-Rad, Waltham, MA, USA) was as follows: initial denaturation (94 °C for 5 min); amplification (40 cycles of: 94 °C denaturation for 30 s, 60.5–67.0 °C annealing for 30 s, and 72 °C extension for 40 s); final extension (72 °C for 7 min); final denaturation and reannealing (94 °C for 30 s and 25 °C for 30 s) (Supplemental Table S1). To avoid evaporation, the 96-well plate with PCR reactions was

Table 1
List of DNA variations in HMBS gene tested by HRM (gDNA GenBank M95623.1; cDNA GenBank X04808.1).

Amplicon comprising exon	Mutation	Number(s) of positive samples	Reference(s)	Polymorphism	Reference
1	–	–	–	g.-64C/T (rs 589925)	[18]
3	c.76C>T (p.Arg26Cys), c.77G>A (p.Arg26His)	1, 1	[9,10]	–	-
4	c.95G>C (p.Arg32Pro)	1	[21]	g.3581A/G (rs17075)	[19]
5/6	c.176C>T (p.Thr59Ille)	1	[22]		
10	c.[518G>A;c.610C>A] (p.[Arg173Gln; p.Gln204Lys])[a]	1	[13]	g.6479T/G (rs1131488)	[20]
11	–	–	–	g.7064C/A (rs1784304)	[5]
12	c.675delA (p.Ala226ProfsX28), c.750A>T (p.Glu250Asp), c.771+1G>T (r.spl?)	3,[b] 2,[b] 1	[7,13]	–	–
15	c.965_966insA (p.Asn322LysfsX36), c.972_973insG (p.Arg325AlafsX33)	1, 1	[14,23]	–	–

[a] Complex mutation.
[b] Found in members of one family.

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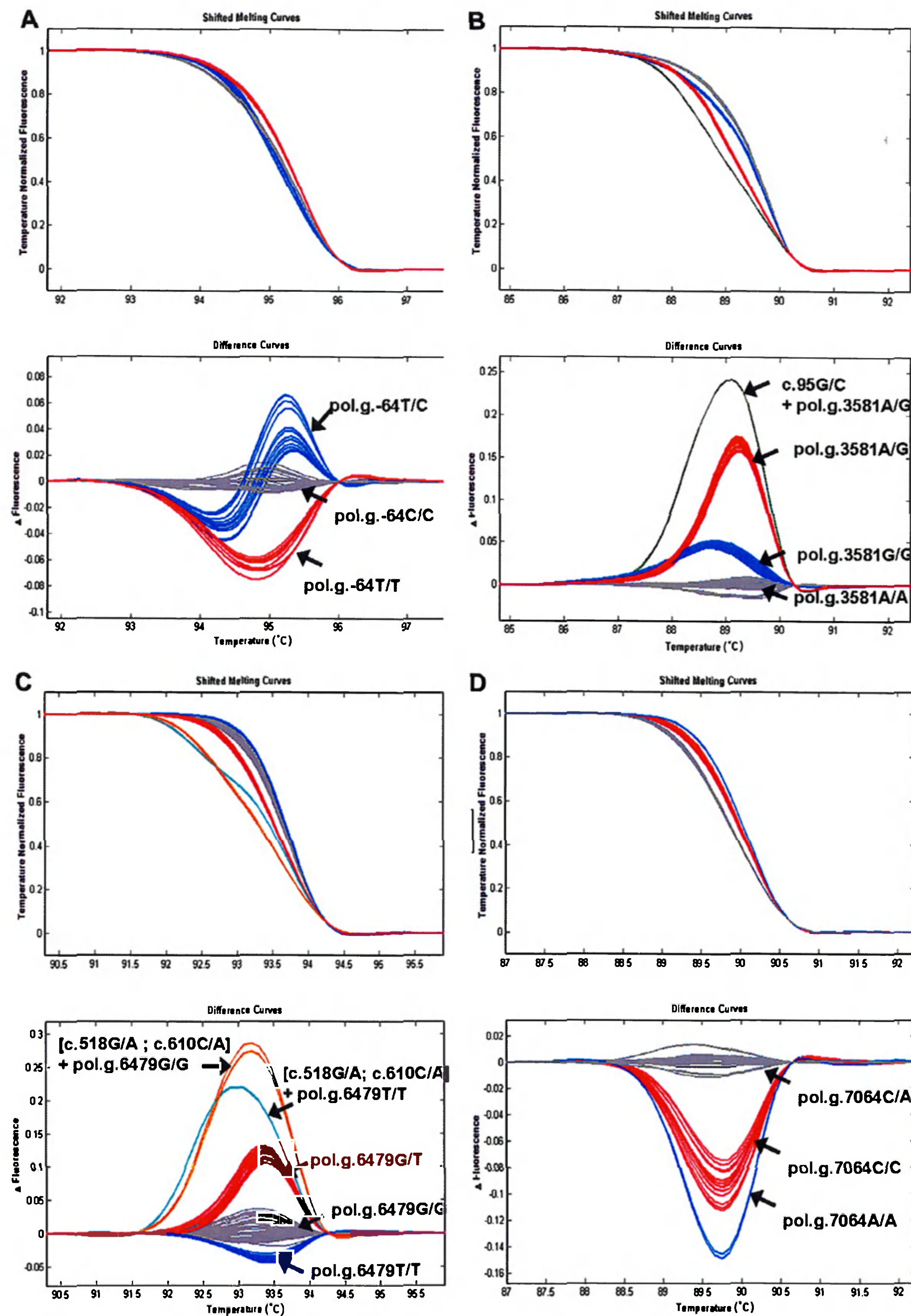

Fig. 1. HRM curves for the HMBS gene amplicons with polymorphic DNA variations obtained using the LightScanner. The upper charts show the normalized and temperature-shifted melting curves. The lower charts show the difference curves of the amplicons with exons 1 (A), 4 (B), 10 (C), and 11 (D). Four different polymorphic DNA variations—g.-64T/C (rs 589925) (A), g.3581A/G (rs17075) (B), g.6479T/G (rs1131488) (C), and g.7064C/A (rs1784304) (D)—are shown. All possible genotypes, two homozygous and one heterozygous, of each polymorphic amplicon were detected by the difference in the curve shape. Moreover, control pathogenic mutations c.95G>C (p.Arg32Pro) in amplicon with exon 4 and c.[518G>A; c.610C>A] (p.[Arg173Gln; p.Gln204Lys]) in amplicon with exon 10 were detected. /, allelic organization.

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overlaid with 15 µl of mineral oil (Sigma–Aldrich, St. Louis, MO, USA). Then the PCR plate was transferred to the LightScanner (Idaho Technology) in which the HRM analyses were performed. The samples were melted by gradually increasing the temperature from 65 to 98 °C at a rate of 0.1 °C/s. All reactions were performed in doublet. The single run time for the melting segment was approximately 15 min, including the instrument's cool-down time.

HRM analyses

Collected data were analyzed with the commercial LightScanner software 1.5 using the Call-IT function supplied with the LightScanner. This patented software intuitively and critically analyzes each sample, defines and groups genotypes, and provides automated results. The melting curves were normalized (87–93 °C for the lower temperature range and 90–98 °C for the upper temperature range). The melting temperature shift was performed automatically by the software using a default adjustment value of 5% for all analyses. Using a curve shape-matching algorithm, samples were automatically clustered into groups and the melting curve and difference plots were inspected. The sensitivity level was set at 0.3, which gave sufficiently consistent and robust results. Significant differences in the fluorescence in all subsets indicated different genotypes.

