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Strukturní a funkční interakce mitochondriálního systému fosforylace ADP

Structural and Functional Interactions of Mitochondrial ADP-Phosphorylating Apparatus

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ABSTRACT

The complexes of the oxidative phosphorylation (OXPHOS) system in the inner mitochondrial membrane are organised into structural and functional super-assemblies, so-called supercomplexes. This type of organisation enables substrate channelling and hence improves the overall OXPHOS efficiency. ATP synthase associates into dimers and higher oligomers. Within the supercomplex of ATP synthasome, it interacts with ADP/ATP translocase (ANT), which exchanges synthesised ATP for cytosolic ADP, and inorganic phosphate carrier (PiC), which imports phosphate into the mitochondrial matrix. The existence of this supercomplex is generally accepted. Experimental evidence is however still lacking.

In this thesis, structural interactions between ATP synthase, ANT and PiC were studied in detail. In addition, the interdependence of their expression was examined either under physiological conditions in rat tissues or using model cell lines with ATP synthase deficiencies of different origin. Specifically, they included mutations in the nuclear genes *ATP5E* and *TMEM70* that code for subunit ε and the ancillary factor of ATP synthase biogenesis TMEM70, respectively, and a microdeletion at the interface of genes *MT-ATP6* and *MT-COX3* that impairs the mitochondrial translation of both subunit *a* of ATP synthase and subunit Cox3 of cytochrome *c* oxidase.

Functional and structural characterisation of the cell lines with ATP synthase defects revealed that nuclear mutations in the genes *TMEM70* and *ATP5E* (the first reported mutation in a nuclear gene coding for a structural subunit of ATP synthase) lead to a reduced content of fully functional ATP synthase. In contrast, a mutation in *MT-ATP6* is accompanied by a normal amount of incomplete ATP synthase that is non-functional due to the lack of subunit *a*. In this case, the pathological phenotype manifests itself above 90 % heteroplasmy of mutated mtDNA. At all the studied defects, a compensatory up-regulation of ANT and PiC was found, likely due to an adaptive mechanism at the post-transcriptional level. Under physiological conditions, however, the expression of ATP synthase, ANT and PiC appears to be co-regulated at the level of transcription.

Although structural analyses revealed the existence of ATP synthasome in rat heart mitochondria, the majority of ATP synthase, ANT and PiC were found as separate entities. The functional significance of ATP synthasome therefore still remains controversial. The analyses also detected an association of ATP synthase with succinate dehydrogenase that had been previously reported as the so-called mitochondrial ATP-sensitive K^+ channel.

KEYWORDS: Mitochondria; oxidative phosphorylation; ATP synthase; ADP/ATP translocase; phosphate carrier; mitochondrial supercomplexes; mitochondrial disorders.

ABSTRAKT

Komplexy oxidativní fosforylace (OXPHOS) se ve vnitřní mitochondriální membráně sdružují ve vyšší strukturní a funkční celky, tzv. superkomplexy. Jejich význam spočívá v cíleném směrování substrátu z jednoho komplexu na druhý. V případě ATP syntázy byly popsány její dimery i vyšší oligomery a také ATP syntazom, v rámci nějž dochází k seskupení ATP syntázy s přenašečem adeninových nukleotidů (ANT), zajišťujícím výměnu syntetizovaného ATP za cytosolické ADP, a fosfátovým přenašečem (PiC), umožňujícím import fosfátu do matrix mitochondrie. I když je existence tohoto superkomplexu obecně přijímána, experimentální důkazy nejsou dostatečné.

V rámci této práce byly detailně zkoumány strukturní interakce ATP syntázy, ANT a PiC. Jejich vzájemné vztahy byly sledovány nejprve na úrovni exprese jednotlivých komponent ATP syntazomu, ať již za fyziologických podmínek v různých tkáních potkana, nebo na modelu deficiencí ATP syntázy v buňkách pacientů s různými genetickými defekty ATP syntázy. Konkrétně se jednalo o mutace v jaderných genech *ATP5E* a *TMEM70*, které kódují podjednotku ε, respektive pomocný faktor v biogenezi ATP syntázy TMEM70, a o mikrodeleci na rozhraní genů *MT-ATP6* a *MT-COX3*, která negativně ovlivňuje mitochondriální translaci podjednotek *a* ATP syntázy a Cox3 cytochrom *c* oxidázy.

Funkční a strukturní charakterizace buněčných linií s defekty ATP syntázy ukázala, že jaderné mutace v genech *TMEM70* a *ATP5E* (první jaderný gen kódující podjednotku ATP syntázy, v němž byla objevena mutace) mají za následek snížené množství jinak plně funkční ATP syntázy, kdežto v případě mitochondriální mutace v *MT-ATP6* je přítomno normální množství neúplné ATP syntázy, u níž chybějící podjednotka *a* vede k její nefunkčnosti. Patologický fenotyp této mutace se projeví až při překročení 90% heteroplazmie mutované mtDNA. U všech zkoumaných defektů bylo pozorováno kompenzační zvýšení přenašečů ANT a PiC, které je pravděpodobně způsobeno zatím neznámým adaptivním posttranskripčním mechanismem. Za fyziologických podmínek se ovšem zdá, že ATP syntáza, ANT a PiC jsou společně regulovány na úrovni transkripce.

Strukturní analýzy ukázaly přítomnost ATP syntazomu v mitochondriích izolovaných ze srdce potkana. Většina ATP syntázy, ANT a PiC ale vzájemně neasociuje, což ještě více podtrhuje otázku funkčního významu ATP syntazomu. Analýzy odhalily také interakci ATP syntázy se sukcinát dehydrogenázou. Seskupení těchto dvou komplexů už dříve byla přisouzena funkce tzv. mitochondriálního ATP-senzitivního K^+ kanálu.

KLÍČOVÁ SLOVA: Mitochondrie; oxidativní fosforylace; ATP syntáza; přenašeč adeninových nukleotidů; fosfátový přenašeč; mitochondriální superkomplexy; mitochondriální onemocnění.

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CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
ANT	ADP/ATP translocase, adenine nucleotide translocator
ATP	adenosine triphosphate
BAT	brown adipose tissue
BNE	blue native electrophoresis
CAT	carboxyatractyloside
CNE	clear native electrophoresis
Co*Q*	coenzyme *Q*
COX	cytochrome *c* oxidase
cyt *c*	cytochrome *c*
CsA	cyclosporine A
CyPD	cyclophilin D
DDM	n-dodecyl β-D-maltoside
IMM	inner mitochondrial membrane
mitoK$_{ATP}$	mitochondrial ATP-sensitive K^+ channel
mPTP	mitochondrial permeability transition pore
MS	mass spectrometry
mtDNA	mitochondrial deoxyribonucleic acid
nDNA	nuclear deoxyribonucleic acid
OSCP	oligomycin-sensitivity conferring protein
OXPHOS	oxidative phosphorylation
PiC	phosphate carrier
RC	respiratory chain
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	denaturing polyacrylamide gel electrophoresis in the presence of SDS
tRNA	transfer ribonucleic acid
VDAC	voltage-dependent anionic channel
Δp	proton-motive force
ΔpH	proton gradient
$\Delta\mu_H+$	electrochemical proton gradient
$\Delta\Psi_m$	mitochondrial membrane potential

INTRODUCTION

1. Mitochondria

Energy supply is essential for all cells, regardless of whether a cell represents a unicellular organism or a component of a larger multicellular entity. The cell needs to transform the chemical energy released by the metabolic conversion of substrates into a form of energy that the cell itself is able to utilize. Therefore, the final goal of energetic metabolism is to produce ATP (adenosine triphosphate), a molecule that can be regarded as a universal source of energy in the cell. Under anaerobic conditions, any cell is able to gain only two ATP molecules from one glucose molecule in the process of glycolysis, which takes place in the cytosol. If a cell is capable of utilising molecular oxygen in the reaction steps following the glycolysis, it can gain approximately 15-times more ATP using the process called oxidative phosphorylation (OXPHOS). In prokaryotes, it takes place on the plasma membrane. In eukaryotes, however, it is situated in a specialised organelle – the mitochondrion, where glucose oxidation is completed with the production of CO_2 and H_2O. Because of the presence of the OXPHOS enzyme system, mitochondria are the key producer of ATP in the cell, which makes them "cellular power plants".

The origin of mitochondria (as well as plastids, specialised organelles in the plant cell, which include chloroplasts), is explained by the so-called endosymbiotic theory (Margulis, 1968). It defines mitochondria as direct descendants of a eubacterial endosymbiont that was originally taken up by a protoeukaryotic host cell with an anaerobic type of heterotrophic metabolism in the process of endocytosis. Specifically, the rickettsial group of α-proteobacteria were identified as the closest relatives of mitochondria based on the phylogenetic analysis of the mitochondrial genome (Gray et al., 2001).

1.1. Mitochondrial genetics and biogenesis

1.1.1. Mitochondrial genome

The mitochondrial genome is usually a circular DNA molecule of varying size (15–60 kbp), similar to a typical bacterial genome. In many organisms, however, the mitochondrial DNA (mtDNA) adopts other types of architecture different from the circular one (Burger et al., 2003).

Compared to the genome of their microbial relatives, the mitochondrial genome is markedly reduced in its coding capacity since the evolution of mitochondria from the α-proteobacterial endosymbiont

was accompanied by transfer of some genes into the host genome and loss of others due to their redundancy (Gray et al., 2001). Based on the gene content in the mitochondrial genome, the evolutional divergence of an organism can be roughly assessed. The most gene-rich, and hence most ancestral, least derived mitochondrial genome described so far is that of the protist *Reclinomonas americana*. Its 69-kbp mtDNA contains 97 genes in total, 92 with an assigned function, including 44 protein-coding genes (Lang et al., 1997). In contrast, human mtDNA (16 569 bp) only contains 13 protein-encoding genes, altogether membrane-embedded OXPHOS components, 2 rRNA molecules and a set of 22 tRNA molecules, which fully ensures mitochondrial translation.

Unlike the nuclear genome, mitochondria and their DNA are maternally inherited. Cells typically contain hundreds of mitochondria. Since each of them usually possesses multiple mtDNA molecules, the mtDNA copy number varies from about ten to several thousand molecules per cell (Burger et al., 2003). Therefore, mutations can arise only in a mtDNA sub-population, which leads to the co-existence of two or more different mitochondrial genotypes (heteroplasmy) within a single cell, organ, or individual. The proportion of mutated molecules (mutational load) then affects the biochemical phenotype, often with a threshold level of heteroplasmy. Only after getting over the threshold, the cell is not able to further cope with the load of mutated mtDNA (DiMauro, 2013).

1.1.2. Mitochondrial proteome

Mitochondria not only play a key role in energy transduction, but also participate in several other important functions, such as assembly of iron-sulphur clusters, biosynthesis of hemes and other metabolites, calcium homeostasis, and apoptosis. The proteins that execute these functions are mostly encoded by nuclear genes. Therefore, the majority of ~1100–1400 distinct mitochondrial proteins (Calvo & Mootha, 2010) are synthesised on cytosolic ribosomes and then imported into mitochondria in a co- or post-translational manner (Fox, 2012).

