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Molecular pathology of porphyrias

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This thesis is based on the following articles, referred to in the text by their capital letter as indicated here:

A) Lamoril J, Andant C, Gouya L, **Malonova E,** Grandchamp B, Martasek P, Deybach JC, Puy H. **Hemochromatosis (HFE) and transferrin receptor-1 (TFRC1) genes in sporadic porphyria cutanea tarda (sPCT)**. Cell Mol Biol (Noisy-le-grand). 2002 Feb;48(1):33-41.

B) Schmitt C, Gouya L, **Malonova E,** Lamoril J, Camadro JM, Flamme M, Rose C, Lyoumi S, Da Silva V, Boileau C, Grandchamp B, Beaumont C, Deybach JC, Puy H. **Mutations in human CPO gene predict clinical expression of either hepatic hereditary coproporphyria or erythropoietic harderoporphyria**. Hum Mol Genet. 2005 Oct 15;14(20):3089-98.

C) Lee DS, **Flachsova E,** Bodnarova M, Demeler B, Martasek P, Raman CS. **Structural basis of hereditary coproporphyria**. Proc Natl Acad Sci U S A. 2005 Oct 4;102(40):14232-7.

D) Ulbrichova D, **Flachsova E**, Hrdinka M, Saligova J, Bazar J, Raman CS, Martasek P. ***De novo* mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study**. Physiol Res. 2007;55 Suppl 2:S145-54.

E) **Flachsova E**, Verma IC, Ulbrichova D, Zeman J., Raman CS, Martasek P: **New mutation within porphobilinogen deaminase gene leading to the truncated protein as a cause of acute intermittent porphyria in extended Indian family** (submitted for publication).

F) **Flachsová E.**, Keslová P., Sedláček P., Plavka R., Zeman J., Starý J., Martásek P. **Treatment of congenital erythropoietic porphyria (Morbus Günther) by cord blood stem cell transplantation, long term follow-up with 7-11% chimerism and prenatal diagnosis in a sibling** (prior to submission).

G) **Flachsova E,** Robreau A-M, Da Silva V, Deybach J-Ch, Martasek P., Puy H **Search for molecular determinants responsible for different phenotypic expression of porphyria variegata** (manuscript in preparation).

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1. INTRODUCTION

Porphyrias are mostly inherited metabolic disorders caused by decreased activities of the enzymes in the heme biosynthetic pathway[1]. The term porphyria describes the purple-red crystalline porphyrins named from the Greek *"πορφυροσ"* purphuros (purple). Porphyrins are essential components of a wide range of chemical reactions that are required for life. These macrocyclic tetrapyrroles act as cofactors for multiple enzymes that perform a variety of processes within the cell, such as methionine synthesis (vitamin B12), oxygen transport (heme) and photosynthesis (chlorophyll).

Heme is a prosthetic group of a number of cellular hemoproteins which have various biological functions. Among them are hemoglobin, myoglobin, drug metabolizing cytochrome P450, cytochromes involved in oxidative phosphorylation (cytochrome c, cytochrome c oxidase, cytochrome reductase), catalases, peroxidases, nitric oxide synthase and guanyl cyclases.

1.1. PORPHYRINS AND PORPHYRINOGENS

The structural basis of porphyrins is the tetrapyrrole ring (Figure 1). The four single pyrrole rings (A, B, C, D/I-IV) are joined together via methine bridges to form a highly conjugated structure: the porphyrin. Its eight hydrogen side atoms (1-8) are substituted with characteristic side chains.

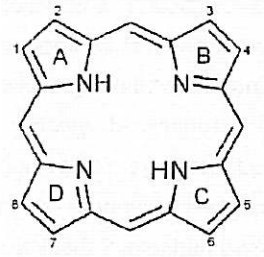

Figure 1: The chemical structure of porphyrins

The biological intermediates of heme synthesis are hexahydroporphyrins, porphyrinogens, with the four methine bridge carbon atoms and the two pyrrolenine nitrogen atoms hydrogenated. They are highly unstable molecules that, when exposed to air, rapidly oxidize to porphyrins. Porphyrinogens are functional precursors of heme, and oxidation to the corresponding porphyrin removes it from the pathway. Protoporphyrin IX is the only oxidized porphyrin that serves as a substrate for heme-pathway enzymes. Porphyrinogens are colorless and do not fluoresce, but highly conjugated porphyrins are colored molecules with unique photochemical features.

The type of the side chain of porphyrinogens determines physico-chemical properties of subsequently oxidized porphyrins. The first of the porphyrinogens synthesized by the heme pathway has eight carboxyle groups. The stepwise decarboxylation of the side chains from eight to two occurs along the pathway. Thus, porphyrins with higher numbers of carboxyl groups are hydrophilic, a quality that facilitates their excretion in urine; while porphyrins with fewer carboxyl moieties as side chains have lipophilic properties and are excreted by the hepatobiliary route.

