ABSTRACT OF PHD THESIS

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Biochemical and Functional Manifestations of Inherited Disorders of the Mitochondrial F_1F_oATP synthase

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Prague, 2007

BIOCHEMISTRY AND PATHOBIOCHEMISTRY

PhD thesis was elaborated during Postgraduate Studies in Biomedicine at the 1st Medicine Faculty of Charles University and Institute of Physiology, Academy of Sciences of the Czech Republic in years 2002-2007.

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PhD Thesis Report sent:

Defence of PhD Thesis is held on September 20th, 2007 in front of the Biochemistry and Pathobiochemistry Commission.

Place of defence:	Děkanát 1.LF UK, Kateřinská 32, Praha 2
Chairman:	Prof.MUDr. Jiří Kraml, DrSc. Ústav lékařské chemie a biochemie 1.LF UK Kateřinská 32, Praha 2

Introduction

Mitochondria and Oxidative Phosphorylation system

Almost 90 % of energetic demands of mammalian organism is covered by ATP generated by the oxidative phosphorylation (OXPHOS) process in mitochondria.

The electrons from oxidized substrates stored in the form of redox equivalents, reduced cofactors NADH and $FADH_2$, are utilized by the OXPHOS. The OXPHOS localized in the inner mitochondrial membrane consists of complexes of the respiratory chain and mitochondrial F_1F_o ATP synthase (ATPase). The respiratory chain is organized into four enzyme complexes of the electron transport chain – Complex I (NADH:coenzyme Q oxidoreductase); Complex II (succinate:coenzyme Q oxidoreductase); Complex III (coenzyme Q:cytochrome *c* oxidoreductase) and Complex IV (cytochrome *c* oxidase). These protein complexes are associated with a variety of redox-active prosthetic groups with increasing reduction potentials transporting electrons from redox equivalents to molecules of oxygen. The electrons are transported by special carriers – coenzyme Q and cytochrome *c*. In addition to the traditional components mentioned above, other dehydrogenases are usually associated with the respiratory chain, e.g. mitochondrial glycerophosphate dehydrogenase (GPDH) [1] and flavoprotein-linked acyl-CoA dehydrogenase [2]. The reactions of complexes I, III and IV are also associated with proton transfer across the mitochondrial inner membrane from matrix into the intermembrane space, creating an electrochemical proton gradient ($\Delta\mu_H^+$). This gradient is subsequently utilized by mitochondrial F_1F_o ATP synthase that synthesizes the ATP from ADP and phosphate and regulates the respiratory rate.

Mitochondrial F_1F_o- ATP Synthase

Structure of F_1F_o- ATP Synthase

The mitochondrial adenosine triphosphate synthase, F_1F_o ATP synthase EC 3.6.3.14 (ATPase) is a large multisubunit enzyme complex situated in an inner mitochondrial membrane. The bovine complex contains 16 different proteins (including the regulatory subunit, IF_1) and has a mass of about 600 kDa [3]. Five subunits in the stoichiometric ratio $\alpha_3\beta_3\gamma\delta\varepsilon$ comprise the F_1 catalytic unit [4], an ATP hydrolysis-driven motor, whereas an additional 10 subunits comprise F_o. The extramembranous F_1 subcomplex is connected with the membrane intrinsic F_o subcomplex by a central stalk [5], the static, non-rotating portions

of F_1 and F_o are attached to each other via the peripheral „stator stalk“. The F_o part of the mammalian enzyme is built of subunits with a total composition - F_o: $abc_{10}defg + F_6 + A6L +$ OSCP [7, 8].

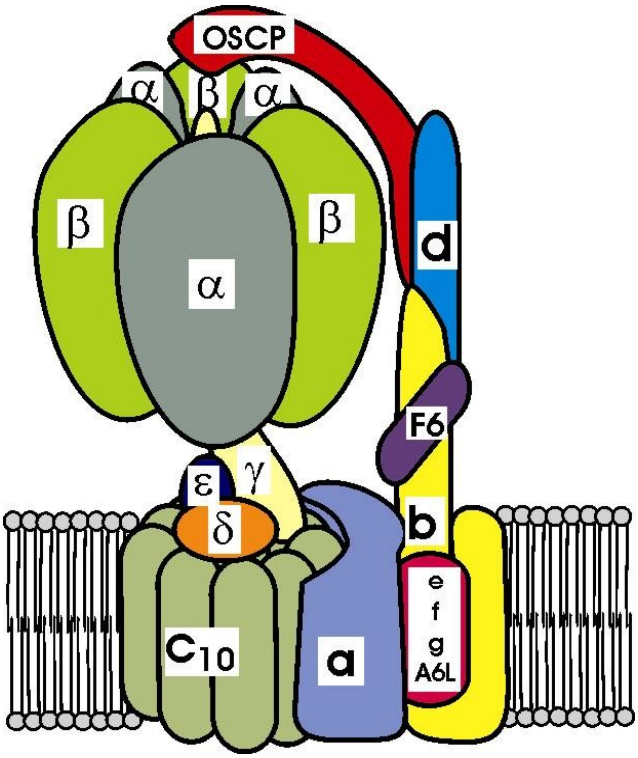

Figure 1. The subunit organisation in mitochondrial F_1F_o-ATPase. *F_1 is the globular catalytic domain made of subunits α, β, arranging heterohexamer $\alpha_3\beta_3$, and the three central stalk subunits, γ, δ and ε. The F_o domain is comprised of the c oligomer (10 copies), subunit a, and the peripheral stalk subunits b, d, F_6 and OSCP. The so-called minor subunits (e, f, g, and A6L) are not shown individually, but they all span the membrane and are probably present in a 1:1:1:1 stoichiometry. The rotor is made up of the central stalk and the c-ring. The remainder of the subunits makes up the stator. The inhibitor protein (IF_1) is not shown; it binds in a catalytic α/β interface near the bottom of $(\alpha\beta)_3$. Adapted from [9].*

Function of Mitochondrial ATP synthase

The mitochondrial F_1F_o- ATP synthase is a nano size rotary engine that produces most of the energy in form of the ATP molecules required to drive many energy demanding reactions in living cells. This multisubunit complex can operate as a reversible rotary motor and exchanges energy by mechanical rotation of the central stalk.