A number of proteins are synthesised as precursors with an N-terminal signalling sequence (mitochondrial targeting sequence, MTS) that is cleaved by an enzyme called signal peptidase in the matrix. In addition to this N-terminal sequence that targets proteins into the mitochondrial matrix, they may contain other inner signalling sequences, cleavable or cryptic, that specify their mitochondrial localisation and target them to a specific mitochondrial compartment. The import of proteins into mitochondria as well as the recognition of signalling sequences is ensured by specialised multisubunit import machineries in both the outer (translocase of the outer mitochondrial membrane, TOM) and inner (translocase of the inner mitochondrial membrane, TIM) membrane (Rehling et al., 2004; Neupert & Herrmann, 2007).

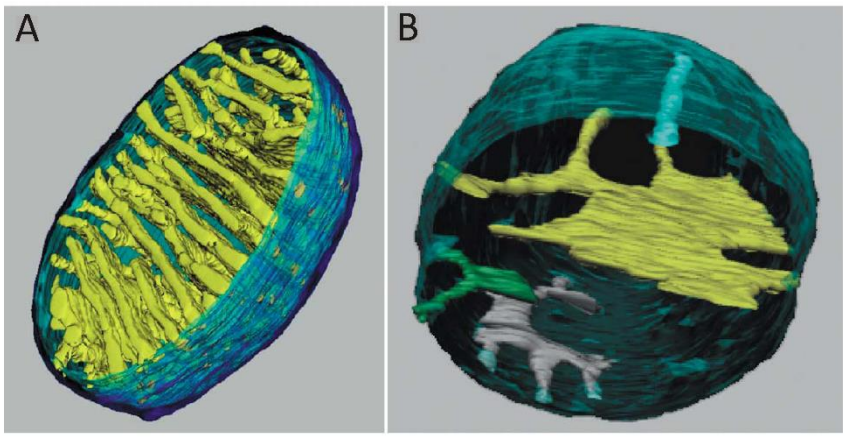
1.2. Mitochondrial structure

Using electron microscopy, mitochondria were revealed to possess a double membrane in the 1950's (Palade, 1953). As double membrane-bound organelles, mitochondria are spatially divided into two functionally distinct inner compartments. The space between the outer and inner membrane is called the intermembrane space. The mitochondrial matrix, where the Krebs cycle, fatty acid oxidation etc. are localised, is completely separated by the inner membrane.

The inner mitochondrial membrane (IMM) is composed of two distinguishable components, as evidenced by the electron tomography (Frey et al., 2002) (**Fig. 1**). The first one, called the inner boundary membrane, follows exactly the shape of the outer membrane. They are connected by contact sites that are formed by a complex of mitochondrial membrane proteins, the MICOS complex (mitochondrial contact site complex) (Harner et al., 2011). The other IMM component creates cristae, membrane projections into the matrix, which profoundly increase the surface of the inner membrane where OXPHOS complexes are localised. The cristae and the inner boundary membrane are connected by cristae junctions. The regulation of cristae junctions is believed to contribute to and maintain the functional compartmentalisation of the IMM. For example, it has been shown that the cristae membrane is enriched especially in the cytochrome *c* oxidase (COX). Furthermore, ATP synthase dimers appear to be localised predominantly in the flexures of cristae membrane. In fact, they most likely play a role in shaping the cristae membrane and hence in establishing the morphology of the IMM (Davies et al., 2012).

Fig. 1 A three-dimensional reconstruction of the mitochondrion.

The 3D model of the mitochondrion was created using electron tomography. (A) The outer membrane displayed in translucent dark blue, the inner boundary membrane in translucent light blue, and the cristae in yellow. (B) Four cristae displayed in different colours to illustrate the variety of their shapes (e.g. lamellar, tubular, or a combination of both). Adapted from Frey et al. (2002).



Mitochondria used to be schematically depicted as isolated elongated cylindrical structures in the cytoplasm, with a diameter of 0.5–1.0 μm. In reality, they often form highly dynamic networks that constantly fuse and divide (Bereiter-Hahn, 1990). Mitochondrial dynamics is a combination of two antagonistic processes – mitochondrial fusion and fission (Westermann, 2010). These alter the mitochondrial morphology in order to optimize the mitochondrial function while meeting the immediate metabolic requirements of the cell (Westermann, 2012). Mitochondrial dynamics also ensures proper inheritance and distribution of mitochondria and quality control (Narendra et al., 2012; Stotland & Gottlieb, 2015), leading to autophagic degradation of damaged mitochondria that are recognised and excluded from the networking process (Westermann, 2010, 2012).

1.3. Oxidative phosphorylation system (OXPHOS)

The oxidation of substrates releases electrons that are captured in the form of reduced coenzymes NADH and $FADH_2$. Being transported via the so-called respiratory chain (RC), these electrons reach their final destination, the molecular oxygen. The RC is composed of four enzyme complexes localised in the IMM (**Tab. 1**) and two mobile electron carriers coenzyme *Q* (Co*Q*) and cytochrome *c* (cyt *c*) (Saraste, 1999).

Tab. 1 Components of the respiratory chain.

The features of four complexes of the mammalian RC are presented: their enzymatic function, the number of subunits in the enzyme complex, including the number of subunits encoded by mitochondrial genes, and the molecular weight of fully assembled complex.

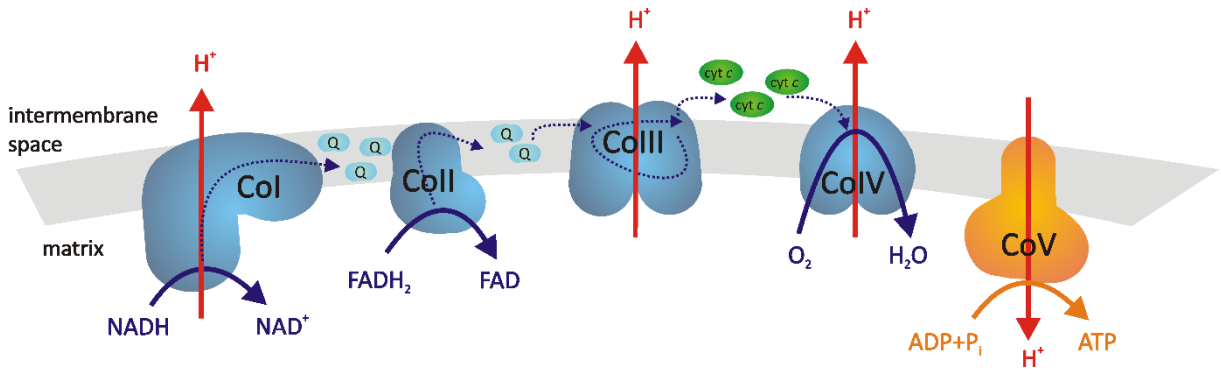
	Complex I (Sazanov, 2015)	**Complex II** (Sun et al., 2005)	**Complex III** (Iwata et al., 1998)	**Complex IV** (Tsukihara et al., 1996)
	NADH dehydrogenase	succinate dehydrogenase	cytochrome bc_1 complex	cytochrome *c* oxidase
Catalytic function	NADH:ubiquinone oxidoreductase	succinate:ubiquinone oxidoreductase	ubiquinol: ferricytochrome *c* oxidoreductase	ferrocytochrome c: oxygen oxidoreductase
Number of subunits	~45	4	11	13
mtDNA encoded	7	0	1	3
Molecular weight (kDa)	~1000	124	~240	204

Electron transport via the RC complexes runs in the direction of rising redox potential. It is accompanied by a release of free energy that is transformed into an electrochemical proton gradient across the IMM. Specifically, three of the four enzyme RC complexes (Complex I, III, and IV) couple the electron transport to pumping of protons from the mitochondrial matrix, which leads to the establishment of a proton gradient on the IMM that is impermeable for ions. This proton gradient has two compounds – chemical (a higher concentration of protons in the intermembrane space compared to the matrix, ΔpH) and electrical (the mitochondrial matrix is negatively charged compared to the intermembrane space where the protons are accumulated, $\Delta\psi_m$). This electrochemical gradient ($\Delta\mu_{H+}$) is used by the F_1F_o-ATP synthase (also referred to as Complex V) for ATP synthesis from ADP and inorganic phosphate (Mitchell, 1961). The RC complexes and ATP synthase together form the so-called OXPHOS system (Hatefi, 1985) (**Fig. 2**). In addition to the key electron pathway via the RC, other dehydrogenases also participate in the supply of electrons to the pool of reduced Co*Q* (e.g. glycerol-3 phosphate dehydrogenase, or electron transferring-flavoprotein dehydrogenase).

Besides ATP synthesis, the energy in the form of $\Delta\mu_{H+}$ can be used for heat dissipation by uncoupling proteins or for the transport of ions, metabolites, and other macromolecules across the IMM.

Fig. 2 The OXPHOS system.

The key components of the oxidative phosphorylation (OXPHOS) system are four RC complexes (Complex I–IV, CoI–CoIV) and ATP synthase (Complex V, CoV).



2. Mitochondrial ADP-phosphorylating apparatus

The electrochemical proton gradient generated by the RC enzyme complexes is utilised by F_1F_o-ATP synthase in the key process of energy metabolism in the cell, i.e. mitochondrial ATP synthesis (Boyer, 1997; Walker, 2013). F_1F_o-ATP synthase catalyses the actual phosphorylation of ADP. To fulfil this function, it requires supply of the substrates, ADP and inorganic phosphate, which is mediated by two specialised carriers in the IMM. ADP/ATP translocase (also referred to as adenine nucleotide translocator, ANT) transports ADP into the mitochondrial matrix in exchange for newly synthesised ATP (Itoh et al., 2004). Inorganic phosphate carrier (PiC) ensures supply of inorganic phosphate from the cytosol, utilising symport with protons or antiport with hydroxyl ions (Seifert et al., 2015). Together, they form the mitochondrial ADP-phosphorylating apparatus.

2.1. F_1F_o-ATP synthase

The mitochondrial F_1F_o-ATP synthase (EC 3.6.3.14) in mammalian mitochondria is a large multisubunit enzyme complex with a molecular weight of ~650 kDa (**Fig. 3**). It consists of about 30 subunits of 18 different types (Lee et al., 2015).

2.1.1. Structure of ATP synthase

In the multisubunit complex of ATP synthase, two large subcomplexes can be distinguished – the globular catalytic F_1 domain, that has been described in detail and is conserved among different species, and the proton-pumping membrane-embedded F_o domain, whose subunit composition depends on the evolutionary progress of a specific species. These two main subcomplexes are interconnected via two stalk domains – the central stalk works as a rotor that couples pumping of protons by the transmembrane F_o domain and ATP synthesis by the catalytic F_1 domain and the peripheral stalk plays a role of a stator that anchors the F_1 domain to the F_o domain (Walker, 2013).

Until 2007, it was widely accepted that the mammalian ATP synthase consists of 15 different structural and one regulatory subunit. Then, two other accessory subunits, DAPIT (diabetes associated protein in insulin sensitive tissues; also known as USMG5, up-regulated during skeletal muscle growth 5 homolog) and 6.8PL (6.8 kDa proteolipid; also known as MLQ according to its N-terminal sequence Met-Leu-Gln), were identified in the membrane-embedded F_o domain (Chen et al., 2007; Meyer et al., 2007). The contemporary view on the subunit composition of mammalian ATP synthase takes into account 17 different structural subunits (**Tab. 2**) and one regulatory subunit (inhibitory factor 1, IF_1). Only two of them (A6L and *a*) are encoded in the mammalian mtDNA (Fearnley & Walker, 1986).