An important characteristic of porphyrins is metal-binding capability. The most commonly bound metals are iron and magnesium. Heme, the iron-containing complex, is central to most biological oxidation and oxygen

transport. Chlorophyl is a magnesium porphyrin compound, which is critical in utilization of solar energy in the biosphere. The Nobel Laureate Hans Fischer described porphyrins as the compounds that "made grass green and blood red".

1.1.1. PHOTOCHEMICAL FEATURES OF PORPHYRINS

The porphyrin macrocycle is highly conjugated. The presence of this conjugated double-bond system makes porphyrins resonating compounds, with a typical dark red color and characteristic absorption bands in the near ultraviolet and visible regions of the electromagnetic spectrum [2].

Porphyrins have absorption spectra consisting of a major band near 400 nm (the Soret band) and multiple smaller bands (depending upon the solvent), between 500 and 630 nm. Porphyrin absorption spectra vary with the solvent in which the porphyrin is dissolved. Solutions of porphyrins in acid have two peaks of absorbance compared to four peaks for porphyrins dissolved in chloroform.

When excited by specific wavelengths within the Soret band, porphyrins emit a characteristic red fluorescence. In acid solution, they show two strong emission bands, one between 580 and 610 nm and the other between 640 and 680 nm. Absorbance and fluorescence of porphyrin solutions are also altered by changes in metal binding and side chains.

1.2. HEME BIOSYNTHESIS

Heme synthesis is one of the essential pathways of life, and occurs in nearly all metabolically active cells. The same pathway is also used by plants, bacteria, and algae to make the other principal pigments of life, chlorophyll and vitamin B_{12}. For anaerobic organisms or facultative

organisms growing under anaerobic conditions, an oxygen independent enzyme exists that catalyzes the antepenultimate and penultimate step in heme biosynthesis [3].

Heme biosynthesis is most active in erythropoietic tissue, where hemoglobin synthesis takes place, and in the liver where heme forms the basis of various heme-containing enzymes such as cytochromes P450, catalase, tryptophan pyrrolase and others. These two major sites of heme production differ in heme synthesis regulation[4]. In erythropoietic tissue heme biosynthetic enzymes are regulated to permit a high steady-state level of synthesis and regulation is tied to the availability of iron. On the contrary, in the liver heme biosynthesis is turned over rapidly, to be able to quickly answer changes in metabolic requirements.

Heme is built from the simple raw materials glycine and succinic acid from which δ-aminolevulinic acid (ALA) is formed. In the next steps, six enzymes catalyze formation and tetramerization of porphobilinogen (PBG), and in the final step, ferrous ion is inserted to form heme[4]. The heme biosynthetic pathway consists of a series of eight functionally interlocking enzymes. The first and the 3 last enzymatic reactions take place in mitochondria, while the remaining four reactions occur in cytosol (Figure 2).