DNA sequencing

The PCR products were purified from an agarose gel using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). The amplicons were sequenced in both directions on the automatic sequencer ABI PRISM 3100/3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the ABI PRISM BigDye Terminator (version 3.1, Applied Biosystems). Identified DNA variations were confirmed by independent sequencing of the second PCR product. The GenBank M95623.1 and GenBank X04808.1 sequence (for gDNA and complementary DNA [cDNA], respectively) was used as the relevant wild-type HMBS sequence.

Results

In this study, we tested the HRM on the LightScanner instrument as a method to screen DNA variations in the polymorphic HMBS gene. We first determined whether the presence of previously detected polymorphisms would adversely interfere with further testing of the gene. To date, only four polymorphisms have been consistently detected in our patients. By HRM, we tested the following variations: g.-64T/C (rs589925) [18], g.3581A/G (rs17075) [19], g.6479T/G (rs1131488) [20], and g.7064C/A (rs1784304) [5] (Table 1). In all cases, we detected three discrete groups of genotypes (two homozygous and one heterozygous) (Fig. 1). Moreover, we defined the allelic frequency in 100 healthy donors using the RFLP assay (Table 2).

Subsequently, we examined whether 10 randomly distributed different mutations, consisting of single-base changes, base deletions, and insertions, could be detected by HRM analysis. For this purpose, we used mutations detected in our laboratory by DGGE and/or DNA sequencing. The complete coding sequence of 10 affected individuals was sequenced to eliminate the possibility of the presence of other mutations. The following mutations were tested: c.76C>T (p.Arg26Cys) [9], c.77G>A (p.Arg26His) [10], c.95G>C (p.Arg32Pro) [21], c.176C>T (p.Thr59Ille) [22], a complex monoallelic mutation c.[518G>A; c.610C>A] (p.[Arg173Gln; p.Gln204Lys]) [13], c.675delA (p.Ala226ProfsX28) [13], c.750A>T (p.Glu250Asp) [13], c.771+1G>T (r.spl?) [7], c.965_966insA (p.Asn322LysfsX36) [14], and c.972_973insG (p.Arg325AlafsX33) [23] (Table 1). The mutation c.95G>C (p.Arg32Pro) and the complex mutation c.[518G>A; c.610C>A] (p.[Arg173Gln; p.Gln204Lys]) were localized in the amplicons together with the known polymorphisms g.3581A/G (rs17075) and g.6479T/G (rs1131488), respectively. All amplicons displayed an altered melting curve profile compared with the normal sample (Figs. 1 and 2). In cases where more mutations were present in the same amplicon, the shapes of the curves were very similar. For example, similar curves were displayed by the mutations c.76C>T (p.Arg26Cys) and c.77G>A (p.Arg26His) in amplicon with exon 3 (Fig. 2), c.750A>T (p.Glu250Asp) and c.771+1G>T (r.spl?) in amplicon with exon 12 (Fig. 2), and c.965_966insA (p.Asn322LysfsX36) and c.972_973insG (p.Arg325AlafsX33) in amplicon with exon 15 (Fig. 2). Only the mutation c.675delA (p.Ala226ProfsX28) in amplicon with exon 12 displayed a difference in curve shape compared with other mutations in the same amplicon (Fig. 2). Where mutations were closely located to other polymorphisms within amplicon with exon 4, four distinct curves were displayed, representing three polymorphic genotypes and the mutation c.95G>C (p.Arg32Pro) in combination with the g.3581A/G (rs17075) genotype (Fig. 1). In amplicon with exon 10, four different curve shapes were identified, representing three polymorphic genotypes and the complex mutation c.[518G>A; c.610C>A] (p.[Arg173Gln; p.Gln204Lys]) with the genotype g.6479G/G (rs1131488).

Finally, we evaluated the HRM method on the group of 97 subjects with suspected porphyria. The data indicated that in 44 subjects diagnosed with AIP (including family members), there were four amplicons with altered melting curve profiles. In the first subject, we detected the curve alteration within amplicons with exons 2/3, 3, and 13/14. Direct sequencing of these samples confirmed DNA variation in all cases: a polymorphism g.3119G/T (rs1006195) in amplicon with exons 2/3 (Fig. 2 and Supplemental Fig. S1 [see supplementary material]) [19], a mutation c.70G>A (p.Gly24Ser) in amplicon with exon 3 (Fig. 2 and Supplemental Fig. S1) [7,8], and a rare polymorphism g.7998G/A in amplicon with exons 13/14 (Fig. 3) [5]. In 2 other related subjects, different curve shapes in amplicon with exon 10 were identified. Direct sequencing confirmed the presence of the complex mutation c.[518G>A; c.610C>A] (p.[Arg173Gln; p.Gln204Lys]) in both samples [13]. Five distinct curve shapes represented three polymorphic genotypes and the complex mutation c.[518G>A; c.610C>A] (p.[Arg173Gln; p.Gln204Lys]) with the genotype g.6479G/G (rs1131488) and c.[518G>A; c.610C>A] (p.[Arg173Gln; p.Gln204Lys]) with g.6479T/T (rs1131488) (Fig. 1). In the remaining 2 subjects, we detected curve alterations within amplicons with exons 2 and 13/14. Direct sequencing of these samples confirmed DNA variations in both cases: a novel rare DNA variation g.3059G>A in amplicon with exon 2 (Supplemental Fig. S1) and a novel mutation c.899_900delinsTGCCTGCATCTG (p.His300LeuFsX10) in amplicon with exons 13/14 (Fig. 3). Of 53 subjects diagnosed with other acute porphyrias, 3 had altered melting curve shapes. In all cases, direct sequencing confirmed DNA variations: a novel rare DNA variation g.2922T>G in amplicon with exon 2 (Fig. 3), a mutation c.87+5G>T (r.spl?) in amplicon with exon 3 (Fig. 2) [24], and another previously unidentified rare DNA variation g.7175A>G in amplicon with exon 11 (Fig. 3).

Table 2
Consistently detected polymorphisms and their allelic frequency (gDNA GenBank M95623.1; cDNA GenBank X04808.1).

Polymorphism	Reference[a]	RFLP	References
g.-64C/T (rs 589925)	0.63/0.37	0.61/0.39	[18,33]
g.3581A/G (rs17075)	0.75/0.25	0.67/0.33	[19,33]
g.6479G/T (rs1131488)	0.69/0.31	0.83/0.17	[20,33]
g.7064C/A (rs1784304)	0.75/0.25	0.79/0.21	[5,33]

[a] Caucasian origin.