2.1.1.1. F_1 domain

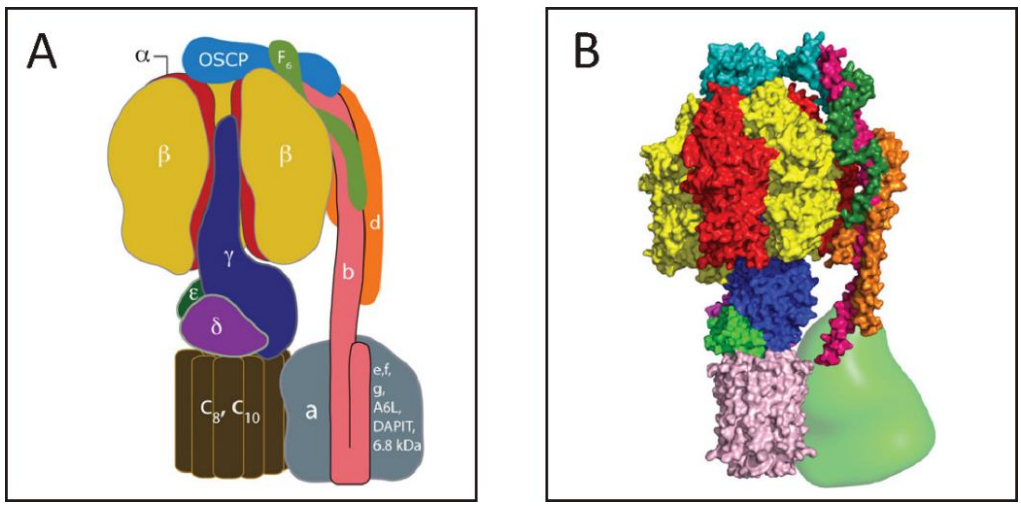
The F_1 domain (~350 kDa) is composed of 5 different subunits with the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ (Walker et al., 1995; Orriss et al., 1996). It is localised on the matrix side of the IMM. All of the three α/β interfaces form the catalytically active sites in the subunit β where ATP synthesis takes place. The subunit γ, specifically its two antiparallel helices with the coiled-coil structure, forms the asymmetric central stalk that interacts with the heterohexamer $\alpha_3\beta_3$ on the one end and with the membrane-embedded ring of subunits *c* on the other end (Gibbons et al., 2000).

2.1.1.2 F_o domain

The name of this domain reflects the fact that this part of the enzyme confers sensitivity to oligomycin. The mammalian F_o domain consists of 12 different subunits of the membrane domain and a peripheral stalk. For the structure of the membrane-embedded domain, the phospholipids in the lipid bilayer play an important role, especially cardiolipin (Eble et al., 1990). Its core is formed by a ring of 8 subunits *c* (there can be up to 15 of them in other species). Each subunit *c* forms an α-helical hairpin and the matrix interhelical loops are in close contact with the subunits γ and δ (Stock et al., 1999).

Fig. 3 Structure of mammalian F_1F_o-ATP synthase.

*The upper part of the model contains the subunits (*α, β, γ, δ, ε*) of the F_1 catalytic domain that protrudes into the mitochondrial matrix. Subunit* γ *is in contact with the F_o membrane domain that contains the* c*-ring and the associated subunit* a*. The number of* c *subunits in the* c*-ring differs between species. The F_o domain contains a number of supernumerary subunits with single transmembrane α-helices (*e, f, g, *A6L, DAPIT, 6.8PL). The peripheral stalk is on the right (subunits OSCP,* b, d, *and F_6). (**A**) One of the three subunits* α *has been removed to expose the elongated α-helical structure of subunit* γ*. (**B**) The molecular structure of the bovine ATP synthase has been determined by X-ray crystallography. The green region is the residual region of the membrane domain where no high-resolution structures have been determined. This region contains subunit* a*, the membrane domain of subunit* b*, and subunits A6L,* e, f, g*, DAPIT, and 6.8PL. Adapted from Walker (2013).*



Other subunits are closely associated to the *c*-ring, especially subunit *a* that is essential for proton pumping by ATP synthase. The structure of this subunit has been very recently partially described by single-particle electron cryomicroscopy in the mitochondrial ATP synthase of *Polytomella*. Subunit *a* is composed of six α-helices and four of them are arranged almost horizontally in the membrane and form two aqueous half-channels that participate in proton translocation (Allegretti et al., 2015).

Other membrane subunits that associate closely with the *c*-ring and subunit *a* contain a single transmembrane α-helix (A6L, *e*, *f*, *g*, DAPIT, 6.8PL). They are connected to the peripheral stalk and attach to the membrane. These subunits of F_o domain, however, are very difficult to crystalize due to their hydrophobicity. Therefore, their structure and arrangement within the domain is not yet clear (**Fig. 3B**) (Walker, 2013). Their topology and mutual proximity has been studied by cross-linking. They are oriented with their N-termini into the matrix with the exception of mitochondrially encoded A6L.

Tab. 2 Structural subunits of mammalian F_1F_o-ATP synthase.

The structural subunits of the mammalian enzyme are listed together with their respective yeast counterparts from Saccharomyces cerevisiae *(Ackerman & Tzagoloff, 2005; Antoniel et al., 2014).*

	Protein	Gene	Molecular weight (kDa)	Yeast homologue	
				Protein	Gene
F_1 domain	*α*	*ATP5A1*	55.1	*α*	*ATP1*
	β	*ATP5B*	51.6	*β*	*ATP2*
	γ	*ATP5C1*	30.2	*γ*	*ATP3*
	δ	*ATP5D*	15.1	*δ*	*ATP16*
	ε	*ATP5E*	5.7	*ε*	*ATP15*
F_o domain	*c*	*ATP5G1,2,3*	14.7	Atp9 (*c*)	*MT-ATP9*
	a	*MT-ATP6*	24.8	Atp6 (*a*)	*MT-ATP6*
	OSCP	*ATP5O*	23.2	OSCP	*ATP5*
	b	*ATP5F1*	24.6	Atp4 (*b*)	*ATP4*
	d	*ATP5H*	18.4	*d*	*ATP7*
	e	*ATP5I*	7.0	*e*	*TIM11/ATP21*
	f	*ATP5J2*	10.7	*f*	*ATP17*
	g	*ATP5L*	11.4	*g*	*ATP20*
	F_6	*ATP5J2*	12.6	*h*	*ATP14*
	A6L	*MT-ATP8*	7.9	Atp8	*MT-ATP8*
	DAPIT (USMG5)	*USMG5*	6.4	—	—
	6.8 kDa proteolipid (MLQ)	*C14ORF2*	6.8	—	—
	—	—	6.7	*i* (*j*)	*ATP18*
	—	—	7.5	*k*	*ATP19*

Its C-terminus extends into the peripheral stalk where it interacts with subunit *b*. This way, it likely stabilises the connection between the F_o domain and peripheral stalk (Lee et al., 2015). In yeast, subunits *e* and *g* were shown to play an essential role in the formation of ATP synthase dimer (Arnold et al., 1998) and some evidence suggest that they also have a similar function in mammals (Bisetto et al., 2008). In general, the role of the supernumerary membrane subunits of ATP synthase is still unknown.

The peripheral stalk is composed of single copies of subunits OSCP (oligomycin-sensitivity conferring protein), *b*, *d*, and F_6. It interacts with the heterohexamer $\alpha_3\beta_3$ via the N-terminal part of OSCP. The C-terminal part of OSCP then associates with subunit *b* (Carbajo et al., 2007).

2.1.2. ATP synthase function

The mitochondrial ATP synthase usually produces the majority of ATP in the cell. It can, however, work in two opposite modes, which depends on the energy state and energy demands of the cell. As a nanoscopic rotational motor, it is able to perform both ATP synthesis and ATP hydrolysis. For ATP synthesis, it utilises the electrochemical proton gradient across the IMM generated by the RC complexes. Proton translocation in the direction of proton gradient generates rotation of the central stalk, which, in turn, generates conformational changes of the heterohexamer $\alpha_3\beta_3$ that catalyses ATP synthesis. So, the proton-motive force is coupled indirectly to ATP synthesis by a mechanical rotary mechanism (Xu et al., 2015). On the contrary, ATP hydrolysis triggers rotation of the central stalk that drives proton pumping from the matrix to the intermembrane space against the proton gradient.

2.1.2.1. ATP synthesis and its molecular mechanism

The mitochondrial ATP synthase works as a molecular motor that is composed of both a stator – the peripheral stalk, and a rotor – the central stalk associated with the *c*-ring. The rotation of the *c*-ring is driven by protonation and deprotonation of its amino acid residues.

The anticlockwise rotation of the asymmetrical subunit γ occurs in three 120° steps and makes the catalytic α/β interfaces form 3 different conformations cyclically (Abrahams et al., 1994). These conformations of the α/β interface differ in their affinity to adenine nucleotides as well as catalytic properties. In the "loose" conformation ($\beta_{DP\text{-}}$), ADP (in complex with Mg^{2+}) and P_i bind to the subunit β in this α/β interface. After adopting the "tight" conformation ($\beta_{TP\text{-}}$), the synthesis of a macroergic bond between ADP and P_i is catalysed. Another turn of subunit γ leads to adopting the conformation that is known as the "empty" or "open" state (β_E) and the newly synthesised ATP is released from the α/β interface (Walker, 2013). The rotation was directly visualised *in vitro* as a movement of actin filaments

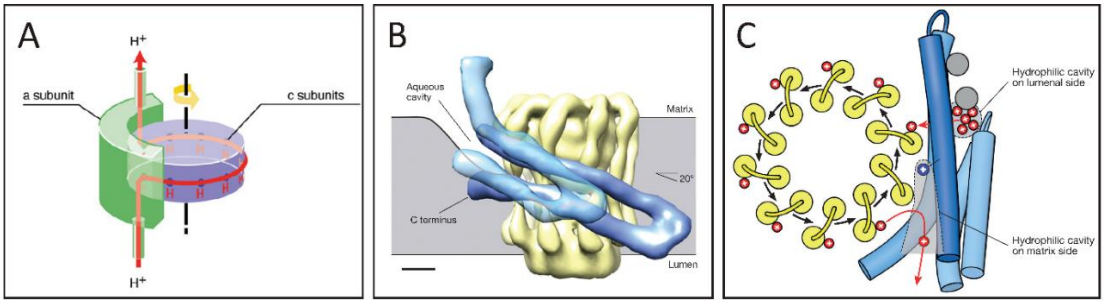
fixed to subunit *γ* of bacterial F_1 domain (Noji et al., 1997) and subsequently proven by demonstration of ATP synthesis driven by physical rotation of the rotor (Itoh et al., 2004; Rondelez et al., 2005).

Each 360° rotation provides the energy to generate three ATP molecules in the F_1 domain. The number of protons required to generate each 360° rotation of the *c*-ring corresponds to the number of subunits *c* that form the ring. The most efficient one is the *c*-ring composed of eight subunits *c*. In this case, the synthesis of three ATP molecules requires eight translocated protons, which corresponds to the cost of 2.7 protons per one ATP. The octameric *c*-ring is found in all vertebrates and the majority of invertebrates. ATP synthases in other organisms (fungi, eubacteria, plant chloroplasts) are less efficient and their *c*-rings contain 10–15 subunits *c*, which corresponds to the energetic cost of up to five protons per one ATP (Watt et al., 2010).