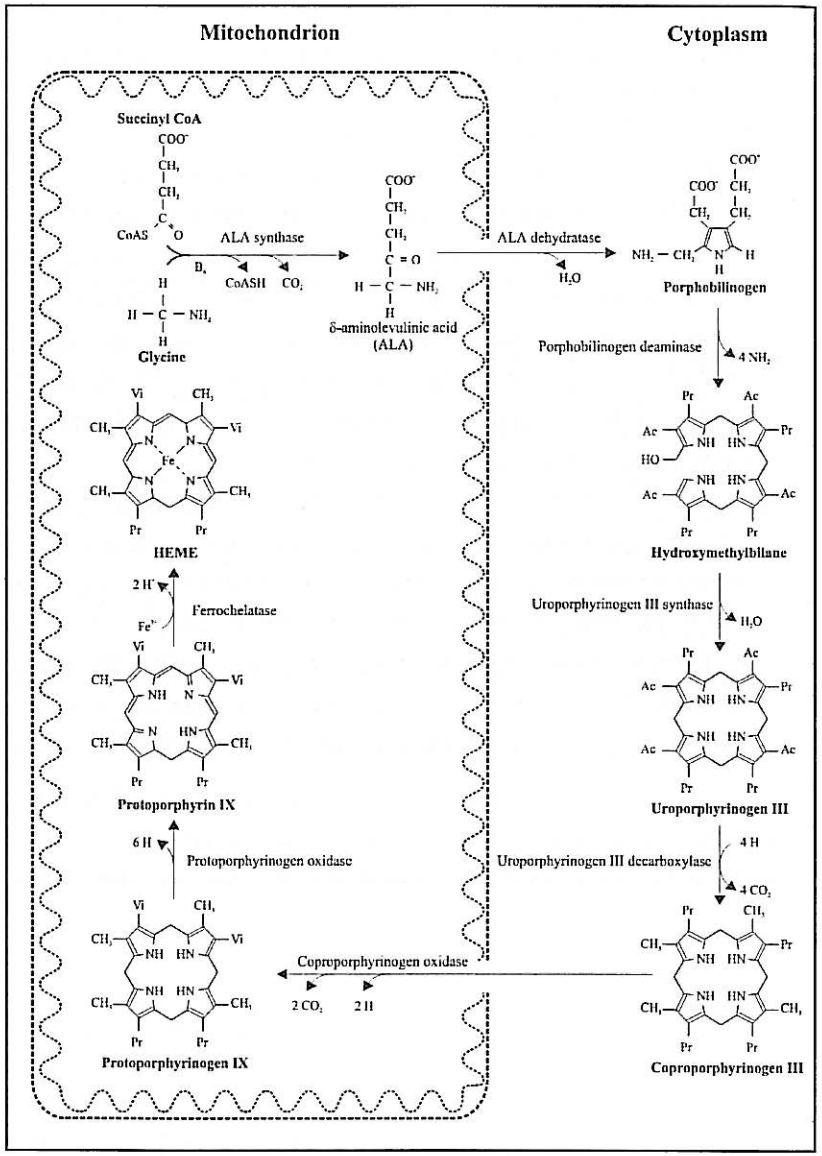

Figure 2: Enzymes and intermediate products in the heme biosynthetic pathway.
Ac= - CH_2COOH, Pr= -CH_2CH_2COOH, Vi= -$CH{=}CH_2$[5].

1.3. PORPHYRIAS

Porphyrias are heterogenous disorders caused by inherited or acquired deficiency of the enzymes in the heme biosynthetic cascade. With the exception of δ-aminolevulinic acid synthase (ALAS) the deficiency of each of the enzyme of heme synthesis can be assigned to a certain type of porphyria[6]. Each one is characterized by a typical spectrum of accumulated and excreted porphyrins and porphyrin precursors, PBG and ALA. Characteristic spectrophotometric and fluorometric properties of porphyrins allow the diagnosis of porphyrias by their analyses in urine, plasma and feces[7]. Genes encoding all 8 enzymes of the heme biosynthetic pathway were cloned and the molecular background of all inherited porphyrias was established. There is a profound genetic heterogeneity at the molecular level in porphyrias. Most of them are inherited in autosomal dominant fashion with low penetrance. The knowledge of molecular bases of the disease permits more accurate diagnoses[8].

The porphyrias can be classified and subdivided in several ways, and a variety of names have been used to describe each particular disorder (Table 1). Based on the site of major production of porphyrins and their precursors they can be classified as hepatic or erythropoietic. Another practical classification is due to pathogenesis of the disease as either acute or chronic based on the ability of certain drugs to precipitate so-called acute porphyric attack.

Table 1: – Classification of porphyrias

Type		Disease	Enzyme	Symptoms
Hepatic porphyrias	Acute	δ-aminolevulinate dehydratase deficiency porphyria	δ-aminolevulinic acid dehydratase	Neurologic
		Acute intermittent porphyria	Porphobilinogen deaminase	Neurologic
		Hereditary coproporphyria	Coproporphyrinogen oxidase	Neurologic + cutaneous
		Porphyria variegata	Protoporphyrinogen oxidase	Neurologic + cutaneous
	Chronic	Porphyria cutanea tarda	Uroporphyrinogen decarboxylase	Cutaneous
Erythropoietic		Congenital erythropoietic porphyria	Uroporphyrinogen III synthase	Cutaneous + hematologic
		Erythropoietic protoporphyria	Ferrochelatase	Cutaneous

Acute hepatic porphyrias are considered to be molecular regulatory diseases of the heme biosynthetic pathway characterized in general by derepression of hepatic ALAS by various stimuli such as drugs, sex hormones, fasting, alcohol etc[9]. Induction factors cause decrease of regulatory liver heme which is followed by maximized PBG synthesis to provide sufficient hepatic heme biosynthesis. An induction of ALAS causes overproduction of ALA and PBG, but their metabolic conversion into hydroxymethylbilane is limited due to decreased activity of porphobilinogen deaminase (PBGD) [10]. This limiting function of hepatic PBGD explains

increased levels of porphyrin precursors in all types of acute hepatic porphyrias. Knowledge of negative feedback regulation of ALAS allows the specific therapy of acute attacks by heme[11,12].

The clinical features may be divided into two main categories: acute porphyric attack and photosensitivity. An acute porhyric attack can present with heterogenous manifestations [13]. In almost all attacks, severe abdominal pain without signs of peritonism is seen. Other symptoms are nausea, vomiting, constipation, and signs of partial ileus hypertension, tachycardia, excessive sweating, electrolyte dysregulation (hyponatremia), peripheral neuropathy and psychiatric symptoms.

Four different acute hepatic porphyrias exist[14-16]: **acute intermittent porphyria**, which is among them the most common, **porphyria variegata** (PV), **hereditary coproporphyria** (HC) and **δ-aminolevulinate dehydratase deficiency porphyria**. In AIP skin photosensitivity is never present. PV is characterized by acute porphyric attacks usually associated with the presence of a vesiculoerosive photodermatitis. Skin photosensitivity may be the only manifestation of the disease. In HC, skin photosensitivity occurs in a minority of cases.