In synthesis mode, the F_o motor converts the electrochemical gradient of protons into torque to force the F_1 motor to act as an ATP generator when molecules of ADP and P_i are substrates for the formation of the terminal phospho-anhydride bond of ATP. On the other hand, in hydrolysis mode, F_1 converts the chemical energy of ATP hydrolysis into torque, causing the membrane-embedded F_o motor to act as an ion pump. The F_1 complex, consisting of $\alpha_3\beta_3$ hexamer around a central coiled-coil subunit γ, is intrinsically asymmetric, owing to different interactions of the central subunit γ with each of the catalytic subunits β, and provides them with different conformations and nucleotide affinities at their catalytic sites. On rotation

of the subunit γ, the conformations of the three subunits β change sequentially such that each subunit β successively adopts the same conformations of varying affinity during one rotational cycle. An anticlockwise rotation of the γ chain, looking from below F_1 and towards the bilayer is associated with ATP synthesis. It is envisaged that the α and β chains are held stationary by a stator.

It was shown that Mg^{2+} plays an important role in transition state formation during ATP synthesis [10]. As a result, three molecules of ATP are synthesized. This model is called as the binding change mechanism [11].

Gene Organization of the Mitochondrial Genome

Mitochondria contain their own DNA, which is thought to be a remnant from the time that they were free-living organisms before forming a symbiotic relationship with eukaryotes. Human mtDNA is a 16.5 kb (16569 base pairs) circular minichromosome, composed of two complementary strands, the heavy (H) and light (L) strands. It is much smaller than most nuclear genes. All of the coding sequences are contiguous with each other with no introns [12]. The only non-coding stretch of mtDNA is the displacement-loop (D-loop). Although human mtDNA encodes the basic machinery for protein synthesis, it remains entirely dependent upon the nucleus for the provision enzymes for replication, repair, transcription and translation.

The human mtDNA contains both protein-encoding genes and protein synthesis genes. The 13 protein encoded genes specify seven subunits of the Complex I (ND1, ND2, ND3, ND4, ND4L, ND5, ND6), three subunits of the Complex IV (COX1, COX2, COX3), subunits 6 and 8 of ATPase, and apocytochrome *b*, which is part of the Complex III. The rest of mtDNA genes encodes two rRNAs (12 and 16S rRNA) and 22 tRNAs that are involved in protein translation.

MtDNA is highly polymorphic, with several differences in sequence between individuals from the same ethnic group and more between those in different groups. MtDNA haplotypes are based upon specific pattern of polymorphism and seem to influence the ageing process, susceptibility to some diseases, and the expression of some mtDNA mutations [13, 14]. MtDNA haplotypes have been used to track population movements across the globe and serve to provide a means to evaluate ethnic descent. A mammalian cell contains multiple mtDNA copies (polyplasmy), since each mitochondria contains 2-10 mtDNA copies and since a cell has up to 10 000 mitochondria, depending on the cell type [18].

Assembly of Mitochondrial ATP Synthase

The multisubunit ATP synthase complex is formed stepwise with the assistance of several assembly factors but the mechanism of how the mammalian ATP synthase assembles from individual subunits is still not well understood.

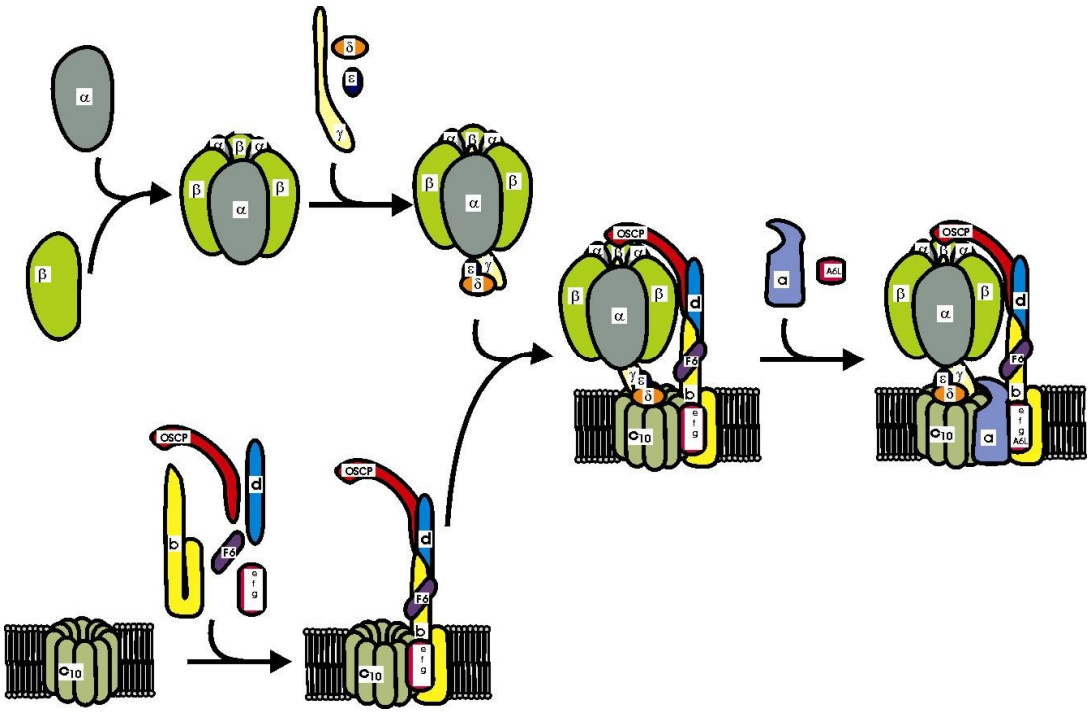

Figure 2. Assembly of the mammalian ATP synthase. *ATP synthase is formed in several successive steps.*

In the case of F_1 part, a close similarity of its structure in all types of energy-transducing membranes suggests an analogous assembly mechanism in mammalian cells, lower eukaryotes and prokaryotes.