According to the two-channel model, protons from the intermembrane space reach a negatively charged aspartate or glutamate of a subunit *c* on the interface of subunit *a* and *c*-ring. This carboxyl group is neutralised and loses an electrostatic interaction with a positively charged arginine of subunit *a*. As a result, it is released into the hydrophobic milieu of lipid bilayer and the *c*-ring is turned around by one monomer. Another negatively charged carboxyl group is revealed and the rotation of *c*-ring is fuelled by its protonation and release (Stock et al., 2000). Subunit *a* was suspected to contain two half-channels, both on the intermembrane and matrix side of the inner membrane, that mediate the transport of protons to and from the *c*-ring (**Fig. 4A**). It was confirmed by electron cryo-tomography when the structure of subunit *a* was solved (Allegretti et al., 2015). Unexpectedly, four α-helices of subunit *a* were found to be localised almost horizontally in the membrane (**Fig. 4B–C**).

Fig. 4 Coupling of proton translocation and rotation of ATP synthase.

*(**A**) The two-channel model of proton translocation in ATP synthase. (**B**) The structure of subunit* a *solved by electron cryo-tomography. (**C**) The model of coupling of proton translocation and ATP synthase rotation utilising the new structure of subunit* a*. Protons (red) reach the conserved glutamate in subunit* c *via the aqueous half-channel in the intermembrane space (lumen in Polytomella). The proton competes with the strictly conserved arginine (blue) for interaction with* c*-ring glutamates, which carry the proton around the* c*-ring. When the* c*-subunit approaches the hydrophilic half-channel on the matrix side, the glutamate becomes hydrated and adopts an open conformation, from which the proton can escape into the matrix. Adapted from Allegretti et al. (2015).*



2.1.2.2. ATP hydrolysis

In cases where the RC function is compromised (for example during oxygen deprivation under ischemia) ATP synthase can operate in a reverse mode as a proton pump, i.e. hydrolyse cytosolic ATP produced in glycolysis, in an effort to maintain the mitochondrial membrane potential. During ATP hydrolysis, the direction of rotation is reversed and it is assumed that the order of structural changes accompanying it is reversed as well (Dummler et al., 1996; Bason et al., 2015).

Since performing the majority of mitochondrial functions requires a sufficient mitochondrial membrane potential, ATP hydrolysis by ATP synthase can help the cell to overcome malignant conditions (Bason et al., 2011; Baker et al., 2012). However, it reduces the pool of available ATP and might turn out to be harmful rather than beneficial (Campanella et al., 2008). Therefore, the cell has evolved mechanisms to avoid excessive ATP hydrolytic activity. Specifically, it can be inhibited by a regulatory protein called inhibitory factor 1 (IF_1) (Faccenda & Campanella, 2012). Furthermore, the reversal of ATP synthase is not always accompanied by the reversal of ANT, which also protects the cell from depletion of ATP that occurs in the cytosol (Chinopoulos et al., 2010; Chinopoulos, 2011).

The active form of IF_1 is a homodimer that can bind two ATP synthase moieties. IF_1 and ATP synthase form a stable complex depending on pH levels (Cabezon et al., 2000; Cabezon et al., 2001). If the pH values in the mitochondrial matrix are lower than 7.0, the affinity of IF_1 to the F_1 subcomplex of ATP synthase rises, and IF_1 binds into the α/β catalytic interface (Bason et al., 2014). At pH values higher than 8.0, the IF_1 dimers form dimers of dimers and higher aggregates, which makes them inactive because the inhibitory regions are masked (Cabezon et al., 2000). Since intramitochondrial pH differs under conditions when ATP synthase works in the synthetic and hydrolytic mode, it likely represents the mechanism that ensures that IF_1 inhibits only ATP hydrolysis and not ATP synthesis (Xu et al., 2015).

2.1.3. ATP synthase biogenesis

The mammalian ATP synthase is composed of 16 subunits encoded by the nuclear genome, which are synthesised on the cytosolic ribosomes and then imported into mitochondria, and 2 subunits (*a*, A6L) encoded by the mitochondrial genome. The biogenesis of ATP synthase complex is therefore very complicated and requires the assistance of several ancillary factors.

As for the mammalian ATP synthase, the process of biogenesis has not been completely elucidated. In yeast, however several assembly and protein-modifying factors have been identified as well as their interacting partners (Ackerman & Tzagoloff, 2005; Ruhle & Leister, 2015). Since some of the yeast biogenetic factors have not been found in mammals and vice versa, it is widely accepted that the

biogenesis of the yeast ATP synthase complex differs in some steps from the biogenesis of the mammalian one, starting with the fact that the yeast subunit *c* (Atp9) is translated from a mitochondrial gene unlike in mammals (Zeng et al., 2008). However, individual yeast strains also differ from each other in some aspects of ATP synthase biogenesis, e.g. the localisation of *ATP9* gene. Therefore, it is important to point out that *Saccharomyces cerevisiae* is the model organism in this field.

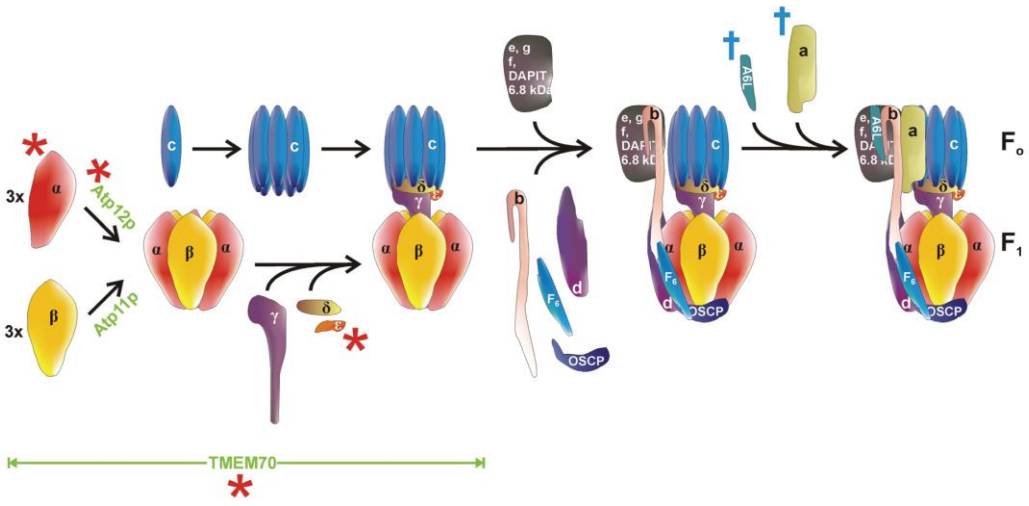
The assembly of heterohexamer $\alpha_3\beta_3$ seems to be a conserved process among eukaryotes, as opposed to the assembly of F_o domain whose both composition and complexity varies among different organisms. Most likely, the F_1 domain and the *c*-ring are built up independently of each other in the matrix and inner membrane, respectively. The formation of a F_1-*c*-ring intermediate is followed by association with the subunits forming the peripheral stalk. In the next step, membrane subunits join the *c*-ring. The mitochondrially encoded subunits *a* and A6L are incorporated last, which likely protects from dissipation of proton gradient by an incompletely assembled ATP synthase (Tzagoloff et al., 2004; Wittig et al., 2010; Rak et al., 2011) (**Fig. 5**).

2.1.3.1. Ancillary factors of ATP synthase biogenesis

As the yeast *Saccharomyces cerevisiae* is a model organism for studying the mitochondrial ATP synthase (Law et al., 1995), the first assembly factors were discovered in this organism.

Fig. 5 The postulated assembly of mammalian ATP synthase.

Red asterisks mark nuclear-encoded structural subunits and ancillary factors of ATP synthase biogenesis whose mutations are responsible for isolated defects of ATP synthase. Blue crosses mark structural subunits of ATP synthase associated with mitochondrial mutations. Adopted from Hejzlarova et al. (2014).



Specifically, Atp11 and Atp12 that participate in the assembly of F_1 domain were described in 1990 (Ackerman & Tzagoloff, 1990; Bowman et al., 1991; Ackerman et al., 1992). A decade later, their homologues were found also in the human genome (Wang et al., 2001). Both genes are extremely conserved among eukaryotes, which most likely reflects their importance for assembly of the heterohexamer $\alpha_3\beta_3$. Atp11 associates specifically with subunits β (Wang & Ackerman, 2000) whereas Atp12 binds subunits α (Wang et al., 2000). As a consequence of binding of free subunits α and β to Atp12 and Atp11, respectively, their aggregation into homooligomers α_n and β_n is prevented (Wang et al., 2000; Ludlam et al., 2009). The release of the chaperones from subunits α and β is most likely initiated by subunit γ in an unknown manner (Ludlam et al., 2009). The assembled heterohexamer $\alpha_3\beta_3$ then associates with the components of the central stalk.

In yeast, two factors have been described (Atp23, Atp10) that participate in the process of subunit *a* maturation and incorporation into the F_o domain (Rak et al., 2009). Atp23, a metalloprotease localised in the intermembrane space (Osman et al., 2007), processes the precursor of subunit *a* (Michon et al., 1988) into a mature protein (Osman et al., 2007; Zeng et al., 2007). ATP10, a protein of the IMM, then assists Atp23 in incorporation of mature subunit *a* into the membrane-embedded F_o domain (Tzagoloff et al., 2004). In mammals, only a partial homologue of Atp23 was found even though subunit *a* is not synthesised as a precursor, which suggests that only its role in association of subunit *a* with the F_o domain is required (Zeng et al., 2007). The function of ATP23 in mammals, however, has not been studied in detail. The self-assembly of *c*-ring seems to be determined by the primary structure of subunit *c* (Arechaga et al., 2002).

In 2008, a new ancillary factor of the mammalian ATP synthase biogenesis, TMEM70, was identified (Cizkova et al., 2008; Houstek et al., 2009). TMEM70 is a protein of the IMM (Hejzlarova et al., 2011) with two transmembrane helices and both N- and C-termini are localised in the matrix (Jonckheere et al., 2011; Kratochvilova et al., 2014). A direct interaction of TMEM70 with the ATP synthase complex has been reported in one study (Torraco et al., 2012). Another study, however, failed to detect such an interaction and described only formation of TMEM70 dimers (Kratochvilova et al., 2014). Its expression is very low compared to other mitochondrial proteins and its role in the ATP synthase biogenesis remains unclear (Hejzlarova et al., 2011). Since the absence of TMEM70 reduces the content of fully assembled ATP synthase, it seems that it is not essential for ATP synthase biogenesis but it profoundly improves its efficiency.

Recently, a protein complex INAC (the inner membrane assembly complex) that is involved in the assembly of the peripheral stalk of yeast F_1F_o-ATP synthase has been described. A model has been proposed where INAC facilitates attachment of the F_1 domain already associated with peripheral stalk

components to the F_o domain (Lytovchenko et al., 2014). However, a further work will be required to validate it. Furthermore, INAC components, Ina17 and Ina22 lack homologues in mammals and thus the assembly of peripheral stalk differs between yeast and mammals.