Contrary to acute hepatic porphyrias, **chronic porphyrias** are porphyrin accumulation diseases where enzymatic defect leads to a massive accumulation of the preceding porphyrins in various tissues. Clinical manifestation is related to the site of porphyrin deposition.

Porphyria cutanea tarda (PCT) is the most common form of porphyria [17]. Clinical manifestation is characterized by skin lesions similar to those seen in VP and HC. An effective treatment is accessible. Either depletion of iron stores or low-dose chloroquine produce prolonged remission in most patients[14].

Erythropoietic porphyrias manifest usually in infancy, contrary to hepatic porphyrias which are present, typically, in adults.

In **erythropoietic protoporphyria** clinical manifestations include skin lesions and liver disease[9,18,19]. Patients complain of a severe burning pain in the sun exposed skin with only small physical signs. In less than 2% of patients, cholestatic liver damage may develop leading to life-threatening liver failure. Recently, there was identified co-inheritance of ferrochelatase (FECH) gene mutation with low output of another normal FECH allele[20].

The clinical severity of **congenital erythropoietic porphyria** is highly variable and ranges from a mild form with only mild cutaneous symptoms to severe disease with non-immune hydrops fatalis, transfusion dependent anemia, severe cutaneous photosensitivity and extreme sensitivity to trauma with consequential mutilations[21]. In severe cases treatment by bone marrow transplantation has been shown to be effective[22].

2. SPECIFIC AIMS OF THE WORK

This thesis is focused on the molecular analyses of porphyrias. The following specific aims were addressed:

1. to investigate the associations between sPCT and mutations in the hemochromatosis (HFE) gene and SNPs in the transferrin receptor-1 gene (TRFC1).

2. to characterize a new case of harderoporphyria, to evaluate iron metabolism in harderoporphyric patients and to investigate the molecular bases of the harderoporphyric phenotype.

3. to characterize the crystal structure of CPO and to explain how naturally occurring mutations in CPO gene alter enzyme activity

4. to identify and characterize the pathognomic mutation within the PBGD gene of a newly diagnosed AIP patient.

5. to perform genetic analysis in the Nepal family suffering symptoms of porphyria, and to characterize the found mutation.

6. to perform the porphyrin analyses and molecular investigations in a first diagnosed Czech patient with congenital erythropoietic porphyria, to monitor the patient after cord blood stem cells transplantation by the decrease in the amount of porphyrins in bodily fluids and to perform the prenatal diagnosis in the next pregnancy

7. to identify and characterize mutations from Czech and French patients with heterozygous and homozygous forms of porhyria variegata and to analyze the factors which can modulate the clinical manifestation and the phenotype of porphyria variegata.

Each specific aim is related to one of the publications, on which my thesis is based (Publications A-G).

3. SUMMARY OF PUBLISHED DATA

3.1. Hemochromatosis (HFE) and transferrin receptor-1 (TFRC1) genes in sporadic porphyria cutanea tarda (sPCT) (Publication A)

The origin of sPCT remains unclear. Among other precipitating factors, iron was implicated in the pathogenesis of sPCT. The association between sPCT and HFE has been suggested, but existing results are ambiguous and other susceptibility genes could be involved. Among them the TFRC1, the receptor used by cells to obtain iron in the form of diferric transferin, could be a candidate gene.

The associations between sPCT and HFE gene mutations in a large cohort of French Caucasian patients was studied and the association between TFRC1 genotypes and sPCT in a case–control study using all known and novel intragenic SNPs was examined.

In the HFE gene, the C282Y, H63D and S65C mutations were studied. The C282Y mutation was present on 12% of chromosomes from patients with sPCT compared with 2% of the controls ($p< 10^4$). No statistically significant difference was found between sPCT patients and controls for the allelic frequencies of H63D and S65C mutations in the HFE gene.

In the TFRC1 gene, three previously reported SNPs were studied: in exon 4, the 519A/G leading to the S142G; in exon 7, the 884C/G; and the X+86G/C in exon 19. In addition, two novel and common SNPs were identified and further analyzed: in intron 4, the IVS4+198G/T and, in exon 19, the X+89A/G. For the IVS4+198G/T SNP, the frequency was 66% of chromosomes from patients with sPCT compared to 46% of chromosomes from controls ($p< 10^3$). No statistical difference was found for the frequencies of the other polymorphisms among sPCT patients and controls.

In summary, our results in French patients underlined the importance of the C282Y mutation as a risk factor in sPCT. We identified a new polymorphism, IVS4+198G/T, in the TFRC1 gene which was found to be associated with sPCT and seems to be a predisposition factor to sPCT as well. Our data suggest that sPCT should be considered as a multifactorial disorder in which several iron metabolism genes could be involved.