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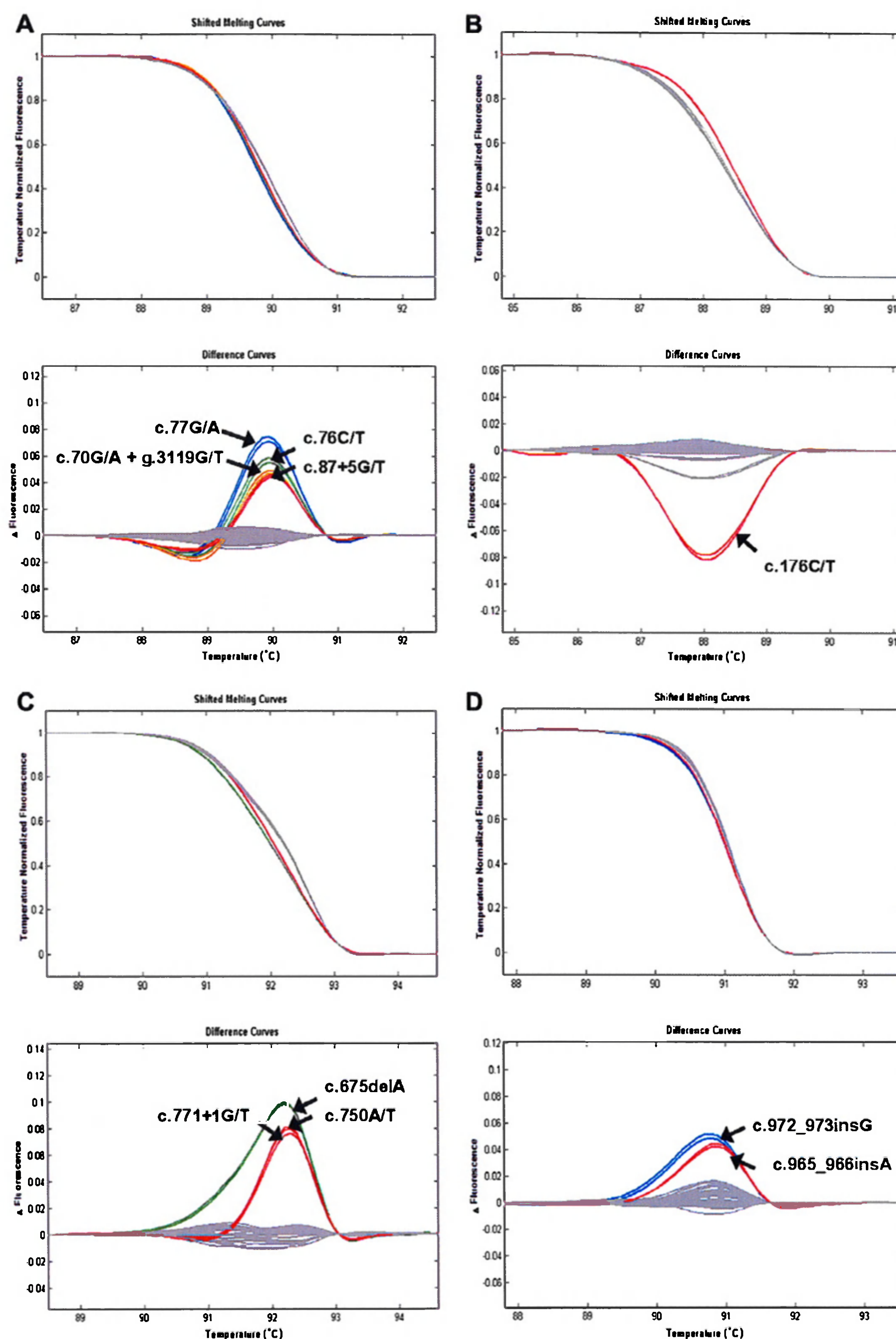

Fig. 2. HRM curves for the HMBS gene amplicons with control pathogenic DNA variations obtained using the LightScanner. The upper charts show the normalized and temperature-shifted melting curves. The lower charts show the difference curves of amplicons with exons 3 (A), 5/6 (B), 12 (C), and 15 (D). On the difference plot, the gray horizontal baseline represents a reference melting curve of the normal samples. A total of 10 different control mutations—c.76C>T (p.Arg26Cys) (A), c.77G>A (p.Arg26His) (A), c.95G>C (p.Arg32Pro) (see Fig. 1B), c.176C>T (p.Thr59Ile) (B), complex mutation c.[518G>A; c.610C>A] (p.[Arg173Gln; p.Gln204Lys]) (see Fig. 1C), c.675delA (p.Ala226ProfsX28) (C), c.750A>T (p.Glu250Asp) (C), c.771+1G>T (r.spl?) (C), c.965_966insA (p.Asn322LysfsX36) (D), and c.972_973insG (p.Arg325AlafsX33) (D)—were tested by HRM. All control mutations were detected by the difference in the curve shape compared with normal controls. Moreover, mutations localized in the same amplicon with known polymorphisms (amplicons with exons 3 and 10) were detected in spite of this background. /, allelic organization.

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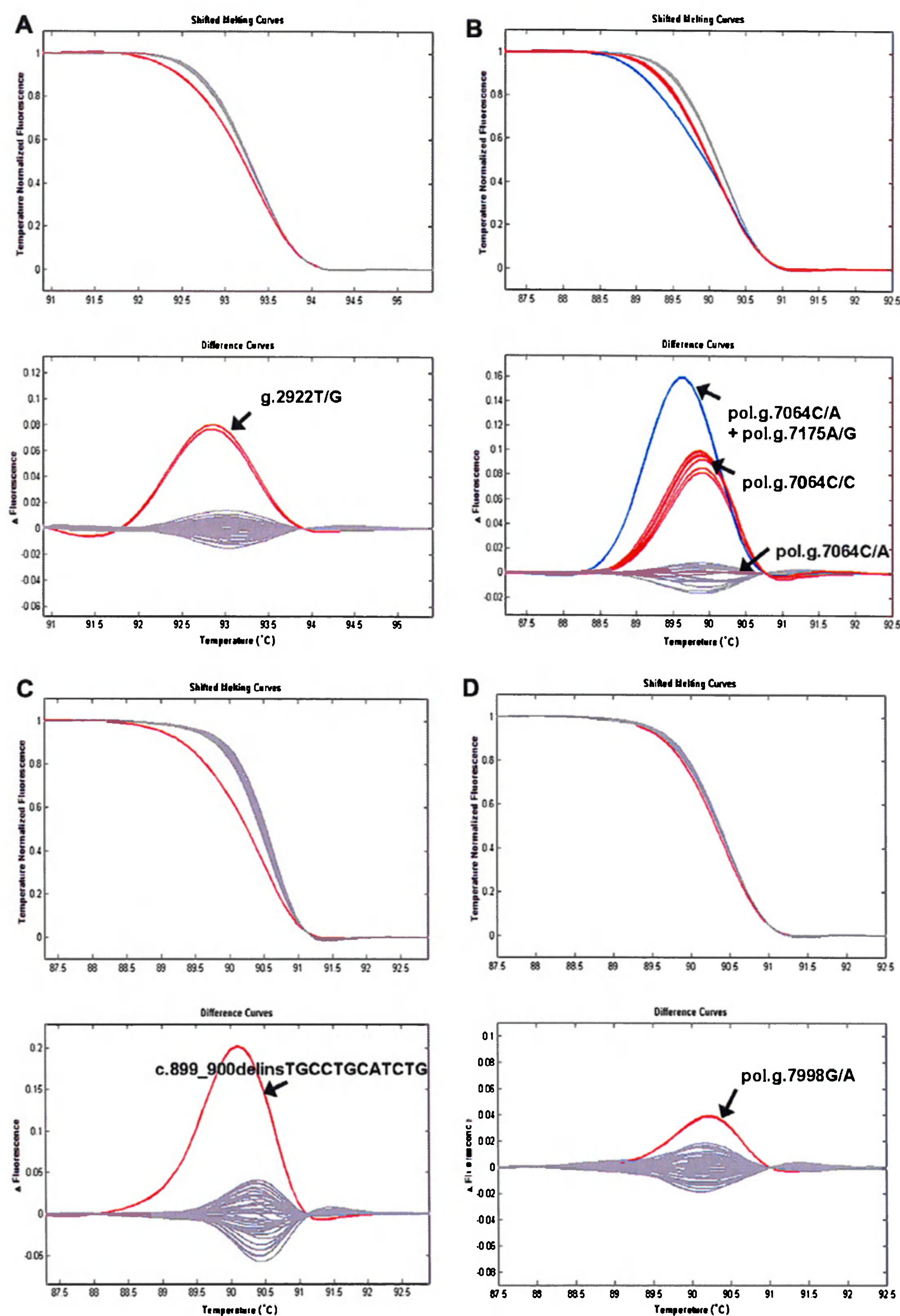