Two proteins, Atp11p and Atp12p, were first described as proteins required for assembly of the F_1 component in yeast *Saccharomyces cerevisiae* [19]. Later human genes for the Atp11p and the Atp12p were characterized [20]. In both yeast and human cells, there was evidence that the Atp11p interacts specifically with the $F_1\beta$ subunit [21], while the Atp12p binds selectively to the $F_1\alpha$ subunit [20, 22]. The assembly of the $\alpha_3\beta_3$ hexamer is a complicated process consisting of interaction sequence where unassembled subunit is replaced by the neighboring subunit [21, 22]. The rest subunits γ, δ and ε are connected subsequently.

Concerning the F_o, the situation is complicated by increasing evolutionary complexity of the F_o structure, which gained 7 new subunits from bacteria to man. Most of the present

knowledge of mitochondrial ATP synthase biogenesis originates from studies in yeast but the assembly process in mammalian cell might be modified as there are substantial differences between higher and lower eukaryotes such as the number and location of F_o subunit c genes, ATP synthase-specific assembly factors, or factors regulating transcription of mtDNA-encoded ATP synthase genes.

It is suggested that primary assembly of the subunit c ring in the membrane is a crucial step for formation of F_o structure [23]. In mammals, subunit c appears to play an important role in the formation of the whole ATPase complex. F_1-c subcomplex (apparent mass, 470 kDa) was identified as a stable assembly intermediate [24]. It is known that association of subunit b with the F_o is important for the subsequent assembly of OSCP and subunit d [25]. However, subunits b and c and subunit 8 of yeast (or A6L) are essential for assembly of subunit a. Subunits e and g are assembled at a late stages [26]. Thus, subunit a, important proton channel component, must be added at the last steps in assembly of F_1F_o. Mutations in mtDNA affecting the *ATP6* gene (for subunit a) are often connected with abnormal accumulation of subcomplexes [27]. In the assembly process of yeast but not mammalian F_o part some factors, namely Atp10p and Atp22p, are involved. The Atp10p is considered to be a chaperone for subunit a. It has been suggested that the Atp22p plays an important role in a post-translational changes in F_o assembly [28, 29]. Presently, a new metalloprotease, termed Atp23p, has been found to be associated with the inner mitochondrial membrane and be conserved from yeast to humans.

The structure of soluble F_1-ATPase from bovine heart reconstituted with IF_1 corresponds to a F_1-F_1 dimer bridged by two IF_1 molecules [30]. Recent data indicate specific associations of ATP synthase with other OXPHOS components and adenine nucleotide (ADP/ATP) translocator (ANT) and Pi carriers (PIC) to build up „ATP-synthasomes“ with a stoichiometry ATPase:ANT:PIC 1:1:1 [31]. This complex is assumed to improve the efficiency of ATP synthesis by substrate-product channeling.

Mitochondrial Diseases

Defects of mitochondrial metabolism cause a wide range of human diseases that include examples from all medical subspecialties. Given its important role in the human body, defects of mitochondrial function can have disastrous consequences. The mitochondrial diseases, namely mitochondrial encephalomyopathies, are defined restrictively as disorders due to defects of mitochondrial energy metabolism, i.e. oxidative phosphorylation [32]. Faulty

oxidative phosphorylation may be due to overall dysfunction of the OXPHOS system or can be associated with single or multiple defects of the five complexes forming the OXPHOS system itself.

Changes in mtDNA sequence can be inherited or somatic (created *in situ*). MtDNA has a mutation rate of 10-20 fold that of nuclear DNA. The reasons for this include relatively high levels of oxygen radicals in mitochondria, the lack of introns, further the lack of protective histones of mitochondrial DNA and the failure of proof-reading by mtDNA polymerases. Over 150 pathogenic point mutations and more than 200 deletions, insertions, and rearrangements have been repeatedly so far identified and new mutations are being described every year (MITOMAP). Most of the sequence variations observed in mtDNA between individuals are simple polymorphisms that carry no risk of disease.

About 60 % of the currently known point mutations affect mitochondrial tRNAs, 35 % affect polypeptide subunits of the OXPHOS system, and 5% affect mitochondrial ribosomal RNAs. The mtDNA depletion syndromes are the result of nuclear gene defects and typically lead to either myopathy or liver and brain disease. Point mutations are frequently inherited whilst deletions and insertions are usually sporadic.

Deficiencies that involve more than one respiratory chain complex are common in mtDNA mutations. All such mutations affect mitochondrial protein synthesis globally, either indirectly; via deletions that remove large segments of the mtDNA (Kearns-Sayre syndrome) or directly, via mutations in specific tRNA and rRNA genes (the most known MELAS and MERRF).

Respiratory chain complexes disorders

Isolated ***Complex I*** deficiency is one of the most common causes of mitochondrial encephalomyopathies. Patients usually present at birth or in early childhood with severe multisystemic disorders dominated by brain dysfunction, most commonly with Leigh syndrome, a devastating neurodegenerative disorder characterized pathologically by bilateral lesions in the brainstem, basal ganglia, thalamus and spinal cord, and presented clinically by psychomotor retardation and brainstem or basal ganglia dysfunction with or without cardiomyopathy [33, 34]. Of the ten known mutations in Complex I genes, six are associated with Leber´s hereditary optic neuropathy (LHON), a maternally inherited cause of blindness [35]. Mutations in mitochondrial tRNA genes also lead to defects of Complex I with

phenotypic presentation MELAS, MERRF, or isolated myopathy (for review see [36]). In general, mtDNA mutations cause only a minority of isolated Complex I deficiencies.