This publication was created in cooperation with the Centre Français des Porphyries in Colombes, France. My particular contribution to this publication was to determine the frequency of SNP in the TFRC1 gene using the denaturing gradient gel electrophoresis and restriction enzyme digestion.

3.2. Mutations in human CPO gene predict clinical expression of either hepatic hereditary coproporphyria or erythropoietic harderoporphyria (Publication B)

The proband developed neonatal icterus, which resolved spontaneously, and during adulthood, he suffered from mild anemia and photosensitive cutaneous lesions. Laboratory investigation showed massive accumulation of harderoporphyrin in feces. The residual activity of CPO was 18% of normal and the molecular analysis showed homozygosity for the previously described point mutation K404E in exon 6.

One of the clinical symptoms of harderoporphyria in this newly diagnosed patient, as well as in other patients diagnosed by us, is iron loading anemia. The reason for iron accumulation in these patients has not been elucidated yet. A detailed study of iron metabolism and possible causes of iron overload was performed. No additional disorder was found in these patients. We conclude that the iron overload is secondary to the marked dyserythropoiesis. Two mechanisms could contribute. First, harderoporphyrin accumulation and heme deficiency in red cell progenitors may cause maturation arrest which is known to stimulate iron absorption. Second,

overproduction of porphyrins may account for hemolytic symptoms from photolysis in dermal capillaries.

Prediction of the 2D structure of CPO using hydrophobic cluster analysis (HCA) was performed. The HCA plot of CPO exhibited a region around residue K404 which formed a hinge between two secondary structures. Five amino acids of this region (D400, R401, G402, T403, and K404) and three surrounding amino acids (F395, Y399, F405) were studied. These eight amino acids in exon 6 of CPO gene were individually mutated by site-directed mutagenesis using the pGEX-2T:CPO bacterial expression system. Six amino acids close to the region of interest (Y399-K404) were replaced by amino acids with different polarity or charge. The Y399L mutant showed only a minor defect without significant harderoporphyrin accumulation. Mutation of the other five amino acids resulted in variable loss of enzymatic activity (1-75%) with major harderoporphyrin accumulation (20-44%). F395 and F405 were replaced by a glycine, which was expected to destabilise the secondary structure. This resulted in loss of enzymatic activity (4 and 1% of residual activity, respectively) without significant accumulation of harderoporhyrin.

To further analyze whether the aberrant harderoporphyrin production is restricted to mutations in the investigated region in exon 6, all point mutations described thus far in CPO patients so far were expressed in E. coli. The residual activities were 1-66%, and no significant accumulation of harderoporhyrin was found, with the exception of mutations R401W and K404E identified in harderoporphyric patients.

Our data strongly support the hypothesis that the region extending from amino acid 400 to 404 is important for the catalyses of oxidative decarboxylation of harderoporphyrinogen. This region may be important in preventing diffusion of harderoporhyrinogen away from the catalytic site during the sequential reaction.

In summary, a new case of harderoporphyria was characterized and the previously described mutation K404E was detected. We propose that iron overload in harderoporphyric patients is secondary due to the marked dyserythropoiesis. An accumulation of harderoporhyrin was found when amino acids in the region 400-405 were mutated, while mutations in other parts of CPO gene did not result in harderoporphyrin accumulation.

This publication was created in cooperation with the Centre Français des Porphyries in Colombes, France. My contributions to this publication were to introduce the particular mutations into the pGEX-2T:CPO bacterial expression system using site-directed mutagenesis and to optimize this system.

3.3. Structural basis of hereditary coproporphyria (Publication C)

The crystal structure of human CPO at 1.58-Å resolution was determined. The tertiary topology is characterized by a large seven stranded β-sheet that is flanked on both sides by α- helices. Contrary to other enzymes containing seven stranded β-sheets which are usually curved, the β sheet in CPO is flat probably due to the high abundance of glycine residues.

As was predicted, the homodimer is the biologically relevant form of CPO. The dimensions of the dimer are 80x60x60 Å. The corresponding β strands from the CPO monomers generate key contacts at the dimer interface in an antiparaller fashion. The interface is made up of 64% nonpolar atoms and 36% polar atoms. Ten intersubunits H bonds also contribute to the dimer stability. Taken together, the interacting surface on the CPO monomer is a hydrophobic patch.

CPO structure lacks a transitional metal center. Neither the energy scans nor the native anomalous differences Fourier maps provide evidence for bound transition metal ions in CPO. The invariant His residues are far apart and cannot serve as ligands.

The structure of the active site and catalytic mechanisms were described. The unexpected finding of citrate at the active site allows us to assign Ser-244, His-258, Asn-260, Arg-262, Asp-282, and Arg-332 as a residues mediating substrate recognition and decarboxylation. We favor a mechanism in which oxygen serves as the immediate electron acceptor, and a substrate radical or carbanion with substantial radical character participates in catalysis.