Fig. 3. HRM curves for the HMBS gene amplicons with DNA variations identified in this study obtained using the LightScanner. The upper charts show the normalized and temperature-shifted melting curves. The lower charts show the difference curves of amplicons with exons 2 (A), 11 (B), and 13/14 (C,D). On the difference plot, the gray horizontal baseline represents the reference melting curve of the normal sample. DNA variations were detected: a new rare DNA variation g.2922T>G (A), a new rare DNA variation g.7175A>G (B), a novel mutation c.899_900delinsTGCCTGCATCTG (p.His300LeuFsX10) (C), and a polymorphism g.7998G/A (rs1799997) (D). All DNA variations were detected by the difference in the curve shape compared with normal controls. DNA variations were confirmed by direct sequencing. /, allelic organization.

In the course of HRM screening, complications occurred with the longest amplicon (2/3) because two melting domains were present (Supplemental Fig. S1). Therefore, this amplicon was divided into two separate ones.

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Table 3
Summary of DNA variations in HMBS gene (gDNA GenBank M95623.1; cDNA GenBank X04808.1) identified by HRM analysis of 97 subjects.

Amplicon comprising exon	DNA variation	Number of positive samples	Localization	Comment and reference
2	g.2922T>G	1	IVS 1	Rare DNA variation (new)
2	g.3059G>A	1	IVS 2	Rare DNA variation (new)
2/3	g.3119T/G (rs1006195)	–[a]	IVS 2	Polymorphism [19]
3	c.70G>A (p.Gly24Ser)	1	E 3	Mutation [7]
3	c.87+5G>T (r.spl?)	1	IVS 3	Mutation [24]
10	c.[518G>A;c.610C>A] (p.[Arg173Gln, p.Gln204Lys])[b]	2[c]	E 10	Mutation [13]
11	g.7175A>G		IVS 11	Rare DNA variation (new)
13/14	c.899_900delinsTGCCTGCATCTG (p.His300LeuFsX10)	1	E 14	Mutation (new)
13/14	g.7998G/A (rs1799997)	1	IVS 14	Polymorphism [5]

[a] Not counted. This amplicon was divided into two separate amplicons.
[b] Complex mutation.
[c] Found in members of one family.

All DNA variations in the HMBS gene identified by HRM analysis Q1 of 97 subjects are summarized in Table 3.

Discussion

To improve molecular testing of the HMBS gene, we searched for a new and modern approach for screening DNA variations. The candidate of choice was the HRM method using the LightScanner instrument. DNA melting analysis of the amplicons generated by PCR using high-resolution instruments was only recently introduced into molecular diagnostics. This method enables screening of genes for different genotypes by generating altered melting curve profiles.

The previously used method for mutation screening in our laboratory was DGGE. It is an effective and relatively sensitive method that can screen up to 384 samples (32 in our case) in one run [13,25]. However, this method is highly time-consuming with respect to processing and separation steps. In contrast to DGGE, HRM analysis combined with the LightScanner has only two sequential steps of PCR amplification and amplicon melting. The main advantage of HRM is its high sensitivity and specificity. Sensitivity and specificity are reportedly better than those using the highly valued, conventional, mutation screening method, namely denaturing high-performance liquid chromatography (HPLC) [26]. Furthermore, HRM is very time- and cost-effective; the LightScanner uses a 96-well plate format (with an optional 384-well plate format). One HRM run proceeds within several minutes, and the additional cost for the LCGreen Plus dye is minimal. HRM also offers a decreased risk of contamination because the analysis immediately follows the PCR reaction and is performed on the same plate.

The sensitivity increases with decreasing size of the amplicon [27]. In our study, we used amplicons with an average length of 300 bp. The only exception was the amplicon with exons 2/3 (634 bp). For heterozygotic amplicons around 300 bp in length, 100% sensitivity and 100% specificity were reported [27]. Improvements in HRM techniques allowed amplicons longer than 500 bp to be screened with the same high sensitivity [28]. For longer amplicons (400–1000 bp), the sensitivity and specificity were 96.1 and 99.4%, respectively [27]. Based on our observations, we believe that increasing the length of the amplicon contributes negatively to the analysis of the melting curve due mainly to the possibility of the presence of more than one melting domain.

The HRM method is suitable for the detection of heterozygous mutations; the homozygous state is more difficult to identify [29]. Analysis of our HRM data indicated that the four consistently detected polymorphisms in HMBS displayed three different melting curve shapes corresponding to the three possible genotypes: two homozygous and one heterozygous (Fig. 1). In spite of this, it can be clearly seen from the standard melting and difference plots (except for amplicon with exon 1) that the two homozygotic genotypes have more similar melting profiles, whereas the homozygotic versus heterozygotic comparison of profiles showed larger differences. Therefore, the two homozygotes are harder to distinguish. In the HMBS gene, nearly all mutations are heterozygous; homozygous mutations are extremely rare events, with only two cases having been reported previously [30,31].

The convenience, effectiveness, and reliability of HRM analysis for genetic screening of the HMBS gene were examined by using 10 known mutations as positive controls. These mutations were randomly located across the gene, and we had previously identified them by DGGE and/or DNA sequencing. All amplicons containing these mutations were readily detected by differences in the melting curve shape compared with the normal sample. When testing the amplicons with the mutations in close proximity to another polymorphism, the mutations displayed distinct curve profiles, indicating that HRM can distinguish among variants with dissimilar polymorphic genotypes. Subsequently, these mutations were included as positive controls for new patient datasets.

After evaluating the method, we tested gDNA of 97 individuals with suspected acute porphyria. Initially, we screened recently diagnosed AIP cases. We included the cases that were diagnosed with other acute hepatic porphyrias but were not supported by molecular evidence (i.e., possible misdiagnoses). The diagnosis of porphyria is based on a combination of clinical and biochemical analyses. The biochemical findings are often inaccurate, especially when tested during the latent phase of the disorder. Moreover, no specific symptom is common to all patients. Therefore, misdiagnoses within the hepatic porphyria family occur occasionally, and the subject is then tested for more than one type of porphyria. From the analyzed set, eight DNA variations were identified: three previously described mutations c.70G>A (p.Gly24Ser) [7,8], c.87+5G>T (r.spl?) [24], and c.[518G>A; c.610C>A] (p.[Arg173Gln; p.Gln204Lys]) [13], one novel mutation c.899_900delinsTGCCTGCATCTG (p.His300LeuFsX10), two previously described polymorphisms g.3119T/G (rs1006195) [19] and g.7998G/A (rs1799997) [5], and two novel rare DNA variations g.3059G>A and g.7175A>G (found in 1 subject). The finding of these two novel rare DNA variations requires further investigation because these variations are localized in the intronic part of the HMBS gene but were presented in only 1 of 200 alleles.

We found the small in/del mutation interesting because there are only 4 such cases (among 307 mutations in the HMBS gene) registered in the mutation database [6]. At the protein level, the mutation p.His300LeuFsX10 would cause the frame shift leading to the premature stop codon after 10 completely different amino acids.