The pathogenic missense mutations have been found in the flavoprotein subunit of ***Complex II***, causing autosomal-recessive Leigh syndrome. Mutations in both, the small and the large cytochrome b_{560} subunits represent the first errors in a respiratory chain gene associated causally with neoplastic transformation [37].

Even though 10 of the 11 subunits of ***Complex III*** are nDNA encoded (only cyt *b* subunit is specific by mtDNA), no pathogenic nuclear mutations have yet been identified. On the other hand, 12 pathogenic mutations have been found in the cytochrome *b* gene and, remarkably, all cases were sporadic, most of them isolated myopathies with or without myoglobinuria [38].

Patients with autosomal recessive ***Complex IV*** deficiency can present with a number of different clinical phenotypes including classical Leigh syndrome, a French-Canadian form of Leigh syndrome, fatal infantile COX deficiency, hypertrophic cardiomyopathy and myopathy, and a reversible COX deficiency confined to skeletal muscle [39]. Mutations in all three of the mtDNA encoded subunits that constitute core of COX have been identified. Mutations have not been identified in any of the 10 nuclear-encoded structural genes in patients with any of the above phenotypes [40]. However, mutations in different mitochondrially targeted nDNA-encoded proteins that are required for the assembly of the holocomplex – SURF1, SCO2, COX10 and others – have now been associated with recessively inherited Leigh or Leigh-like syndromes and COX deficiency.

Mitochondrial ATP synthase disorders

Generalized decrease in the content of human ATP synthase or alteration of its structure and function are associated with severe pathological states resulting in typical mitochondrial diseases. There are two types of isolated deficiency of ATPase that have been described, differing in the pathogenic mechanism, biochemical phenotype (structural and functional) as well as in clinical presentation.

The first and long well-known type, are qualitative defects of ATPase caused by mutations in mtDNA-encoded ATPase subunit that produce a variable picture of differential onset and differential severity. Up to date, no mutation has been found in *ATP8* gene that codes the second mitochondrially synthesized ATPase subunit. Pathogenic mutations (five missense mutations described so far) found in the subunit a are all associated with “striatal necrosis

syndromes", maternally inherited Leigh syndrome (MILS) (mutations T8993G and T8993C), or NARP (neuropathy, ataxia and retinitis pigmentosa).

In addition to missense mutation in the *ATP6* gene there exists yet another inherited defect, microdeletion of few nucleotide bases. A 2 bp microdeletion at positions 9205 and 9206 (9205ΔTA) was originally discovered in a newborn with transient lactic acidosis [41].

ATPase defect due to nuclear genome mutations was demonstrated for the first time in 1999 in a new type of fatal mitochondrial disorder [42]. A boy with severe lactic acidosis, cardiomyopathy and hepatomegaly died within 2 days of his life. Up to now 14 cases have been described that exhibit quite homologous phenotype consisting of elevated plasma lactate levels, 3-methyl-gltaconic aciduria, hypertrophic cardiomyopathy, psychomotor and various degree of mental retardation [42-45].

Finally, the first mutation in the *ATP12* gene, for assembly factor of mitochondrial ATPase, has been identified in a single infant patient with lactic acidosis, dismorphic features and rapidly progressive encephalopathy [45].

Nuclear defects involved in biogenesis, function and dynamics of OXPHOS

Disorders due to mutations in nDNA are very numerous not only because most respiratory chain subunits are nucleus-encoded, but also – and more importantly – because correct structure and functioning of the respiratory chain requires many steps, all of which are under the control of nDNA. These steps include assembly of respiratory chain complexes associating factors SURF1, SCO1, SCO2, COX10, COX15, LRPPRC, BCS1L, ATP12 and other, and also transport of nDNA-encoded proteins from the cytoplasm into mitochondria. MtDNA integrity and replication requires nDNA-encoded factors - thymidine phosphorylase, adenine nucleotide translocator (ANT1), Twinkle, helicase, polymerase γ (POLG), thymidine kinase 2 or deoxyguanosine kinase. The respiratory chain is embedded in the lipid bilayer of the inner mitochondrial membrane. Alternations of this lipid milieu can cause disease, as illustrated by altered synthesis of cardiolipin in Barth syndrome. Mitochondria are dynamic organelles and move around the cell, divide by fission, and fuse with one another. Disorders of these essential functional can also cause disease, as illustrated mutations in a gene encoding a dynamin-related guanosine triphosphatase

At the end, abnormalities of mtDNA and/or OXPHOS activity have been identified in several different neurodegenerative diseases, such as Friedreich´s ataxia, Wilson disease, Huntington disease, Parkinson disease, amyotrophic lateral sclerosis, and Alzheimer disease [46].

Mitochondria and ROS

In most cell types, mitochondria appear to represent one of the major sources of generation of free radicals or reactive oxygen species (ROS). ROS may cause lipid peroxidation and damage to cell membranes and to DNA, above all mtDNA, so that mitochondria represent not only a major source of ROS generation, but also a major target of ROS induced damage. The major recently demonstrated components of ROS generation are Complexes I, II and III of the respiratory chain cascade, external NADH dehydrogenase, mitochondrial glycerophosphate dehydrogenase, dehydroorotate dehydrogenase, aconitase, α-ketoglutarate dehydrogenase and monoamino oxidase (for references see [47]).

In overall, ROS production is very complicated process depending on redox state of the mitochondria and correlating with values of the mitochondrial membrane potential $\Delta\Psi_m$ [48]. It is also well documented that high levels of potential $\Delta\Psi_m$ above 140 mV lead to an exponential increase of mitochondrial ROS production [49, 50]. In line with this finding, decrease of $\Delta\Psi_m$ via stimulation of ATP synthase, a low AT/ADP ratio, substrate limitation or increased proton permeability lower the amount of ROS produced [51]. It is assumed that the UCP proteins play an important role in oxidative stress in cells. It was shown that mainly UCP2 expression might limit the free radical generation [52].