There are >20 naturally occurring HC mutations, and a majority of these mutations lead to substitution of amino acid residues within the structural framework of CPO. The molecular basis for how these alterations diminish enzyme activity is unknown. We have focused on mutations in the CPO gene that are distant from the active site although with little to no activity. The deletion of residues 392-418 encoded by exon six disrupts dimerization. W427R also affect dimerization, H327R and R327C perturb the interaction between helix α7 and the dimerization helix α9, and R331W substitution abolishes the hydrogen bonds between the guanidinium group and the carbonyl of Leu-446 and Arg-447.

The harderoporphyria - causing mutation K404E precludes a type I β-turn from retaining the substrate for the second decarboxylation cycle. The R401W mutation is also defective in the second decarboxylation step. The enzyme most likely prevents the release of the intermediate by an ionic interaction with a propionate group that does not undergo decarboxylation.

In summary, the crystal structure of CPO at 1.58 Å was determined. We conclude that the homodimer is the biologically relevant form of CPO. CPO catalyses an unusual metal- and cofactor–independent oxidative decarboxylation. The structure of the active site and catalytic mechanisms were described. The effects of naturally occurring mutations in the CPO gene were determined. The deletion of residues 392-418 encoded by exon six disrupts dimerization. The primary role for the 401-405 segment is to deter the premature release of intermediate.

This paper was created in cooperation with the Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, Texas, USA. My contributions to this study were to prepare the CPO expression vectors with the desired mutation and to prepare crystallization quality protein.

3.4. De novo mutation found in the porphobilinogen deaminase gene in Slovak acute intermittent porphyria patient: molecular biochemical study (Publication D)

The proband was a boy having his first attack of AIP at the age of 15 years.

The molecular analyses revealed a novel mutation in exon 15, the 966insA. The proband was the only member of the family having this mutation. The mutation was suggested to be a *de novo* mutation as non-paternity was excluded by DNA microsatellite analyses.

This mutation leads to the formation of a stop codon after 36 completely different amino acids, compared to the original sequence. The truncated mutant protein consists of 357 amino acids as opposed to the normal 361 amino acids.

To investigate the impact of the mutation, we expressed the normal and mutated PBGD in a procaryotic expression system. We inserted the cDNA of PBGD into the pGEX4T-1 expression vector. Using site directed mutagenesis, the mutation was introduced into the construct.

The analysis of the purified mutant protein using the SDS PAGE showed several bands, in contrast to a single homogenous band in the case of normal protein, suggesting low stability of the mutated protein. Both normal and the mutated PBGD enzymes were similar in size, approximately 68 kDa with GST-tag and 42 kDa without GST-tag.

Specific activities were estimated for the thrombin-digested protein and for the fusion protein as well. The fusion protein exhibited approximately 22% higher activity than the thrombin digested protein. The purified mutant enzyme had extremely low residual activity, 0.18% of normal.

The structure of the mutant protein was predicted using computer-assisted structure prediction. The mutation is located in the $\beta 3_3$ sheet; two helices, $\alpha 2_3$ and $\alpha 3_3$, of domain 3 are completely missing due to the truncation. In the normal protein, the C-terminal helices protect the β strands from being exposed to solvent. This is in agreement with the severely impaired stability of mutant PBGD, as detected from SDS PAGE results.

In summary, the de novo mutation 966insA was found in a Slovak patient with AIP. Due to the 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.

This study was performed in cooperation with Department of Pediatrics in Prague, Second Pediatric Department in Košice and Laboratory of Structural Biology in Houston. My contribution to this study was to identify the mutation in the proband´s PBGD gene using DGGE analysis and direct sequencing. I performed this investigation in all members of the family, and not find the mutation in either one of the parents. This led to conclusion that the mutation found in the young proband is a *de novo* one. Such a situation always complicates diagnostic outcome in a disease normally inherited in an autosomal dominant fashion.

3.5. New mutation within porphobilinogen deaminase gene leading to the truncated protein as a cause of acute intermittent porphyria in extended Indian family. (Publication E)

On the basis of the internet search we were contacted by a 50 years old proband from Nepal suffering typical abdominal form of porphyria. He, not being a health professional, diagnosed himself as having an acute porphyric attack, asked for the hemearginate (Normosang, Orphan Europe) treatment with excellent clinical affect, and arranged sending blood or genomic DNA from 15 family members to our laboratory.

Molecular analyses revealed a novel mutation in exon 15, the 973insG. Subsequently, genetic analysis was performed in all accessible members of this extensive family. The mutation leads to inclusion of four different amino acids and, further, the protein 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 analysis of the purified mutant protein using the SDS PAGE showed several bands, in contrast to a single homogenous band in the case of normal protein, suggesting very low stability of the mutated protein. The purified mutant enzyme had very low residual activity, 0.5 % of the average level of protein expressed by the normal allele. The loss of C-terminal helix in mutant protein would be expected to expose the β -strand core that could make the protein prone to agregation and loss of enzymatic function.