In an attempt to detect rare cases of dual porphyria [32], we screened patients with previously identified mutations within genes linked specifically to hepatic porphyria. A rare novel DNA variation g.2922T>G in 1 subject with porphyria variegata was found. This finding requires further investigation because this var-

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iation is localized in the regulatory segment of the erythroid promoter of HMBS.

Robust and effective screening methods are particularly useful and important for genetic consultation in families with genetic defects. Once a mutation is detected in a patient, molecular testing is offered to family members. Identification of subjects and testing within the affected family can be particularly important because of the high prevalence of asymptomatic carriers (90%). Knowledge of the disorder status can assist in preventing life-threatening acute attacks brought on by specific environmental factors.

In conclusion, we have found that the HRM method using the LightScanner is a fast and cost-effective prescreening method for detecting DNA variations in the HMBS gene. During the pilot phase of our study, all 10 mutations in the polymorphic HMBS gene were correctly detected, providing 100% sensitivity. Moreover, screening of the HMBS gene in 97 subjects with suspected acute porphyria revealed nine different DNA variations, four of which were novel. We showed here that the HRM method can serve as a useful screening tool for gene variations even in amplicons with different polymorphisms.

Acknowledgments

We appreciate the participation of the patients and their relatives in the study. This research was supported by the Ministry of Education, Sport, and Youth of the Czech Republic and the Grant Agency of Charles University (GAUK 257540 54007, MSM0021620806, 1M6837805002). We thank American Journal Experts (AJE) for proofreading this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.07.050.

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Please cite this article in press as: D.U. Douderova P. Martasek Detection of DNA variations in the polymorphic hydroxymethylbilane synthase gene by high-resolution melting analysis Anal. Biochem. (2009), doi:10.1016/j.ab.2009.07.050

PUBLICATION G

Lichen sclerosus et atrophicus in a patient carrying a novel hydroxymethylbilane synthase mutation *(Ulbrichova Douderova D et al., prior to submission)*

Lichen sclerosus et atrophicus in a patient carrying a novel hydroxymethylbilane synthase mutation

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For submission to: *Blood Cells, Molecules and Diseases*.

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Abstract

A 48-year-old female patient presented with skin lesions typical for a cutaneous porphyria. Biochemical and enzymatic analyses revealed a slightly elevated concentration of aminolevulinic acid (ALA) and porphobilinogen (PBG) in urine and a ~50% reduction in hydroxymethylbilane synthase (HMBS) activity in erythrocytes. Sequencing of the *HMBS* gene identified a novel missense mutation c.601C>G, p.Arg201Gly. Upon *in vitro* expression and purification, mutant p.Arg201Gly displayed a residual activity of 5.9% of that of the wild-type enzyme, a reduced thermo-stability and a shift in optimal pH. Based on the 3-D structure of human enzyme, ion-pairing between Arg201 and Asp178 plays an important role in maintaining the structural stability. Thus, the replacement of Arg201 by a glycine residue will disrupt the ion-pairing and in turn, destabilize the enzyme. The results of both *in vivo* and *in vitro* studies confirmed a latent status of acute intermittent porphyria (AIP) in this patient since she had never experienced any symptoms of AIP although one of her sisters did suffer from acute abdominal pain. The skin condition in this patient was subsequently diagnosed as lichen sclerosus et atrophicus (LSA) by biopsy. An association between porphyria and autoimmune skin disease is well known. A specific association of AIP and LSA cannot be excluded.

Keywords:

acute intermittent porphyria

hydroxymethylbilane synthase

lichen sclerosus et atrophicus

mutation

in vitro expression

enzyme structure

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Introduction

Porphyrias are a group of seven metabolic disorders in the heme biosynthesis. They can be classified into acute and cutaneous porphyrias based on clinical symptoms. A common symptom of acute porphyrias is the acute abdominal pain, whereas photosensitivity of the skin occurs in cutaneous porphyrias that are comprised of both acute and non-acute porphyrias [1]. Acute intermittent porphyria (AIP, OMIM #176000), a dominantly inherited disorder, is the result of a ~50% deficiency in the hydroxymethylbilane synthase activity (HMBS, EC 2.5.1.61). Mutations in the *HMBS* gene are responsible for the enzyme deficiency. However, not all mutation carriers will develop AIP clinical symptoms, the so called incomplete clinical penetrance. Pre-symptomatic mutation carriers are at risk for developing acute attacks when exposed to provocative factors such as exogenous or endogenous hormones, stress, fasting, drugs and alcohol [1]. These individuals can be readily identified within an AIP family provided that the family-own mutation was known. In fact, an accurate identification of pre-symptomatic mutation carriers so that they can be advised to avoid the provoking agents, is an essential element in the management of families with AIP [2].

Lichen sclerosus et atrophicus (LSA) is a chronic inflammatory dermatosis that results in white plaques with dermal atrophy. It occurs more frequently in women than in men. Although the exact cause of LSA is unknown, several risk factors have been proposed, including autoimmune disease, infections and genetic predisposition [3].

In this study, we report a latent AIP patient incidentally identified during clarification of a skin disorder that was eventually diagnosed as LSA.

Methods

Biochemical and enzymatic assays in the diagnosis of porphyrias

Analysis of porphyrins and their precusors aminolevulinic acid (ALA) and porphobilinogen (PBG) in urine, feces, plasma and erythrocytes were routinely performed in our laboratory. All methods have been previously described [4]. In addition, HMBS activity was measured in packed erythrocytes from blood samples [4]. The biochemical and enzymatic assays enabled differential diagnosis of all porphyrias.

Genetic analysis and in vitro characterization of HMBS mutant

The patient gave an informed consent for genetic testing, and the study was carried out in accordance with the principles of the Declaration of Helsinki. Mutational analysis in both the *HMBS* gene and the *PPOX* genes was performed according to previously described methods [5,6]. Mutant HMBS was generated by site-directed mutagenesis and expressed in the *p*GEX-4T-1 vector [7]. Purification and enzymatic assay in mutant and wild-type enzymes were performed as previously described [7].

To analyze structure-function correlation, 337 UniProt (www.uniprot.org) and 32 Ensembl (www.ensembl.org) HMBS protein sequences were aligned with MUSCLE software [8]. The data-set contained 46 eukaryotic and 323 prokaryotic diverse sequences; a subset of 216 sequences showed amino acid identity smaller then 90%, and a subset of 59 sequences identity smaller then 60%. The three-dimensional structure was displayed and examined with MBT platform using the coordinates of the PDB structure ID 1pda [9,10].

Case report

A 48-year-old female Swiss patient was referred to the Outpatient Clinic of Dermatology in Triemli Hospital with skin lesions. The patient described point-shaped skin rash, itching and blistering in the face and dorsa of hands after sun exposure that occurred initially at the age of

12. Since then, she frequently experienced recurrences of the skin symptoms. On physical examination, sclerotic whitish macules and plaques with brownish to livid margins were found on the left cheek (Fig 1A) and left breast (Fig 1B). Multiple erythematous papules with partial scarring appeared on the neck and the back of hands (Fig.1C). In addition, atrophic pigmentation changes were visible on the hands (Fig.1C) and in the genital area. None of the family members of the patient had similar skin lesions.

The skin symptoms in this patient were atypical for cutaneous porphyrias. However, further inquiry into the family history revealed that patient's sister suffered from acute abdominal pain of unknown nature. Biochemical and enzymatic analyses were therefore conducted in order to exclude porphyrias, in particular variegate porphyria (VP), an acute porphyria of which patients may present cutaneous symptoms and/or neurovisceral attacks.