2.1.3.2. Tissue-specific regulation of ATP synthase biogenesis

In mammals, the content of subunit *c* seems to be indicative of the total content of ATP synthase complex. Brown adipose tissue (BAT), a specialised mammalian thermogenic organ that utilises a high oxidative capacity to produce heat instead of ATP, is characterised by a low content of ATP synthase (Cannon & Nedergaard, 2004). This reduction in the total amount of ATP synthase correlates with a down-regulated expression of subunit *c* whereas transcript levels of other ATP synthase subunits, both nuclear and mitochondrial, are high (Houstek et al., 1995). Subunit *c* is encoded by three genes (*ATP5G1*, *ATP5G2*, and *ATP5G3* coding for the isoforms P1, P2, P3, respectively) that differ in the transport pre-sequence. Their expression, however, results in identical mature proteins (De Grassi et al., 2006). P2 and P3 mRNA appears to be expressed constitutively whereas P1 isoform responds to a number of physiological stimuli as a means of regulating the relative content of ATP synthase (Andersson et al., 1997; Sangawa et al., 1997). In a transgenic mouse, overexpressing of P1 isoform in BAT led to an increase in the total amount of functional ATP synthase in this tissue, thus confirming that the levels of P1 isoform of subunit c are crucial for defining the final content of ATP synthase in BAT (Kramarova et al., 2008).

2.1.4. Genetic defects of ATP synthase

Deficiencies in mitochondrial energy metabolism are usually associated with severe multisystem diseases. They are often referred to as mitochondrial encephalo-cardiomyopathies since the nervous system, heart and skeletal muscle are the most affected organs. Mitochondrial dysfunction has been, however, recognised also as a major contributing factor in metabolic and neurodegenerative diseases, ageing and cancer (Greaves et al., 2012).

Primary mitochondrial diseases are caused by mutations in both mitochondrial and nuclear genes. The pathogenesis of mtDNA mutations does not follow the rules of Mendelian genetics. Instead, they are maternally inherited and the resulting phenotype depends on the level of heteroplasmy (DiMauro, 2013). On the other hand, nuclear mutations follow the Mendelian genetics for gene transfer to another generation and their inheritance is often autosomal recessive. They are more frequent since, firstly, the majority of OXPHOS components is encoded by the nuclear genome; and secondly, many processes that govern the correct structure and function of OXPHOS complexes are under control of nuclear genome (Tuppen et al., 2010).

Isolated defects of ATP synthase can be classified into two groups with different pathogenic mechanisms, biochemical phenotypes and clinical presentations. On the one hand, qualitative defects are characterised by normal levels of incomplete and non-functional ATP synthase and have been described for mtDNA mutations. Quantitative defects, on the other hand, underlie mitochondrial disorders of nuclear genetic origin and are characterised by a reduction in the content of fully assembled and functional ATP synthase (Houstek et al., 2006). In both cases, ATP synthase is not able to utilise the mitochondrial membrane potential, and consequently, its increase stimulates mitochondrial reactive oxygen species (ROS) production. Therefore, energy deprivation and ROS production represent important factors in the pathogenesis of isolated defects of ATP synthase (Mracek et al., 2006).

2.1.4.1. Mutations in mitochondrially encoded subunits of ATP synthase

Point mutations in the mtDNA gene *MT-ATP6* encoding subunit *a* are a predominant cause of maternally inherited ATP synthase defects. The most frequent missense mutations are *m.8993T>G* (p.L156R) (Holt et al., 1990) and *m.8993T>C* (p.L156P) (de Vries et al., 1993). The clinical phenotype manifests as NARP (neuropathy, ataxia, retinitis pigmentosa) or the more severe MILS (maternally inherited Leigh's syndrome), depending on the level of heteroplasmy (Jonckheere et al., 2012). In addition to the numerous point mutations in *MT-ATP6* that usually lead to substitution of amino acids involved in the function of the proton channel, two cases with a unique microdeletion of two base pairs (*m.9205delTA*) at the interface of *MT-ATP6* and *MT-COX3* genes in the polycistronic mitochondrial transcript *MT-ATP8*/*MT-ATP6*/*MT-COX3* have been reported (Seneca et al., 1996; Jesina et al., 2004). This microdeletion removes the stop codon in the *MT-ATP6* gene, which impairs the translation of both subunit *a* of ATP synthase and subunit Cox3 of cytochrome *c* oxidase (Jesina et al., 2004).

Mutations in the other mtDNA gene associated with ATP synthase, *MT-ATP8*, coding for the subunit A6L are extremely rare – only three have been reported so far: a nonsense mutation (Jonckheere et al., 2008) and two missense mutations (Ware et al., 2009; Mkaouar-Rebai et al., 2010), one of them localised in the region of the bicistronic transcript *MT-ATP8/MT-ATP6* where these two genes overlap and hence affecting both subunits *a* and A6L (Ware et al., 2009).

Neither missense nor nonsense mutations in the mtDNA genes encoding subunit *a* and A6L usually lead to a reduction in the content of ATP synthase. However, ATP synthesis by this kind of ATP synthase is impaired. Biochemical and clinical manifestations depend on the mutation load and the majority of cases develop some symptoms only if the heteroplasmy of mutated mtDNA reaches 80–90 % (Hejzlarova et al., 2014).

2.1.4.2. Mutations in nuclear encoded subunits and ancillary factors of ATP synthase

The biochemical manifestations of these defects are very homogenous. They are characterised by a reduced content of fully assembled and functional ATP synthase complex (Hejzlarova et al., 2014). Lactic acidosis and methyl glutaconic aciduria are major clues in the diagnosis (De Meirleir et al., 2004). The clinical manifestation, however, differs between individual cases.

Since the first demonstration of an ATP synthase defect of unknown nuclear origin in 1999 (Houstek et al., 1999), mutations in four nuclear genes have been associated with an isolated deficiency of ATP synthase so far. Two of them, *ATP5A1* and *ATP5E*, code for the structural subunits *α* and *ε*, respectively, and are discussed in the following parts of this thesis (Mayr et al., 2010; Jonckheere et al., 2013). The other two, *ATPAF2* and *TMEM70*, encode ancillary factors that play specific roles in the biogenesis of ATP synthase (Hejzlarova et al., 2014).

A homozygous mutation in *ATPAF2* coding for the assembly factor ATP12 was the first reported pathogenic mutation in a nuclear gene associated with an isolated defect of ATP synthase (De Meirleir et al., 2004). No other patient with a mutation in this gene has been found since then. On the other hand, mutations in *TMEM70* represent one of the most frequent causes of ATP synthase deficiencies. In fact, TMEM70 as an ancillary factor of ATP synthase biogenesis was identified on the basis of an analysis of a group of patients with an unknown defect that presents itself with a reduced content of assembled ATP synthase (Cizkova et al., 2008). The *TMEM70* gene contains 3 exons. In these patients, the substitution 317-2A>G leads to a loss of the splice site in the second intron, so it is not recognised by the splicing machinery. As a result, aberrant *TMEM70* transcripts are produced that are not translated. For a recent review of all reported *TMEM70* mutations, see Hejzlarova et al. (2014).

2.2. ADP/ATP translocase (ANT)

ANT belongs to the mitochondrial carrier family (MCF) encoded by the *SLC25* genes (Palmieri, 2004; Palmieri et al., 2011; Gutierrez-Aguilar & Baines, 2013). The size (30–34 kDa) and structure of all SLC25 transporters are very similar. They contain three tandem-repeated homologous domains, and each of these consists of two transmembrane helices (Pebay-Peyroula et al., 2003; Palmieri, 2004) (**Fig. 6**). The members of SLC25 family are extremely hydrophobic proteins with several tissue-specific isoforms. In mammals, each isoform is usually encoded by a unique nuclear gene (Rondelez et al., 2005).

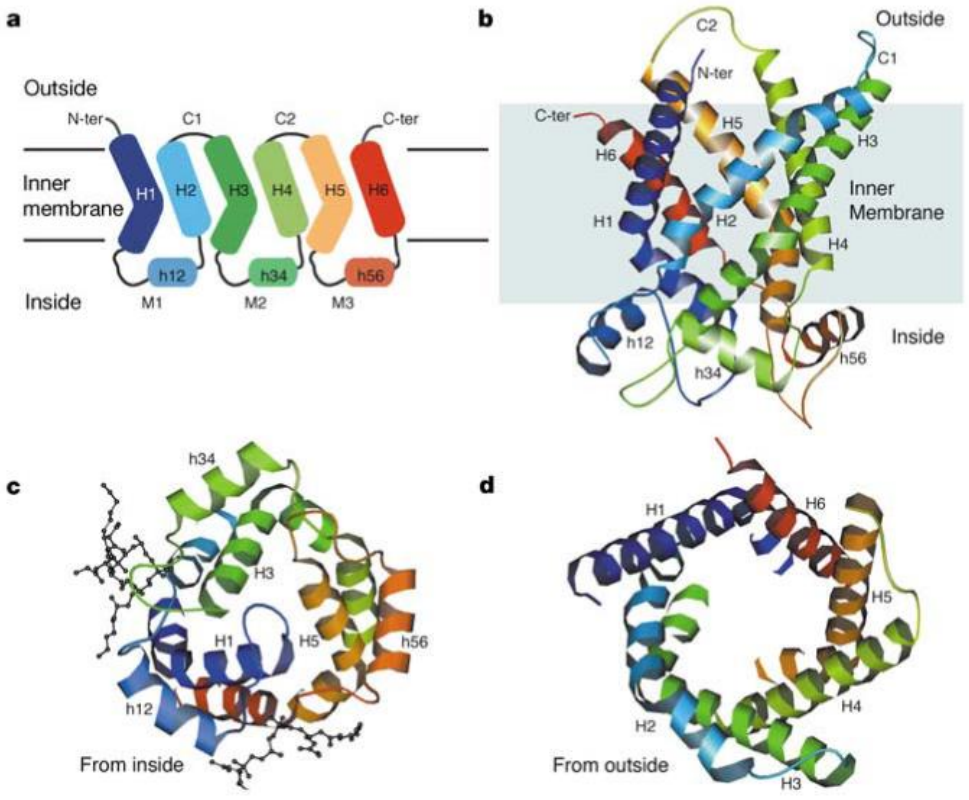
Specifically in rodents, three genes coding for tissue-specific ANT isoforms have been described whereas four genes have been identified in humans so far (Dahout-Gonzalez et al., 2006) – *SLC25A4* (ANT1, a heart-type isoform), *SLC25A5* (ANT2, a liver-type isoform, expressed ubiquitously), *SLC25A6*

(ANT3, expressed in highly proliferative cells, present only as a pseudogene in rodents), and *SLC25A31* (ANT4, a testes-specific isoform) (Dolce et al., 2005; Brower et al., 2007). Each ANT isoform has a tissue-specific expression pattern that may be related to specific energy requirements (Stepien et al., 1992). For example, the transient expression of ANT1, possessing a higher catalytic activity than ANT2, in the neonatal liver has been associated with increased supply of mitochondrial ATP to the cytosolic ATP consumption initiated at birth (Xu et al., 2015).

Moreover, expression of the same isoform may be regulated by different transcriptional mechanisms in different tissues. For example, the expression of ubiquitous ANT2 under stimulation by thyroid hormones is increased in rat heart and liver but not in other tissues (Dummler et al., 1996). It seems that one isoform is not able to completely substitute loss of function of another one since *ANT1* knock-out mice are characterised by mitochondrial myopathy and cardiomyopathy and manifest severe exercise intolerance (Graham et al., 1997). However, loss of ANT1 function is still compatible with adult

Fig. 6 Structure of the ADP/ATP carrier solved by X-ray crystallography at a resolution of 2.2 Å.