In summary, a novel mutation within the PBGD gene (973insG), which leads to truncated protein, resulting in instability of the protein and loss of enzymatic function was identified and characterized in an extensive Indian family from Nepal. The proband diagnosed himself based on internet information.

This study was performed in cooperation with Department of Genetic Medicine, Sir Ganga Ram Hospital, New Delphi, India and Laboratory of

Structural Biology in Houston. My contribution to this study was to identify the proband´s mutation in PBGD gene using DGGE analyses and direct sequencing.

3.6. Treatment of congenital erythropoietic porphyria (Morbus Günther) by cord blood stem cell transplantation, long term follow-up with 11% chimerism and prenatal diagnosis in a sibling. (Publication F)

The proband was a boy who developed severe photosensitivity in the neonatal period after phototherapy for an icterus. The porphyrin analysis showed an excessive amount of porphyrins in urine and plasma and mildly increased fecal porphyrins. The identification of the porphyrin isomer in urine showed nonphysiologically increased isomer I, which is typical for Günther disease. Molecular investigation of the UROS gene revealed homozygosity for "the hotspot" mutation C73R and thus confirmed the diagnosis. Parents of this child were heterozygous for this mutation.

At the age of 8 ½ months, the boy underwent the cord blood stem cells transplantation (CBST). After the transplantation he prospered well. No new marks of photosensitivity appeared, hepatosplenomegaly resolved, and his psychomotor development continues.

Detailed examination of changes in body fluid porphyrin content was performed. We found a fluctuation in the excretion of total porphyrins in the first 5 months after transplantation; after that period, the quantity of excreted porphyrins was low, but did not reach the reference range, remaining about 100-200 times higher. In samples of urine from the 5th and 11th months after CBST, we analyzed isomers of excreted COPRO. The ratio of isomer I and III remained pathological. In plasma, total porphyrins descended continuously but 13 months after CBST, they still exceeded normal value, by approximately 10-fold. Eleven months after CBST, we measured the activity of UROS in

erythrocytes. The activity of UROS in erythrocytes of the child was within reference range, while the activity of UROS of his mother was slightly decreased.

Prenatal diagnosis was performed in the next pregnancy. The examination of porphyrins in amniotic fluid, as well as DNA analysis showed that the fetus was a heterozygous carrier of the mutation C73R and thus excluded from the clinical expression of CEP.

In summary, the first patient with CEP was diagnosed in the Czech Republic. Successful treatment by cord blood stem transplantation was documented by porhyrin analyses in long-term follow-up. A prenatal diagnosis was performed in the next pregnancy within the family.

This study was performed in cooperation with Departement of Pediatric Haematology and Oncology, Second Faculty of Medicine, Prague. My contribution was to identify the mutation in the UROS gene in proband and his extended family, to analyze porphyrins in body fluids during long-term monitoring after CBST, and to link 7-11% chimerism with the residual metabolic defect.

3.7. A search for molecular determinants responsible for different phenotypic expression of porphyria variegata (Publication G)

DNA from eight patients with "classical" heterozygous PV and from three patients with the homozygous form was analyzed. In all cases, the pathognomic mutation was identified. The list of mutations found is in table 3A in publication G.

To further investigate the functional consequence of these naturally occurring mutations in PPOX, we expressed normal and mutated human PPOX in Escherichia coli using the pGEX-2T vector. We analyzed in detail these mutations: a) new missense mutations in patients with the heterozygous

form of PV, b) all missense mutation found in ours patients with homozygous PV, c) two already described SNP R304H and P256R and these two polymorphism together in one allele.

The activities of the mutated proteins are summarized in table 3B in this publication. Except for the mutation Y422C, all missense mutations found in patients with heterozygous porphyria variegata lack PPOX residual activity. The point mutation Y422C had residual activity 3%. All of the examined mutations found in patients with homozygous PV demonstrated some residual activity (ranging from 0.42%-10.3%). Both analyzed SNPs demonstrate decreased activity in comparison with wt PPOX. The substitution R304H preserved about 85 % of activity and the allelic variation P256R had 65%. The coexistance of the polymorphism R304H together with P256R in one PPOX allele led to further diminution of the activity, to approximately 10%.

The frequencies of the two SNPs, as well as that of the newly identified SNP IV10+9 G→A, were established in the Czech and French populations. The frequency of the P256R variant was 2.3% in Czech and 4.5 % in French population, that of R304H was 4.5% in Czech and 27% in French population, and that of IV10+9 G→A was 0.9% in both analyzed cohorts.

In summary, a search for the molecular determinants responsible for different phenotypic expression of porphyria variegate led to the identification and characterization of two polymorphisms within the PPO gene that result in lowering PPO activity in addition to the original mutation. Once the critical threshold for decreased activity is reached, phenotypic expression of an acute attack may occur.