The results of biochemical analyses showed normal fecal porphyrin content, a normal protoporphyrin concentration in erythrocytes and a negative plasma fluorescence scan. However, urinary ALA, PBG and porphyrins were slightly increased. Repeated measurement of erythrocyte HMBS activity subsequently revealed a ~50% reduction in the enzyme activity (Table 1). The biochemical findings were compatible with the latent status of AIP and largely excluded cutaneous porphyrias.

Later, the patient presented to the Dermatological Clinic of University Hospital of Zürich where she received further work-up. Autoimmune antibodies including antinuclear antibodies (ANA), anti-double stranded DNA, anti-SSA, anti-SSB, anti-phospholipid-antibody, and anti-neutrophil cytoplasmatic antibodies (ANCA) were all within normal limits. A punch biopsy from the cheek showed atrophic epidermis and slight edema in the upper dermis. In the lower dermis, a zone of hyalinization is delineated by diffuse mainly lymphocytic and histiocytic

infiltrates with a few interspersed plasma cells (Fig.1D & E). No deposits of immunoglobulins or complement were found on direct immunofluorescence of a fresh-frozen skin section. ECM1 antibody was not measured. Combining clinical and histological aspects, a diagnosis of lichen sclerosus et atrophicus was established.

Results

Although the biochemical test results particularly the negative result in plasma fluorescence scan, largely excluded PV, the *PPOX* gene of the patient was sequenced with no mutations identified in its entire coding region as well as the exon-intron boundaries.

The unexpected finding of a decreased HMBS activity lead to a search for possible mutations in the *HMBS* gene. Direct sequencing of PCR-amplified *HMBS* gene fragments unveiled a C to G transversion at nucleotide position 601 in exon 10 (c.601C>G) resulting in the substitution of Arg201 by a glycine residue (p.Arg201Gly). The patient was heterozygous for the p.Arg201Gly mutation (Fig. 2A). This mutation was however, absent in 100 chromosomes from non-porphyric individuals.

The novel missense mutation p.Arg201Gly was subsequently characterized *in vitro*. A p.Arg201Gly mutant was generated by site-directed mutagenesis and expressed in a prokaryotic system (*E. coli BL21 DE3*). Both the mutant and wild-type enzymes were purified to homogeneity before analyses. A residual activity of 5.9% of that of the wild-type enzyme was measured in the p.Arg201Gly mutant. Comparing to the wild-type enzyme, the mutant was extremely unstable towards heat treatment with an estimated half-life of 15 mins at 65°C (Table 1). In addition, the mutant enzyme exhibited an optimal pH of 7.8, lower than the value determined in the wild-type enzyme (pH 8.2) (Table 1; Fig. 2B).

In the 3-D structure of human HMBS published recently, two lobes of the central domain are joined by ion-pairing between Arg201 and Asp178 (Fig. 2C) [11]. The same ion pair exists in the structure of *E. coli* enzyme which is very similar to that of the human enzyme [7,12]. Residues equivalent to Asp178 and Arg201 are conserved among all 46 known eukaryotic HMBS sequences, and are highly conserved (i.e., ~80%) among prokaryotic enzymes as well [7]. Such high degree of conservation suggests that the ion pair plays an important role in the enzyme structure. The loss of enzyme activity and thermo-stability in the p.Arg201Gly mutant can be explained by the disappearance of positive charge, which leads to a disruption of the ion-pair and destabilization of the central domain.

Two other known mutations in the ion pair, p.Arg201Trp and p.Asp178Asn, were also shown to be thermolabile and enzymatically impaired, but to a lesser degree than the p.Arg201Gly mutant (Table 1) [7,13]. In addition, the activity of p.Asp178Asn was pH-dependent with also a shift in optimum (Fig. 2B) [7]. Since both mutations introduced residues capable of a weak Coulombic interaction, the ion pairs Arg201-Asn178 and Trp201-Asp178, although weakened functionally, remained intact.

Discussion

The result of *in vitro* characterization of the p.Arg201Gly mutant suggested a deleterious effect on HMBS, which was in accordance with the ~50% reduction in the erythrocyte HMBS activity *in vivo* as the patient was heterozygous for the mutation. Thus, a latent status was granted in our patient taken into account that she has so far not shown any symptoms of AIP. Retrospectively, the cause of acute abdominal pain in patient's sister was likely AIP-related.

One of the unknown aspects in the pathogenesis of AIP is the mechanism of incomplete penetrance. According to the literature, only 10-20% of the mutation carriers develop clinical

symptoms [14]. To prevent the onset on AIP symptoms, our patient was advised to avoid taking porphyrinogenic drugs and other precipitating factors.

This study demonstrated that a comprehensive laboratory analyses is essential for the differential diagnosis of porphyrias even among latent cases. With respect to a particular enzyme deficiency, the combination of mutational analysis and *in vitro* enzymatic studies offers the most convincing evidences on the molecular nature of the defect.

LSA is an autoimmune disease of unknown origin that is closely related to other inflammatory and autoimmune skin diseases such as lupus erythematosus or lichen planus [15,16]. Associations between photosensitive autoimmune skin diseases such as lupus erythematosus and photosensitizing porphyrias such as porphyria cutanea tarda have long been recognized [17]. AIP normally does not photosensitize affected individuals. In the setting of renal insufficiency, accumulation of metabolites may induce photosensitization [18]. Our patient lacks any indication for renal insufficiency. She reported, however, the appearance of skin lesions following sun exposure and exhibits a photodistribution of the skin lesions on sun-exposed area. An association between AIP and LSA in this patient can thus not be excluded.

Acknowledgement

The authors wish to thank Mr. Sulejman Ahmetovic for his excellent technical assistance. This work was supported by the Ministry of Education, Sport and Youth of Czech Republic, the granting agency of Charles University; contract grant number: MSM0021620806, 1M6837805002 and GAUK 257540 54007 to D.U. and P.M. E.I.M. is a recipient of grants from the Hartmann-Müller Stiftung (No. 1187) and from the Velux Stiftung (Project No. 480).

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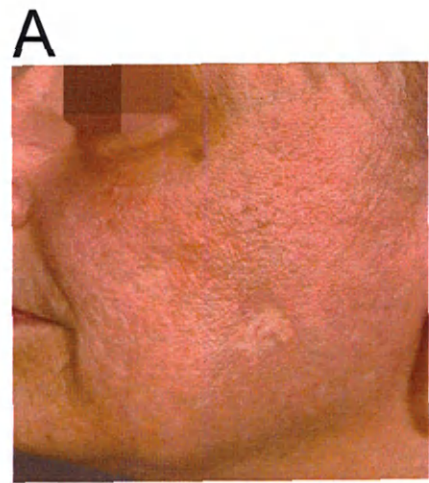
[18] *ask Elisabeth*

Legend to figures

Fig. 1. On photoexposed skin of the face (**A**), the cleavage (**B**) and the dorsa of both hands (**C**), whitish atrophic and at times slightly retracted macules and with a brownish to reddish border can be observed. A punch biopsy from the cheek shows atrophic epidermis and slight edema in the upper dermis. In the lower dermis, a zone of hyalinization is delineated by diffuse mainly lymphocytic and histiocytic infiltrates with a few interspersed plasma cells. Hematoxylin and eosin stain shown at low (**D**) and high (**E**) magnifications.