Mitochondria are equipped with series of antioxidant defenses. Mitochondria contain a high concentration of glutathione, a variant of superoxide dismutase, catalase and glutathione peroxidase. In addition to cytochrome *c*, other electron carriers, namely ubiquinol QH_2, appear to have a detoxifying role against ROS.

The aims of the thesis

The ATPase is a key component of mitochondrial energy conversion in the mammalian organism. The isolated defects of the ATPase result in mitochondrial diseases which are deleterious and manifest primarily in children, very often shortly after birth.

The labor techniques to determine defects of ATPase are still not routinely used in many diagnostic laboratories. It is therefore probable that ATPase disorders may escape diagnosis in accordance with our opinion that the incidence of isolated ATPase deficiency would be much higher than originally believed.

The present thesis is focused on the diseases caused by various etiopathologies of mitochondrial ATP synthase. The primary aim was to characterize pathophysiological consequences as well as clinical manifestation of isolated defects of ATPase that are caused by mutations in mitochondrial genome or in nuclear genes.

The specific aim of this work was:

a) to characterize structural and functional changes in ATPase and cytochrome *c* oxidase due to a 9205ΔTA microdeletion in the mtDNA *ATP6* gene;
b) to elucidate the consequences of the replacement of the initiation codon AUG for GUG in the *ATP6* gene due to homoplasmic A8527G mutation;
c) to characterize and correlate biochemical data and clinical presentation on 14 patients with isolated ATPase deficiency caused by nuclear genetic defects;
d) to characterize pathological mechanism of the isolated ATPase disorders with respect to energy deprivation and increased ROS production.

Summary of results

The thesis consists of 5 attached articles that all have already been published. They have presented many results of our studies describing several deficiencies of mitochondrial ATPase due to various mutations and defects. In these papers we have analyzed pathogenic mechanisms and shown structural and functional consequences as well as clinical features of the ATPase disorders.

1. **Diminished synthesis of subunit a (ATP6) and altered function of ATP synthase and cytochrome *c* oxidase due to the mtDNA 2bp microdeletion of TA at positions 9205 and 9206; P.Ješina**, M.Tesařová, D.Fornůsková, A.Vojtíšková, P.Pecina, V.Kaplanová, H.Hansíková, J.Zeman and J.Houštěk: *Biochem J.* 383, 2004: 561-71 ***(IF = 4,278)***

The first paper describes a very rare mtDNA mutation, 2 bp deletion of TA at positions 9205 and 9206 (9205ΔTA), which affects the STOP codon of the *ATP6* gene and the cleavage site between the RNAs for *ATP6* and *COX3*. In the affected boy with severe encephalopathy, a homoplasmic mutation was present in blood, fibroblasts and muscle.

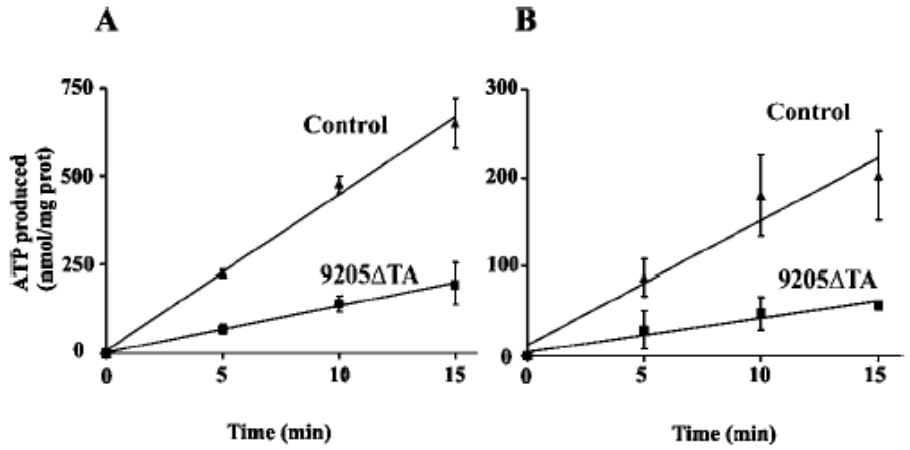

Fig 3. Production of ATP *by control and 9205ΔTA fibroblasts with pyruvate + malate or succinate as substrates.*

In 9205ΔTA fibroblasts, ATP production was observed to be approx. 30 % of control values (Fig. 3) that are in accordance with the oxygraphic measurements. The content of subunit a was decreased 10-fold compared with other ATPase subunits, and [^{35}S]-methionine labeling showed a 9-fold decrease in subunit a biosynthesis (Fig. 4). The content of COX subunits I, IV and VIc was decreased by 30-60 %. Northern Blot and quantitative real-time

reverse transcription-PCR analysis further demonstrated that the primary *ATP6-COX3* transcript is cleaved to the *ATP6* and *COX3* mRNAs 2-3 fold less efficiently. Structural studies by Blue-Native and two-dimensional electrophoresis revealed an altered pattern of COX assembly and instability of the ATPase complex, which dissociated into subcomplexes.