***a**. A schematic diagram of the secondary structure. **b**. A view from the side. **c**. A view from the inside. Two cardiolipin molecules are depicted. **d**. A view from the outside.*
Transmembrane helices, surface helices, intermembrane space loops and matrix loops are labelled H, h, C, and M, respectively. Adopted from Pebay-Peyroula et al. (2003).



life (Palmieri, 2008), as in the case of a patient with a homozygous mutation in ANT1 (Palmieri et al., 2005). Beside insufficient energy provision in heart and skeletal muscle mitochondria with predominant expression of ANT1 isoform, loss-of-function mutations of ANT1 are associated also with increased ROS production in these tissues, which leads to accumulation of mtDNA rearrangements and deletions (Esposito et al., 1999; Palmieri et al., 2005).

Under physiological conditions, ANT catalyses the electrogenic exchange of cytosolic ADP^{3-} for mitochondrial ATP^{4-}, which is driven by the mitochondrial membrane potential (Dahout-Gonzalez et al., 2006). A potent inhibitor of ANT is carboxyatractyloside (CAT). As the CAT-ANT complex can be relatively easily purified, ANT was the first carrier from the SLC25 family that was isolated and characterised (Brandolin et al., 1974; Riccio et al., 1975). These purification studies also revealed that ANT is one of the most abundant mitochondrial proteins and represents up to 12 % of the total mitochondrial protein mass (Boxer, 1975; Riccio et al., 1975; Klingenberg et al., 1978; Hackenberg & Klingenberg, 1980). The atomic structure of bovine ANT was also solved in complex with CAT, namely at a resolution of 2.2 Å (Pebay-Peyroula et al., 2003) (**Fig. 6**.). Because CAT binds into ANT from the side of intermembrane space, the resolved ANT structure is in the so-called cytosolic conformation where ANT is opened to the intermembrane space.

Whether mitochondrial carriers function as monomers or dimers is still a matter of debate (Trezeguet et al., 2008; Monne & Palmieri, 2014). The early ANT purification studies indicated that ANT molecules might associate into dimers in the lipid bilayer (Klingenberg et al., 1978; Hackenberg & Klingenberg, 1980). Kinetic studies also favours the dimeric organisation of ANT since a positive cooperativity in the binding of substrates has been observed (Dahout-Gonzalez et al., 2006). The most recent evidence however supports the monomeric state of ANT (Bamber et al., 2006; Bamber et al., 2007; Nury et al., 2008; Crichton et al., 2013).

2.3. Phosphate carrier (PiC)

The physiological role of PiC is to catalyse the uptake of phosphate (P_i) into the mitochondrial matrix, either by proton co-transport or in exchange for hydroxyl ions at the expense of the electrochemical proton gradient (Palmieri, 2004). Either way, the uptake of P_i is electroneutral (Seifert et al., 2015).

Phosphate carrier, as well as ANT, belongs to the *SLC25* gene family (Runswick et al., 1987), but unlike ANT, its two tissue-specific isoforms, PiC-A (a heart-type isoform) and PiC-B (a liver-type isoform that is expressed ubiquitously), originates from alternative splicing of a single gene transcript (*SLC25A3*) (Dolce et al., 1996; Palmieri, 2004). *SLC25A3* contains nine exons and two of them are alternatively spliced in a mutually exclusive manner, which results in two isoforms that differ from each other by

nine amino acids in the N-terminal part and share >70 % homology (Dolce et al., 1994). Their catalytic Michaelis constants K_m are different and most likely reflect energy demands of the tissues that they are expressed in (Dolce et al., 1996; Fiermonte et al., 1998). Indeed, the cell can modulate the intensity of mitochondrial ATP production by changing the level of expression of individual PiC isoforms. PiC-B, expressed ubiquitously, takes care of the routine energy demands of the cell. PiC-A, characterised by a lower substrate affinity (higher K_m) and a lower maximum transport rate (V_{max}) (Fiermonte et al., 1998) is required to operate under increased energy demands when PiC-B is overwhelmed by high cytosolic concentrations of P_i (e.g. during muscle contractions) (Dolce et al., 1996; Fiermonte et al., 1998; Palmieri, 2004).

Upon PiC depletion in mouse models, cardiac hypertrophy is observed, which is likely a consequence of insufficient P_i uptake and inadequate mitochondrial ATP production (Gutierrez-Aguilar et al., 2014; Kwong et al., 2014). In human, mutations that affect the heart-specific PiC-A isoform have been also reported to lead predominantly to cardiomyopathy (Mayr et al., 2007; Mayr et al., 2011). The cardiac phenotype is shared by mice as well as patients with a loss of function for the *SLC25A4* gene encoding the ANT1 protein (Graham et al., 1997; Palmieri et al., 2005; Narula et al., 2011).

3. Mitochondrial supercomplexes

The RC complexes associate into larger structures that are called RC supercomplexes (respirasomes). They were reported both in the bacterial plasma membrane and in the IMM of eukaryotes (Dudkina et al., 2015). The existence of supercomplexes significantly reduces the distances between the consecutive RC complexes that their substrates, i.e. Co*Q* and cyt *c*, must surpass using only diffusion. Consequently, the efficiency of the RC is increased due to substrate channelling (Bianchi et al., 2004) and ROS production by the RC complexes is attenuated (Acin-Perez et al., 2008; Lenaz & Genova, 2009). Similarly, the key OXPHOS component, ATP synthase, is also able to organise itself into more complex structures, such as dimers and higher oligomers (Wittig & Schagger, 2009; Seelert & Dencher, 2011; Habersetzer et al., 2013). Furthermore, substrate channelling was suggested to favour the assembly of ATP synthase, ANT and PiC into a supercomplex called ATP synthasome. Supercomplexes of ATP synthase with other mitochondrial proteins have been reported to perform an array of mitochondrial functions, including mitochondrial permeability transition (Bernardi & Di Lisa, 2015; Bernardi et al., 2015) or mitochondrial ATP-sensitive K^+ import (Ardehali et al., 2004).

3.1. Supramolecular structures within the respiratory chain

To describe the organisation of RC enzymes in the IMM, two models have been suggested – the so-called solid state model (Chance & Williams, 1955) and more recently, the fluid state model (Hackenbrock et al., 1986). The fluid state model is defined by free movement of individual OXPHOS components in the IMM. It is accompanied by their spontaneous and transient interactions with each other and also with the mobile electron carriers Co*Q* and cyt *c*. This model regards electron transport as a diffusion-based random collision process. On the contrary, the solid state model suggests a stable, at least for a limited period of time, organisation of RC complexes into higher molecular structures that ensure substrate channelling from one complex to another. This model is supported, among others, by repeated purifications of the same supercomplexes with the same composition and even stoichiometry. In reality, mutual co-existence of isolated RC complexes and supercomplexes is expected with dynamic conversions between each other in order to optimize OXPHOS performance (Boekema & Braun, 2007). Therefore, the plasticity model has been developed as a combination of both extreme situations (Enriquez & Lenaz, 2014). In fact, the content of supercomplexes in the cell was shown to depend on its energy demands and growth conditions in yeast (Schagger & Pfeiffer, 2000).

Among eukaryotes, several types of supercomplexes have been described. They differ from each other in their protein composition, their stability and/or the stoichiometry between individual RC complexes.

Supercomplexes are composed of the RC complexes I, III, and IV. The RC complex II (succinate dehydrogenase) that represents an alternative input of electrons into the RC is believed not to be involved in the formation of RC supercomplexes (Schagger & Pfeiffer, 2001), possibly because of its concomitant involvement in the tricarboxylic acid cycle (Chaban et al., 2014).

In mammals, two types of functionally active RC supercomplexes have been described – I_1III_2 and $I_1III_2IV_{1–4}$ (the latter one being referred to as a respirasome) (Schafer et al., 2006). The first and also smaller one was even the first supercomplex whose existence was reported. With the molecular weight of approximately 1500 kDa, it is regarded as a building block of the larger respirasome. It is mostly studied in *Arabidopsis thaliana* (Dudkina et al., 2005a) since this supercomplex is much more abundant and stable in this model plant in comparison to the mammalian mitochondria (Eubel et al., 2004). The respirasome with the molecular weight of 1700–2100 kDa, depending on the number of complex IV monomers involved in the structure, represents the largest detected form of association of OXPHOS complexes that can autonomously carry out respiration in the presence of Co*Q* and cyt *c* (Schagger & Pfeiffer, 2000).

Information on what determines the structure and composition of these supercomplexes is very limited. In the case of the mammalian supercomplex I_1III_2, the complex I is stabilised by the mutual interaction with the complex III dimer, which might be the reason for an almost complete absence of free complex I in the mammalian mitochondria (Schagger & Pfeiffer, 2000). There is still approximately a third of complex III that is not bound to complex I, hence available as an electron acceptor for other dehydrogenases, such as complex II or glycerol-3 phosphate dehydrogenase (Schagger & Pfeiffer, 2001). In the most studied yeast, *Saccharomyces cerevisiae*, that lacks complex I, the majority of complex IV is associated with the complex III dimer to form III_2IV_1 or III_2IV_2 supercomplexes (Cruciat et al., 2000; Schagger & Pfeiffer, 2000).

Characterisation of supercomplexes relies on their solubilisation as an intact structure from the IMM. The efficiency of solubilisation depends on the type of detergent used. To study supercomplexes, digitonin and Triton X-100 are usually applied because they are mild enough not to disrupt the non-covalent protein-protein interactions inside supercomplexes (Schagger & Pfeiffer, 2000; Eubel et al., 2004). The composition of solubilised supercomplexes can then be revealed by using a type of native electrophoresis (Schagger & Pfeiffer, 2000; Eubel et al., 2004), immunoprecipitation (Cruciat et al., 2000), or sucrose density ultracentrifugation (Dudkina et al., 2005a).

3.2. Supramolecular organisation of ATP synthase

3.2.1. ATP synthase oligomerization

The ATP synthase dimers were first described in yeast (Arnold et al., 1998). Afterwards, not only stable dimers in different species (Schagger & Pfeiffer, 2000; Eubel et al., 2004; Dudkina et al., 2005b; van Lis et al., 2007) but also higher oligomers of ATP synthase were characterised (Allen et al., 1989; Giraud et al., 2002; Dudkina et al., 2006; Strauss et al., 2008).

In yeast, four subunits of ATP synthase that associate with its F_o domain were identified to contribute to the stability of dimeric organisation, namely the subunits *e*, *g*, *k*, and *i* (*j*) (Arnold et al., 1998; Wittig et al., 2008; Wagner et al., 2010). In fact, the subunits *e*, *g* and *k* seem to be dimer-specific in yeast since they were not found in the monomeric form of ATP synthase (Arnold et al., 1998; Arselin et al., 2003). Furthermore, subunits *e* and *g* were proposed to be the actual dimerization elements connecting two ATP synthase monomers. Both of these subunits contain a conserved dimerization motif GXXXG within their transmembrane segment that was proposed to be involved in their heterodimerization (Arselin et al., 2003; Bustos & Velours, 2005). In addition, subunit *e* also contains a coiled-coil region in its C-terminal segment in the intermembrane space that might be involved in the homodimerization of two subunits *e* (Everard-Gigot et al., 2005). However, ATP synthase oligomers were proposed to display two different intermolecular interfaces (Dudkina et al., 2006). In the other one, Atp4 (*b*) most likely plays a key role (Spannagel et al., 1998).

What is the purpose of ATP synthase dimerization? Dimers seem to possess a higher catalytic efficiency but it is not their intrinsic characteristic (Bisetto et al., 2007). Dimerization might enhance the stability of ATP synthase since the monomer appears to be more labile and susceptible to proteolysis (Arnold et al., 1998). Since the *c*-rings in ATP synthase dimers rotate in opposite directions with respect to the dimerization axis, it was proposed that dimerization could play a role in balancing the forces generated in individual ATP synthase molecules due to the torque and also strengthen peripheral stalks connected within the dimer (Chaban et al., 2014).