This publication was created in cooperation with the Centre Français des Porphyries in Colombes, France. This work was the main subject of my PhD thesis. My contribution was to optimize, prepare and to express individual mutated PPOX proteins in prokaryotic expression systems, to measure the activity of mutated PPOX proteins, and to estimate the frequency of mutations and polymorphisms in the PPOX gene in the Czech population.

4. GENERAL DISCUSSION

Porphyria was mentioned for the first time probably as early as 460 BC by Hippocrates [23] but the greatest discoveries date from the end of the 19th century. As in other metabolic diseases, the research in porphyrias began in clinical description and headed towards the understanding of the basis of this magical disease to facilitate better care of the patients and to comprehend heme synthesis. The porphyria research history can be divided into several periods regarding the dominant methodology (Table 10).

Table 10: Dominant Disciplines in Porphyria Research[24]

1937-1952	Chemistry
1952-1967	Biochemistry
1967-1982	Enzymology
1982-1997	Molecular genetics
1997-	Functional genomics and proteomics, gene therapy, animal trangenic models, structural aspects of proteins of heme synthesis

The end of 20th century was the great entrance of molecular biology. During a 15-year period, the genes coding all enzymes of heme biosynthesis were cloned and expressed. Consequently, the methods for identification of mutations and their screening were optimized for each porphyria. The detection of pathognomic mutations greatly improved the diagnostics and ameliorated the therapy.

Nowadays even more profound discoveries have come involving the heme biosynthetic pathway. Successful crystallization of the enzymes of heme biosynthesis allows us to learn about the tertiary and quaternary structures of these enzymes. The study of the consequence of mutations in different

regions of the proteins provides better understanding of the function of the enzyme. This is important, not only for the care of patients with porphyrias, but also for understanding the regulation of heme biosynthesis and the importance of heme in a wide variety of essential biological processes. The introduction of animal models of certain porphyrias helps us better understand the pathogenesis of these mysterious diseases and improves the therapeutic possibilities. Laboratory trials of bone-marrow transplantation (BMT) on murine models were followed by successful BMT in human patients with CEP. Presently, the promising gene therapy is in progress.

The outcomes from this PhD dissertation deepen our knowledge about porphyrias and the structure and function of heme biosynthetic pathway enzymes. In publication A, we analyzed the relationship between sPCT and mutations in the HFE gene and SNPs in the TRFC1. In publication B, we studied the molecular bases of harderoporphyria, the consequence of the localization of mutations in the CPO gene on phenotype variant manifestation, either as HC or as harderoporhyria. In publication C, we report the crystal structure of CPO, we describe the catalytic mechanism of the enzyme and hypothesize how naturally occurring mutations in the CPO gene alter enzyme activity. In publication D and E, we investigated the consequences of the mutation found in patients with AIP on the structure and function of PBGD. In publication F, we present the outcome of the patient with CEP after CBST. Until now only ten successful transplantations of haematologic stem cell have been reported. In publication G, we investigate the effect of mutations in PPOX gene found in patients with PV on the function of the enzyme and we analyze the modulation of the phenotype of PV by an additional SNP in PPOX gene.

In conclusion, our work has provided previously uncharacterized insights for understanding porphyrias at the molecular level.

5. CONCLUSION

1. **An association between the HFE C282Y mutation and sPCT was validated and the new polymorphism IVS4+198G/T in TFRC1 gene, which seems to be predisposition factor to sPCT, was identified in a French population.**

2. **A new case of harderoporphyria was characterized. Harderoporphyric patients exhibit iron overload secondary due to dyserythropoiesis. Only a few missense mutations, restricted to five aminoacids in exon 6, may cause accumulation of significant amounts of harderoporphyrin, thus this region is important for catalysis of oxidative decarboxylation of harderoporphyrinogen.**

3. **The crystal structure of CPO at 1.58 Å was determined. The structure of the active site and catalytic mechanisms was proposed. The consequence of naturally occurring mutation in CPO gene was determined.**

4. **One *de novo* mutation in the PBGD gene (966 insA) was found in a Slovak patient with AIP. This mutation leads to an almost complete loss of enzymatic function and a decrease in the stability of the protein structure.**

5. **A novel mutation within the PBGD gene (973insG), which leads to truncated protein, resulting in instability of the protein and loss of enzymatic function was identified and characterized in an extensive Indian family from Nepal. The proband diagnosed himself based on internet information.**

6. **The first patient with CEP was diagnosed in the Czech Republic. Successful cord blood stem transplantation was documented by porhyrin analyses. A successful prenatal diagnosis was performed in the next pregnancy within the family.**

7. **A search for the molecular determinants responsible for different phenotypic expression of porphyria variegate led to the identification and characterization of two polymorphisms within the PPOX gene that result in a protein variant lowering PPOX activity in addition to the disease-causing mutation. Once the critical threshold for decreased activity is reached, phenotypic expression of an acute attack may occur.**

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