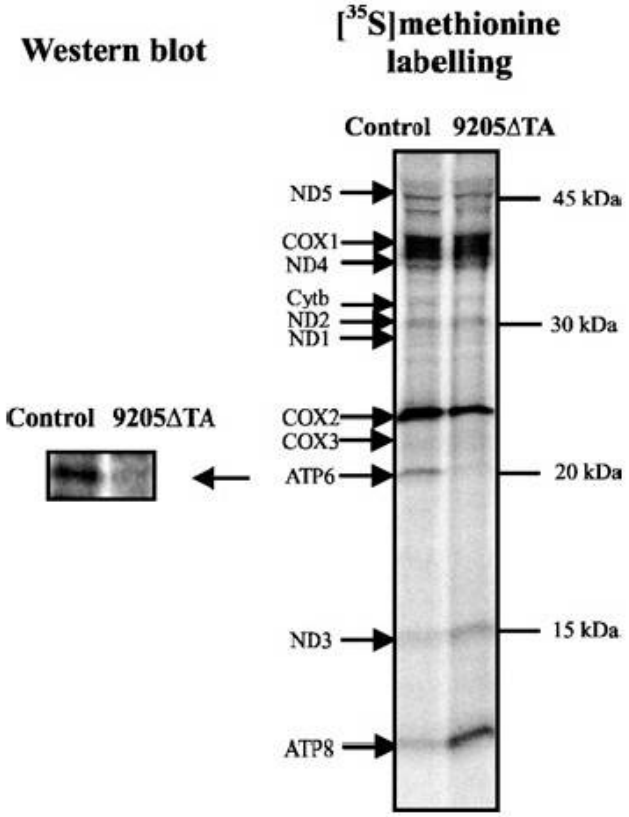

Fig 4. [^{35}S]methionine labeling and electrophoretic analysis of subunit a. *Radioactive proteins were separated by SDS-PAGE and detected by phosphorimaging. On the left side, the Western Blot from the same SDS-PAGE using an antibody against subunit a.*

The results indicate that the 9205ΔTA mutation prevents the synthesis of ATPase subunit a, and causes the formation of incomplete ATPase complexes that are capable of ATP hydrolysis but not ATP synthesis. The mutation also affects the biogenesis of COX, which is present in a decreased amount in cells from affected individuals. The only other known case of the 9205ΔTA homoplasmic mutation described Seneca et al. [204-207], presented with very mild phenotype and minimum biochemical changes. An explanation for this difference might be due to some compensatory mechanism affecting the post-transcriptional processing and/or translation of the *ATP6* and *COXIII* mRNAs.

2. **GUG is an efficient initiation codon to translate the human mitochondrial *ATP6* gene**; A.Dubot, C.Godinot, V.Dumur, B.Sablonniere, T.Stojkovic, J.M. Cuisset, A.Vojtíškova, P.Pecina, **P.Ješina** and J.Houštěk: *Biochem Biophys Res Commun*. 313(3), 2004:687-93; ***(IF = 2,904)***

The second paper presents another novel mutation in *ATP6* gene. In contrast to the mutation in the STOP codon presented in previous article, a maternally inherited and practically homoplasmic mitochondrial mutation, 8527A>G, changing the initiation codon AUG into GUG, normally coding for a valine, was observed in the *ATP6* gene encoding the ATPase subunit a. In patient fibroblasts, no effect of 8527A>G mutation was demonstrated on the biosynthesis of mtDNA-encoded proteins, on size and the content of ATPase subunit a (Fig. 5), on ATP hydrolysis and on mitochondrial membrane potential. In additional, ATP synthesis was barely decreased (Table 1).

	Muscle homogenate		Skin fibroblasts	
	Controls	Patient P	Controls	Patient P
Complex I	3–11	6		
Complex I + III	3–9	9	11–38	21
Complex II	19–40	55	8–28	14
Complex II + III	4–12	4	16–40	24
Complex IV	40–100	127	50–120	72
ATPase (oligomycin-sensitive)			127–166	143–199
ATP synthesis (succinate)			37–51 (46 ± 8)	31–42 (37 ± 6)
ATP synthesis (P + M)			37–48 (44 ± 6)	30–37 (34 ± 3)
Citrate synthase	91–200	120	27–81	48

Table 1. Oxidative phosphorylation complex enzyme activity. *Activities are expressed as nmol substrate/min/mg protein in muscle homogenate or skin fibroblasts.*

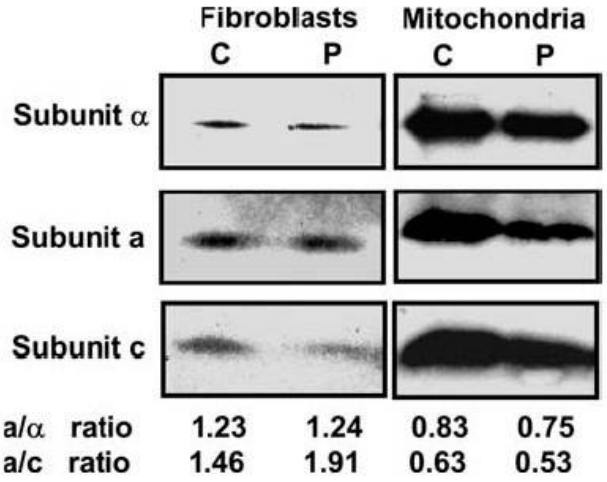

Fig 5. Western Blot analysis of ATPase subunit α, a, and c. *Relative ratios of ATPase subunit ae shown.*

The main conclusion of this paper is that, in human mtDNA, GUG can be used as an alternative initiation codon to produce a normal amount of ATPase subunit a. To our knowledge, GUG has never been reported to be able to initiate the translation of human mtDNA-encoded proteins.

3. **Mitochondrial Membrane Potential and ATP Production in Primary Disorders of ATP Synthase;** A.Vojtíšková**, P.Ješina,** M.Tesařová, M.Kalous, A.Dubot, C.Godinot, D.Fornůsková, V.Kaplanová, J.Zeman a J.Houštěk: *Toxicol.Mech.Methods* 14, 2004:7-11; ***(IF = 0,464)***

In this article, we summarize our analysis of the functional consequences of various types of primary ATPase defects at the level of mitochondrial ATP production and maintenance and discharge of the mitochondrial membrane potential $\Delta\Psi_m$.