In the last decade, other physiological roles of ATP synthase dimers have been proposed, such as mitochondrial membrane permeability transition (Bernardi et al., 2015) (see chapter 3.2.5.) and shaping of the IMM. The association of two molecules of ATP synthase happens via the F_o domains that are in the angle of 35–90° to each other. This angle between two ATP synthase molecules in the dimer appears to be specific to each species (Dudkina et al., 2006) and might trigger bending of the IMM, and hence affects to establishment of the IMM morphology. Yeast mutants carrying deletions in the subunits *e*, *g*, *b*, and *h* lack ATP synthase dimers (Arnold et al., 1998; Arselin et al., 2003) and exhibit

an altered morphology of the IMM (Paumard et al., 2002; Arselin et al., 2003; Arselin et al., 2004; Goyon et al., 2008), which might be further proof that the ATP synthase dimers play a role in shaping the mitochondrial cristae (Giraud et al., 2002; Paumard et al., 2002; Arselin et al., 2004; Dudkina et al., 2005b; Strauss et al., 2008; De los Rios Castillo et al., 2011). This is supported by alterations of mitochondrial morphology in human pathological states that are characterised by a reduced content of ATP synthase dimers (Mourier et al., 2014).

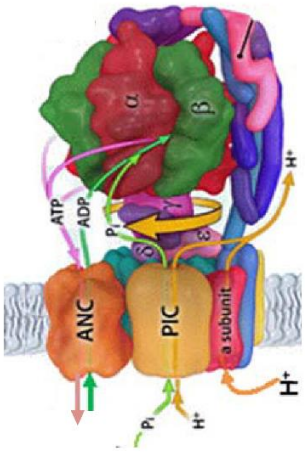
Using advanced electron microscopic techniques, e.g. electron cryo-microscopy (cryo-EM), the globular F_1-domains are recognised as 9-nm projections into the mitochondrial matrix in *Paramecium* (Allen et al., 1989), *Saccharomyces cerevisiae (Dudkina et al., 2006), Polytomella* (Dudkina et al., 2006), or bovine mitochondria (Rubinstein et al., 2003). These techniques allow us to observe ATP synthase dimers in a double row along the curve of a cristae (Strauss et al., 2008; Thomas et al., 2008; Davies et al., 2011; Davies et al., 2014). In tubular-shaped cristae, the rows of ATP synthase dimers appear to be arranged in a helical fashion (Allen et al., 1989; Dudkina et al., 2006). Some cryo-EM studies suggest that the ability to induce the membrane curvature is an intrinsic feature of ATP synthase monomer (Baker et al., 2012).

3.2.2. ATP synthasome

While the association of ATP synthase with a 29 kDa protein (likely ANT) in bovine heart was already reported at the end of the 1970's (Galante et al., 1979; Montecucco et al., 1980), ATP synthasome, a putative supercomplex of ATP synthase, ANT, and PiC (**Fig. 7**), was first described by Pedersen and his

Fig. 7 ATP synthasome.

ATP synthasome, a supercomplex composed of ATP synthase, ANT (also known as ANC, adenine nucleotide carrier), and PiC (phosphate carrier). Adapted from Timohhina et al. (2009).



co-workers in 2003. They detected co-localisation of its components in highly purified vesicles of mitochondrial cristae membrane isolated from rat liver (Ko et al., 2003; Chen et al., 2004). Since then, ATP synthasome has been reported in digitonin-solubilised bovine heart mitochondria on clear native (Wittig & Schagger, 2008) or blue native gels (Seelert & Dencher, 2011) as well as in the protozoan, *Leishmania (Detke & Elsabrouty, 2008)*. This would suggest that the ATP synthasome is an evolutionarily conserved structure that would form a single catalytic unit responsible for mitochondrial ATP production. However, a targeted search for ATP synthasome in a close relative of *Leishmania*, *Trypanosoma brucei*, was unsuccessful (Gnipova et al., 2015). The results of complexome profiling, a recently developed approach to detect novel mitochondrial supercomplexes, are also heterogeneous. In mitochondria from HEK293 cells solubilised with DDM, ATP synthasome was found (Wessels et al., 2013). On the other hand, another profiling analysis of digitonin-solubilised rat heart mitochondria failed to detect any association of ATP synthase with neither ANT nor PiC (Heide et al., 2012). Since complexome profiling is a method based on resolution of solubilised proteins on blue native gels followed by mass spectrometric (MS) analysis of individual parts of the gel and both ANT and PiC are highly hydrophobic, which complicates their detection by MS, it is difficult to conclude that their absence in the region of the gel containing ATP synthase is a proof against the existence of ATP synthasome. The existence of this supercomplex therefore remains controversial.

Not only has the structural association of ATP synthase, ANT, and PiC been questioned, but also the functional significance of ATP synthasome. The proposed 1:1:1 stoichiometry of the ATP synthasome constituents may not be so beneficial for substrate channelling in the process of ATP synthesis (Chen et al., 2004), given the slow catalytic turnover of ANT as compared with other carriers (e.g. PiC) that seems to be compensated for by its high abundance in mitochondria (Clemencon, 2012),

3.2.3. Mitochondrial interactosome

Yet another supercomplex, mitochondrial interactosome, was suggested to contain ATP synthasome in the inner membrane, creatine kinase in the intermembrane space, and voltage-dependent anion channel (VDAC) in the outer membrane and reported in cardiac, skeletal, and brain cells. Such a complex would physically link ATP synthesis and production of another high-energy compound, phosphocreatine (Saks et al., 2010; Guzun et al., 2012). Cytoskeletal proteins tubulins were shown to interact with VDAC and selectively restrict its channel permeability for adenine nucleotides but not for phosphocreatine or creatine, which would favour the phosphocreatine pathway for energy transfer to the cytosol (Timohhina et al., 2009; Guzun et al., 2012). In cancer cells, however, creatine kinase is down-regulated and tubulin is replaced by hexokinase, which results in direct utilisation of

mitochondrial ATP for glycolytic lactate production and hence contributes to the manifestation of Warburg's effect (Saks et al., 2010).

3.2.4. Mitochondrial K_{ATP} channel

ATP-sensitive potassium channels (K_{ATP}) in the plasma membrane have been reported to play a role in a number of processes in different tissues. Their activity is regulated by adenine nucleotides, namely ATP inhibits their opening (Szabo & Zoratti, 2014; Tinker et al., 2014). For many years, a mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel, especially its opening, has been proposed to play an important role in cardioprotection and ischemic pre-conditioning, without its precise molecular identity being known (O'Rourke, 2004; Szabo & Zoratti, 2014). Among others, a large multiprotein complex whose components include ATP synthasome, succinate dehydrogenase (RC complex II) and one ABC transporter (mitochondrial ATP-binding cassette protein 1, mABC1) has been suggested to confer the mitoK_{ATP} channel activity (Ardehali et al., 2004), however, it was never validated. The pursuit of structural information on the mitoK_{ATP} channel, nevertheless, continues even though its existence is still controversial (O'Rourke, 2004; Garlid & Halestrap, 2012; Szabo & Zoratti, 2014).

3.2.5. Mitochondrial permeability transition pore

Mitochondrial permeability transition is defined as a non-selective increase in the permeability of the IMM to solutes with molecular masses up to 1.5 kDa that is usually induced by intramitochondrial Ca^{2+} accumulation (Bernardi et al., 2015). This process was linked to reversible opening of an unknown high-conductance channel (Dahout-Gonzalez et al., 2006; Bernardi & Di Lisa, 2015). As a consequence of mitochondrial permeability transition, the transmembrane proton gradient is dissipated, ATP synthesis stops, and substrates and nucleotides are lost from the matrix due to mitochondrial swelling and rupture. Eventually, these events lead to cell death through necrosis (Szabo & Zoratti, 2014).

The molecular nature of mitochondrial permeability transition pore (mPTP) has been a mystery for a long time, although studied intensively. A desensitising effect of cyclosporine A (CsA) directed the research to its mitochondrial target – cyclophilin D (CyPD), mitochondrial peptidyl-prolyl *cis-trans* isomerase that assists in protein folding. Several proteins were suggested to contribute to the pore formation. At first, the peripheral benzodiazepine receptor (today called mitochondrial translocator protein, TSPO) and the VDAC, both proteins of the outer membrane, in association with ANT were assigned the mPTP function. An alternative model was represented by a complex of PiC, ANT and CyPD (Szabo & Zoratti, 2014). With the exception of CyPD that was confirmed as an important modulator, genetic deletions of the respective genes excluded their involvement in the mPTP formation (Kokoszka et al., 2004; Gutierrez-Aguilar et al., 2014; Kwong et al., 2014). CyPD sensitises mPTP to Ca^{2+} and

confers sensitivity to CsA, but it is not an essential structural component of mPTP (Bernardi & Di Lisa, 2015).

In 2012, a new hypothesis on the molecular nature of mPTP was postulated. As the channel-forming component of mPTP, the ATP synthase complex was identified due to the interaction of its peripheral stalk with CyPD under permeability transition stimuli (Giorgio et al., 2009). Importantly, CsA counteracted this association. As the actual interacting partner of CyPD, OSCP was identified and characterised (Giorgio et al., 2013; Antoniel et al., 2014). The role of ATP synthase in the mPTP formation was also indirectly supported by other studies. Down-regulation of *c* subunit of ATP synthase using siRNA technology resulted in mPTP inactivation (Bonora et al., 2013; Alavian et al., 2014) whereas its overexpression accelerated mitochondrial permeability transition and onset of its effects on cellular physiology (Bonora et al., 2013). Some even suggested that the actual channel is located within the *c*-ring of F_o-subcomplex after separation from the F_1 domain, between the individual c-monomers (Alavian et al., 2014; Bonora et al., 2015; Jonas et al., 2015).