Fig. 2. (A) Identification of the c.601C>G (p.Arg201Gly) mutation in the *HMBS* gene. The right panel, a segment of exon 10 sequence from the patient; the left panel, the same sequence from a control subject. The mutation is indicated by an *arrow*, the polymorphism g.6479T/G (rs1131488) is indicated by an *asterisk* (*); (B) pH activity profile of mutant p.Arg201Gly (▲), p.Asp178Asn (■), and wild-type (□) enzymes; (C) The left panel, a Ribbon representation of the human HMBS structure. The N-, central and C- domains are in *light blue*, *dark blue* and *green*, respectively; dipyrromethane cofactor is in ball and stick, mutation affected residues are in *red*. The right panel, a zoom-in of the central domain displaying the ion pair between Asp178 and Arg201.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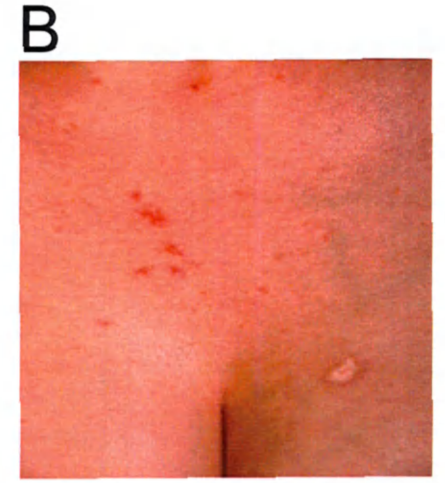
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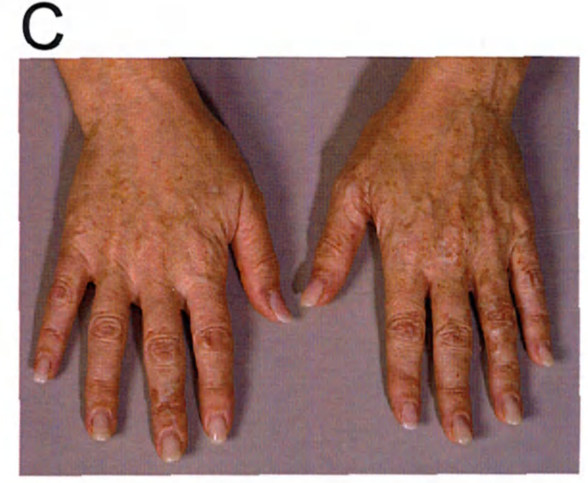
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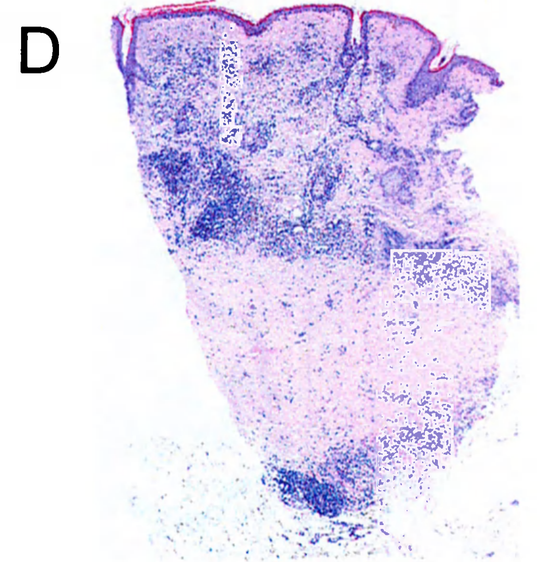
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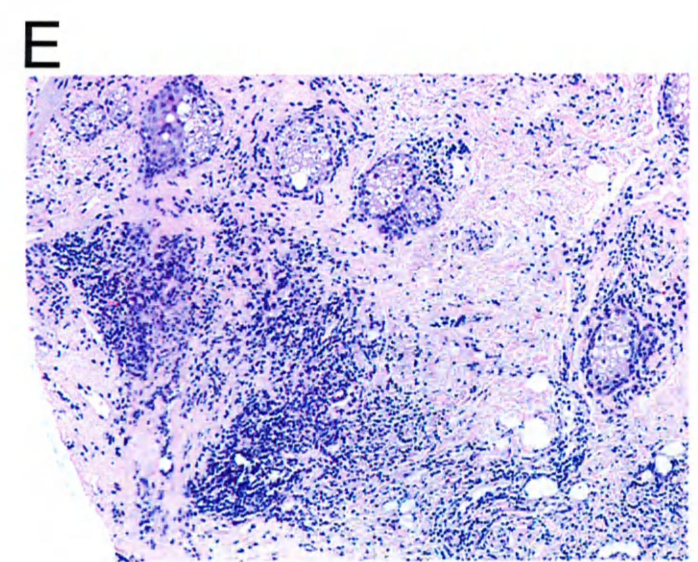
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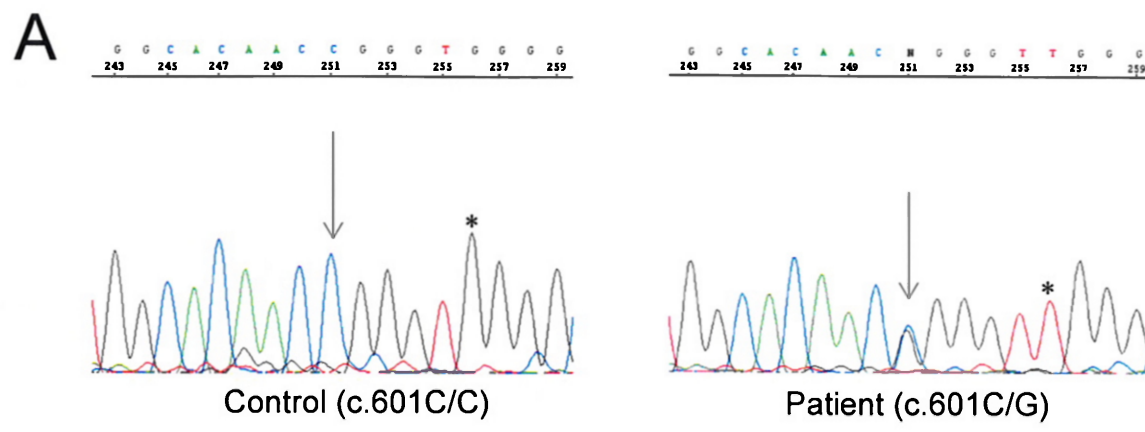
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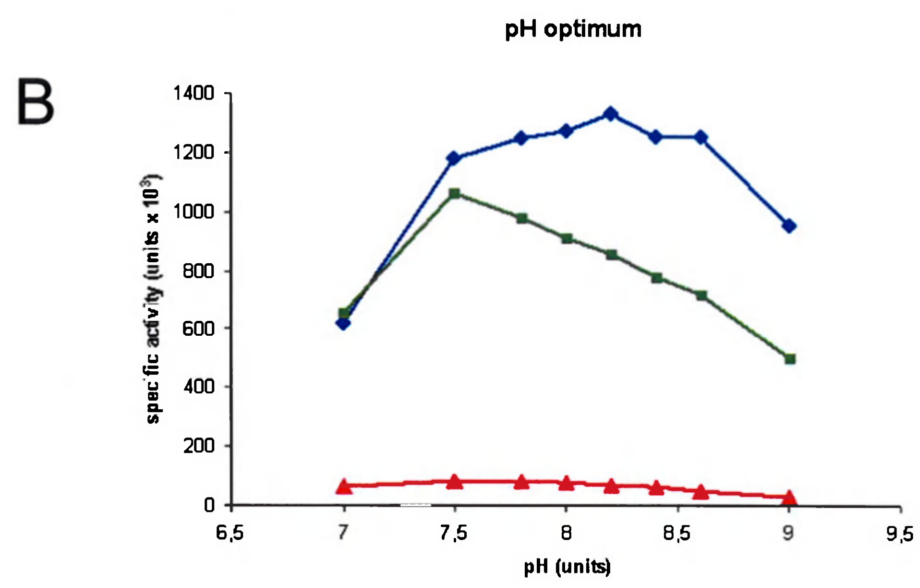
Control (c.601C/C)
Patient (c.601C/G)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pH (units)