ATP production in fibroblasts from three patients with selective deficiency of ATP synthase

Case	I	II	III
Phenotype	LA, CM, H	LA, CM	LA, CM, EM, dev. delay
Onset/survival	Newborn/2 days	Newborn/32 days	Neonate/7 years
ATPase activity in fibroblasts (% of control)[a]	30%	20%	30%
ATP production in fibroblasts (% of control)[b]	28–32%	32–47%	18–23%
ATP production in cybrids (% of control)[b]	102–116%	220–380%	230%

[a]Oligomycin-sensitive ATPase activity.
[b]Antimycin A–sensitive production using succinate, ketoglutarate + malate, or glutamate + malate as a substrate.
CM, cardiomyopathy; EM, encephalomyopathy; H, hepatomegaly; LA, lactic acidosis.
Note: The data for Case I are from the work of Houstek and colleagues (1999); the data for Cases II and III are from two new patients from unrelated families.

Table 2. ATP production in fibroblasts from patients with selective ATPase deficiency.

Studies of fibroblasts with ATPase defects due to heteroplasmic mtDNA mutations in the *ATP6* gene, affecting protonophoric function or synthesis of subunit a, show that at high mutation loads, mitochondrial membrane potential $\Delta\Psi_m$ at state 4 is normal, but ADP-induced discharge of $\Delta\Psi_m$ is impaired and low ATP synthesis is also found when the ATPase content is diminished by altered biogenesis of the enzyme complex (Table 2). Elevated $\Delta\Psi_m$ in primary ATPase disorders could increase mitochondrial production of reactive oxygen species and decrease energy provision. This conclusion was later confirmed by several studies from our group as well as other laboratories.

4. **Deficiency of mitochondrial ATP synthase of nuclear genetic origin.** W.Sperl, **P.Ješina**, J.Zeman, J.Mayr, L.DeMeirleir, R.Van Coster, A.Pícková, H.Hansíková, H.Houšťková, Z.Krejčík, J.Koch, E.Holme and J.Houštěk: *Neuromuscul Disord.* (2006 Dec;16(12):821-9. Epub2006 Oct 17); ***(IF = 3,043)***

In the fourth paper we summarize data on 14 patients with ATP synthase deficiency caused by nuclear genetic defects. We compared and analyzed available information on clinical presentation, tissue involvement, metabolic profiles, and specific methods to the structure and function of ATPase in order to highlight the dominant clinical and laboratory features and the differences from patients with mtDNA-based defects. A quantitative decrease of the ATPase complex was documented by Blue-Native electrophoretic and Western blotting analysis.

Parameter	Proportion
Consanguinity of parents	4/14
Gestational age ⩾37 weeks	9/14
Gender	
Male	6/14
Female	8/14
Birth weight <3rd percentile of corresponding gestational age	10/14
Low Apgar scores <8 in the 5th and/or 10th min	8/8
Neonatal onset	14/14
Died	7/14
Within 1st month of life	4/7
After 1st month of life	3/7
Alive	7/14
Surviving ⩾3 years	3/7
⩾5 years	2/7
⩾10 years	2/7
Age at diagnosis	
Within 1 month	4/14
Within 1 year	6/14
>1 year	4/14
Clinical course	
Neonatal fulminant	5/14
Progressive	5/14
Slowly progressive	4/14
Cardiomyopathy hypertrophic	11/14
Dilated	2/14
Hypotonia (muscular ± central)	12/13
Psychomotor retardation (of longer surviving patients)	10/10
Hepatomegaly	6/14
Facial dysmorphism	5/14
MRI/sonography of brain	
Normal	5/10
Unspecific findings	5/10
Microcephaly	5/14

Table 3. Family history, clinical course, symptoms.

A quantitative decrease of the ATPase complex was documented by Blue-Native electrophoretic and Western blotting analysis (Fig. 7). It was supported by the diminished activity of oligomycin/aurovertin-sensitive ATP hydrolysis in various tissues, namely in fibroblasts, muscle, or liver. All patients had neonatal onset and elevated plasma lactate level. In most tested cases increased lactate in urine and cerebrospinal fluid as well as alanine in blood were also observed. In 12 patients investigated 3-methyl-glutaconic aciduria was detected. Seven patients died, mostly within the first weeks of life and surviving patients showed psychomotor and various degrees of mental retardation. Eleven patients had hypertrophic cardiomyopathy; other clinical signs included hypotonia, hepatomegaly, facial dysmorphism and microcephaly (Tables 3 and 4). In one patient, the gene defect was localized to the assembly factor Atp12p. In other cases the mutated genes remain unknown.

Parameter	Proportion
Hyperlactataemia >2.5 mmol/l	14/14
Lactate in urine >50 mmol/mol creatinine	10/10
Lactate in cerebrospinal fluid >2.5 mmol/l	6/6
Alanine in blood >560 μmol/l	8/10
3-Methyl-glutaconic aciduria >20 mmol/mol creatinine	12/12
ATP synthase hydrolytic activity <30% of control	13/13
Fibroblasts	10/10
Muscle[a]	6/7
Liver	1/1
ATP production in fibroblasts <50% of control	4/4
ATP synthase content <30% of control (detected by Blue-Native electrophoresis)	13/13
Muscle[a]	9/10
Fibroblasts	10
Heart	3
Liver	3
Brain	1

[a] ATP synthase activity and content in patient P4 with ATP12 mutation were 70–75% of control.

Table 4. Biochemical data in 14 patients with ATP synthase deficiency.

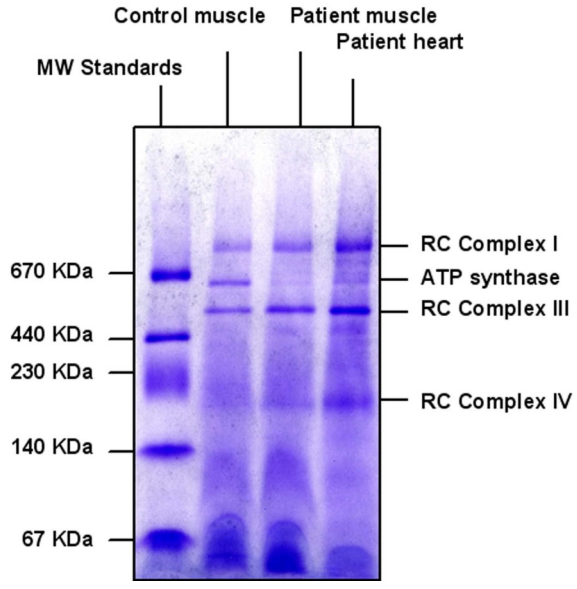

Figure 7. Isolated defect of ATPase in muscle and heart of patient revealed by Blue-Native PAGE electrophoresis.

This phenotype markedly differs from the severe central nervous system changes of ATPase disorders caused by mitochondrial DNA mutations of the *ATP6* gene presenting mostly as NARP and MILS. The increasing number of cases reviewed here indicates that these biosynthetic defects may be more frequent than originally thought.

5. **Mitochondrial diseases and genetic defects of ATP synthase** J.Houštěk, A.Pícková, A.Vojtíšková, T.Mráček, P.Pecina, **P.Ješina**; *Biochem Biophys Acta – Bioenergetics*, 1757, 2006:1400-1405; ***(IF = 4,302)***

This paper describes an overview of isolated defects of ATPase. Alteration of ATPase biogenesis may cause two types of deficiencies that have been described, differing in the pathogenic mechanism, biochemical phenotype (structural and functional) as well as in clinical presentation.

The first, long well-known type are qualitative defects of ATPase caused by mutations in one of mtDNA-encoded ATPase subunits. They are presented with structurally modified ATPase complex but the enzyme does not function properly. Maternally transmitted mutation in the *ATP6* gene are manifested as NARP or as Leigh syndrome. A different form of qualitative ATP synthase deficiency is represented by very rare microdeletion 9205ΔTA in the *ATP6* gene.

The second currently known type of isolated disorders of ATPase are quantitative defects in which the cellular content of the enzyme is selectively reduced. The disorder is of nuclear genetic origin. At present, more patients with ATPase deficiency have been diagnosed.

Both types of ATP synthase disorders result in a decreased ability of mitochondria to produce ATP. Therefore the mainly affected tissues are brain and heart. The low ability of mitochondria to utilize respiration-generated proton gradient for ATPase generally means that the mitochondrial membrane remains hyperpolarized, which may lead to increased generation of reactive oxygen species (ROS) by the respiratory chain (Fig. 8).

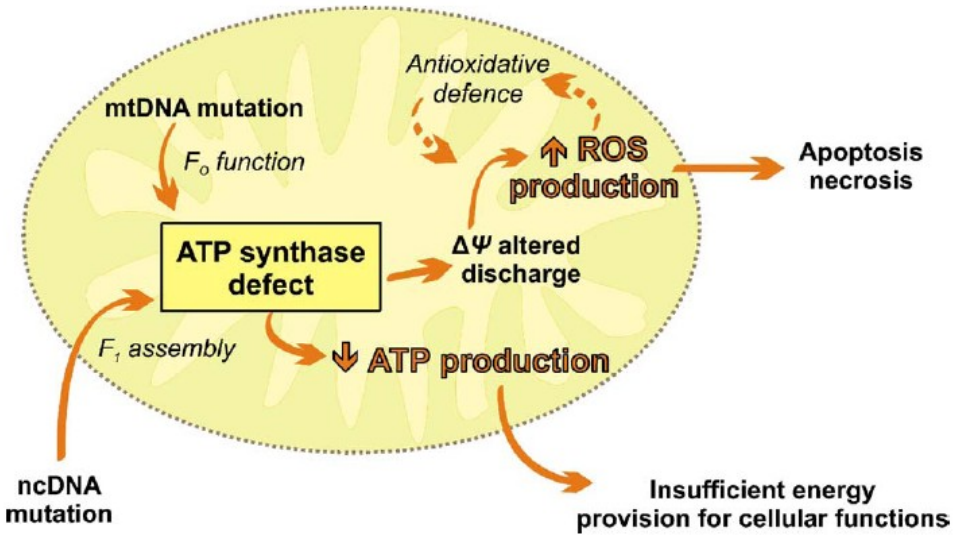

Figure 8. Pathogenic mechanism of ATPase defects – energy deprivation and increased ROS production.

Conclusions

From the results of our studies performed at several different genetic models of isolated disorders of the mitochondrial ATPase and/or correlations of biochemical phenotypes with clinical presentation we conclude that:

- The 9205ΔTA mutation in the *ATP6* gene prevents the synthesis of ATPase subunit a and cytochrome *c* oxidase subunit COXIII, and causes the formation of incomplete ATPase complexes that are capable of ATP hydrolysis but not ATP synthesis. The mutation also affects the biogenesis of COX, which is present in a decreased amount in cells from affected individuals. This mutation is the first described mutation in STOP codon for gene in mtDNA. It affects the cleavage site between the *ATP6* and *COXIII* transcripts and therefore this mutation leads to pathological changes (structural and functional) in both ATPase and COX.

- In human mtDNA, GUG in the *ATP6* gene can be used as an alternative initiation codon to produce a normal amount of ATPase subunit a. The GUG codon is thus able to initiate the translation of human mtDNA-encoded proteins similarly as in few cases of other eukaryotic cells.

- The isolated deficiency of mitochondrial ATPase due to mutations in nuclear genes presents with specific and homogenous phenotype that is characterized biochemically by decreased ATPase activity and quantitative decrease of the ATPase complex and clinically by neonatal onset, elevated plasma lactate levels, 3-methylglutaconic aciduria, psychomotor retardation, hypertrophic cardiomyopathy. On these bases it can be easily differentiated from maternally-transmitted ATPase defects due to mutations in mtDNA *ATP6* gene. The nuclear ATPase defects thus represent a specific entity among mitochondrial diseases.

- Our studies clearly demonstrate that low ATPase content and decreased mitochondrial ATP production lead to high values of $\Delta\Psi_m$ and are associated with activation of ROS generation by the mitochondrial respiratory chain. Both the energetic deprivation and increased oxidative stress are important components of the pathogenic mechanism of ATPase disorders.

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