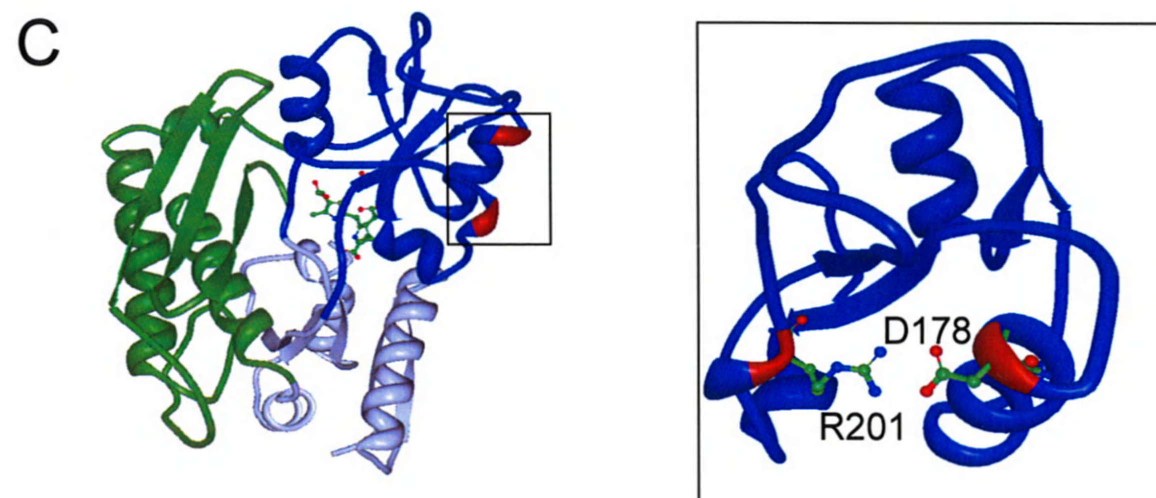
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Table 1. *In vivo* and *in vitro* effects of missense mutations affecting ion pair between Asp178 and Arg201 in the *HMBS* gene.

		In vivo analyses in the patient			*In vitro* analyses in recombinant enzymes			
Gene mutation	Protein mutation	Erythrocyte HMBS activity* (unit)§	Urinary ALA# (µmol/mmol creatinine)	Urinary PBG# (µmol/mmol creatinine)	Residual enzyme activity (% of wild-type)	Thermo-stability half-life at 65°C (min)	pH optimum	Reference
c.601C>G	p.R201G	60, 66, 58	5.6, 2.9	7.2, 11.3	5.9	15	7.8	Present work
c.601C>T	p.R201W	-	-	-	41.6	25	n.d	Chen et al 1994 [13]
c.532G>A	p.D178N	-	-	-	81	60	7.5	Ulbrichova et al 2009 [7]
wild-type	wild-type	66-126	<2.5	<1.25	100	>240	8.2	Minder & Schneider-Yin 2008 [4]; Ulbrichova et al 2009 [7]

*measured in three separate occasions; §one unit is defined as formation of 1pmol of uroporphyrin/hemoglobin (mg)/h; # measured in two separate occasions; n.d., not determined.

PUBLICATION H

Gene symbol: HMBS. Disease: Porphyria, acute intermittent (Ulbrichova D et al., 2008)

Hum Genet (2008) 124:293–324
DOI 10.1007/s00439-008-0551-3

HUMAN GENE MUTATIONS

Novel human pathological mutations

Published online: 12 September 2008
© Springer-Verlag 2008

Gene symbol: G6PD

Disease: Glucose-6-phosphate dehydrogenase deficiency

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Missense/Nonsense Mutations (single base-pair substitutions)

Accession number	Codon number	Nucleotide substitution	Amino acid substitution
HM080001	228	gGAC-AAC	Asp-Asn

Gene symbol: CCM2

Disease: Cerebral Cavernous Malformations

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E-mail: ajimenez@ruberinternacional.es

Small deletions (<21 bp)

Accession number	Codon number/location	Deletion
HD080001	204	AGCAGCTGCTgtcccccactgcag_I6E6_^GTCGCTGCGG

Comments: The first genetic analysis was performed at Prevention Genetics. The mutation was also found in other affected relative at an independent laboratory (data not published). Exon6-14_Exon6-1 del GTCCCCCACTGCAG.

Gene symbol: HMBS

Disease: Porphyria, acute intermittent

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Missense/Nonsense Mutations (single base-pair substitutions)

Accession number	Codon number	Nucleotide substitution	Amino acid substitution
HM080045	30	CTT-CGT	Leu-Arg

Comments: first base of ex. 3/4.

Gene symbol: MECP2

Disease: Rett syndrome

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Small insertions (<21 bp)

Accession number	Codon number/location	Insertion
HI080008	274	CCGGGG^AGTGagtgTGGTGGCAGC

Comments: This mutation causes a frameshift and creates a premature stop codon (V275fsX57) resulting in a truncated protein (330aa).

Gene symbol: TAZ

Disease: Barth syndrome

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PUBLICATION I

Gene symbol: HMBS. Disease: Porphyria, acute intermittent *(Ulbrichova Douderova D et al., sent to Hum Genet 2009)*

Report of the novel mutation identification

Sent to Hum Genet 2009

This mutation of maternal origin in exon 5 of the HMBS gene was identified in one patient, female, with manifests typical for acute intermittent porphyria (AIP). During an acute attack characterized by severe abdominal pain, hyponatremia and behavioral disturbances, the level of porphyrin precursors were highly elevated. Her PBG and ALA levels were 93 and 79 mg/24h, respectively, (normal values: less then 2.5 mg/24h for PBG and less than 7.5 mg/24h for ALA), and a distinct peak in the fluorimetric plasma scan was identified at 404/622 nm. Her PBGD activity measured a month after the acute attack was 71%, (normal: >70%). DNA sequence analyses revealed small insertion c.184_185insT within HMBS gene and confirmed the diagnosis of AIP.
This mutation (p.Lys62IlefsX3) causes a frameshift and creates a stop codon (+3aa) resulting in truncated protein (64aa).

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PUBLICATION J

***Gene symbol: HMBS. Disease: Porphyria, acute intermittent** (Ulbrichova Douderova D et al., sent to Hum Genet 2009)*

Report of the novel mutation identification

Sent to Hum Genet 2009

This mutation of maternal origin in exon 8 of the HMBS gene was identified in one patient, female, with manifests typical for acute intermittent porphyria (AIP) - abdominal pain, dark urine and muscle weakness. During an acute attack the level of porphyrin precursors PBG and ALA were elevated (16.6 mg/100ml and 18.2 mg/100ml, respectively). DNA sequence analyses revealed small insertion c.384_385insT within HMBS gene and confirmed the diagnosis of AIP.
This mutation (p.Val130CysfsX80) causes a frameshift and creates a stop codon (+80aa) resulting in truncated protein.

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