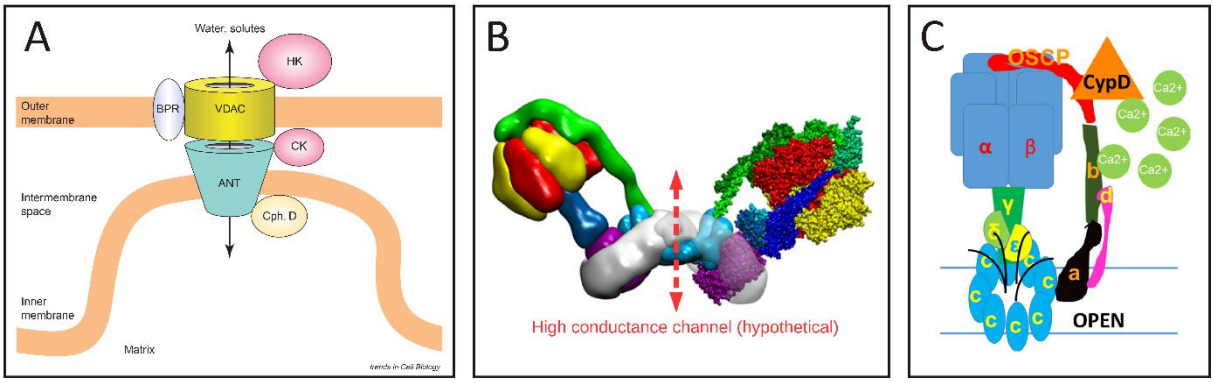
Dimers but not monomers of ATP synthase have been shown to possess the mPTP activity in bovine hearts (Giorgio et al., 2013), *Saccharomyces cerevisiae* (Carraro et al., 2014) and *Drosophila melanogaster* (von Stockum et al., 2015). It indicates that mitochondrial membrane permeability transition and the involvement of ATP synthase is an evolutionarily conserved phenomenon. mPTP might be formed by ATP synthase dimers as a consequence of their conformational change that follows replacement of Mg^{2+} for Ca^{2+} at the catalytic site. The presence of Ca^{2+} instead of Mg^{2+} uncouples the proton pumping and ATP-hydrolytic activities of ATP synthase (Papageorgiou et al., 1998). Binding of CyPD to OSCP in mammalian mitochondria would promote this cofactor exchange on condition that the intramitochondrial Ca^{2+} concentration is sufficiently high. The conformational change would then result in pore opening at the interface between dimers, which is consistent with the phenotype of yeast mutants in the dimer-specific subunits *e* and *g* that are strikingly resistant to mPTP opening (Carraro et al., 2014). This transformation is fully reversible and ATP synthase can return to its basal state after its catalytic site is re-occupied by Mg^{2+}. Therefore, it seems that the mPTP may provide mitochondria with a fast mechanism of Ca^{2+} release and thus take part in physiological regulation of Ca^{2+} homeostasis. In the case of prolonged pore opening, a series of events results in matrix swelling and eventually rupture of the outer mitochondrial membrane. Ca^{2+} induced formation of mPTP might, in the end, lead to release of cytochrome *c* and other pro-apoptotic factors, which contributes to apoptosis (Bernardi & Di Lisa, 2015; Bernardi et al., 2015; Bonora et al., 2015).

The molecular identity of mPTP is, however, still uncertain (**Fig. 8**). Even if the involvement of ATP synthase itself or in association with ANT and PiC in the form of ATP synthasome is validated, it is

unclear whether the pore is formed from a novel conformation of one or more of these proteins or is located at an interface between them (Halestrap & Richardson, 2015).

Fig. 8 Suggested models for mPTP structure.

*(**A**) According to one of the first mPTP models, the pore is formed by ANT in the inner membrane and VDAC in the outer membrane (BRP, benzodiazepine peripheral receptor; CK, creatine kinase; HK, hexokinase; Cph. D, cyclophilin D). Adapted from Desagher & Martinou (2000).*
*In most recent models, the pore-forming component in the inner membrane has changed. The dimer of ATP synthase in association with ANT and PiC (ATP synthasome) that directly interacts with the main mPTP regulator, CyPD via its OSCP subunits has been suggested to form the channel at the interface of two ATP synthase monomers in the dimer (**B**). The channel was also proposed to be formed after an induced change in the ATP synthase conformation in the middle of the c-ring (**C**). Adapted from Bernardi et al. (2015) and Jonas et al. (2015), respectively.*



AIMS OF THE THESIS

The first objective of my thesis was to characterise structural-functional relationships accompanying ATP synthase defects of a different genetic origin. The investigated cell lines were used to achieve my second objective, *i.e.* to study the association of ATP synthase, ANT and PiC into ATP synthasome.

The specific aims of my thesis were:

- To examine in detail structural interactions in the putative supercomplex of ATP synthasome in mammalian mitochondria
- To analyse the expression of ATP synthasome components in rat tissues and to test whether their expression is affected by different genetic defects of ATP synthase in patient cells
- To characterise molecular pathogenic mechanisms underlying the genetic defects of ATP synthase due to mutations in the following genes
    - → *ATP5E* (coding for subunit *ε*)
    - → *MT-ATP6* (coding for subunit *a*)
    - → *TMEM70* (coding for the ancillary factor of ATP synthase biogenesis TMEM70)

SUMMARY OF THE RESULTS

During my studies, I have co-authored fourteen articles published in peer-reviewed journals. Six of them are included and briefly summarised in my PhD thesis. A complete list of my publications in chronological order is detailed at the end of this section.

My first-author publication focuses on the components of mitochondrial ADP-phosphorylating apparatus, their expression and structural interactions (**Article 1**). We showed limited association of ATP synthase not only with ANT and PiC (into a supercomplex called ATP synthasome) but also with succinate dehydrogenase (**Article 2**). To study expression of ATP synthasome components and their interdependence, we used several cell lines with different genetic defects of ATP synthase resulting in a low content of the enzyme or an alteration of its structure. In the other four articles these model cell lines are characterised with regard to impacts of the defect they harbour on mitochondrial functions. Specifically, we described structural and functional consequences of genetic defect of subunit *ε* in cells of a patient (**Article 3**) as well as in model cells with down-regulated expression of this subunit (**Article 4**). The reported missense mutation in the gene *ATP5E* coding for subunit *ε* was the first reported mutation in a nuclear encoded structural subunit of ATP synthase and it inspired us to develop a model cell line with a knock-down of this subunit in an effort to elucidate its function in human cells. Compared to defects in nuclear encoded subunits of ATP synthase, mutations in the mitochondrial gene *MT-ATP6* coding for subunit *a* are reported more regularly. We also characterised one mutation, specifically *m.9205delTA* that does not only affect ATP synthase but also cytochrome *c* oxidase (**Article 5**). In the last article, we investigated adaptive changes in the expression of mitochondrial proteins in patients with a mutation in *TMEM70* coding for an ancillary factor of ATP synthase biogenesis (**Article 6**).

1. **Mitochondrial ATP synthasome: expression and structural interaction of its components**

   Nůsková, H., Mráček, T., Mikulová, T., Vrbacký, M., Kovářová, N., Kovalčíková, J., Pecina, P., Houštěk, J. (2015). Biochem Biophys Res Commun. doi: 10.1016/j.bbrc.2015.07.034
   IF 2.297 (2014)

To characterise the relationships in the expression of ATP synthase, ANT, and PiC in mammalian cells, we used two models of isolated ATP synthase deficiency – rat BAT with a physiological down-regulation of ATP synthase, and fibroblast cultures of patients with different types of ATP synthase deficiency. In rat tissues, both transcript and protein levels of ANT and PiC correlate with the content of ATP synthase. Therefore, ANT and PiC levels appear to be transcriptionally controlled in accordance with the biogenesis of ATP synthase. In contrast, the content of ANT and PiC is increased in the ATP synthase deficient patient fibroblasts compared to control cells. Since there is no significant change in the transcript levels of ANT and PiC, the observed adaptive responses are likely regulated post-transcriptionally, possibly at the level of protein synthesis or stability.

To describe further the structural interactions of ANT, PiC, and ATP synthase, we analysed rat heart mitochondria solubilised with different mild non-ionic detergents. We characterised the association of ATP synthasome components by immunoprecipitation, blue native and SDS polyacrylamide gel electrophoresis combined with immunodetection and MS analysis. Our results indicate that both carriers can be found attached to monomeric and dimeric forms of ATP synthase. However, the majority of immunodetected PiC and especially ANT did not associate with the ATP synthase, suggesting that while ATP synthasome is present in heart mitochondria, most of the PiC, ANT, and also ATP synthase probably exist as separate entities.

2. **High molecular weight forms of mammalian respiratory chain complex II.**
   Kovářová, N., Mráček, T., **Nůsková, H.**, Holzerová, E., Vrbacký, M., Pecina, P., Hejzlarová, K., Kl'učková, K., Rohlena, J., Neužil, J., Houštěk, J. (2013). PLoS One *8*, e71869.
   IF 3.534

While studying the association of ATP synthase, ANT and PiC, we uncovered an unexpected connection between ATP synthase and succinate dehydrogenase (RC complex II). This revelation is included in this publication.

In general, the OXPHOS complexes are arranged into supramolecular structures that can be preserved under solubilisation with mild detergents and resolved by native electrophoretic systems. The involvement of complexes I, III and IV in supercomplex formation and also multimeric forms of ATP synthase have been studied quite thoroughly. However, the association of complex II, which links the RC with the tricarboxylic acid cycle, with other OXPHOS enzymes is questionable.

In this publication, we reported the existence of structures of high molecular weight that contained complex II (CII_{hmw}) and were preserved only under specific conditions, i.e. under the combination of solubilisation by digitonin and resolution by clear native electrophoresis (CNE). We showed that CII_{hmw} structures are enzymatically active and differ in the electrophoretic mobility between rat tissues (500–1000 kDa) and cultured human cells (400–670 kDa). Furthermore, they are destabilised in mtDNA-depleted rho^0 cells whereas their formation is unaffected by isolated defects in the other OXPHOS complexes. Electrophoretic studies and immunoprecipitation experiments of CII_{hmw} did not reveal any specific interactions with the RC complexes I, III or IV or enzymes of the tricarboxylic acid cycle. However, they suggest a specific interaction between complex II and ATP synthase. Their association has been previously reported in a supercomplex that was suggested to confer the activity of mitochondrial ATP-sensitive K^+ channel (Ardehali et al., 2004).

3. **Mitochondrial ATP synthase deficiency due to a mutation in the *ATP5E* gene for the F_1 epsilon subunit.**

   Mayr, J.A., Havlíčková, V., Zimmermann, F., Magler, I., Kaplanová, V., Ješina, P., Pecinová, A., **Nůsková, H.**, Koch, J., Sperl, W., Houštěk, J. (2010). Hum Mol Genet *19*, 3430-3439.
   IF 8.058

This publication was a result of our collaboration with the Paediatric Department of Prof. Wolfgang Sperl at the Paracelsus Medical University in Salzburg, Austria. Similarly to the next publication, it demonstrates the importance of subunit ε for the biogenesis of ATP synthase.

In 2008, a common mutation in the gene *TMEM70* coding for an ancillary factor of ATP synthase biogenesis was identified in a group of patients with an isolated ATP synthase defect (Cizkova et al., 2008). However, there was a single patient with a distinct clinical phenotype and no detected mutation in *TMEM70*. In this publication, we reported that the underlying genetic cause of ATP synthase defect in this patient was a homozygous mutation *c.35A>G* in the nuclear gene *ATP5E* coding for subunit *ε*. The identified mutation was the first mutation reported in a nuclear encoded structural subunit of ATP synthase. It leads to an amino acid substitution (p.Tyr12Cys) that affects a tyrosine residue at the N-terminus highly conserved among eukaryotes.

Biochemical analysis of the patient's fibroblasts showed a reduction of both oligomycin-sensitive ATP-hydrolytic and ATP-synthesising activity to ∼30 % when compared to controls. The mitochondrial content of fully assembled ATP synthase was equally reduced. However, its molecular weight is unchanged due to incorporation of the mutated subunit *ε* and its catalytic activity seems to be preserved. The insufficient capacity of ATP synthase then leads to a decrease in respiration and to an increase in mitochondrial membrane potential under ADP stimulation, compared to control cells. Similarly to the down-regulation of subunit *ε* in the HEK293 cells, a detailed protein analysis revealed a decrease in the content of both F_1 and F_o subunits of ATP synthase with the only exception of subunit *c* that was accumulated in a detergent-insoluble form. Furthermore, the content of RC complexes I, II, III, and IV were unchanged or slightly increased compared to controls.

Finally, data obtained from metabolic labelling were indicative of a decreased *de novo* synthesis of ATP synthase. Therefore, this isolated defect of ATP synthase most likely results from an impairment of ATP synthase biogenesis that leads to a reduced content of ATP synthase that is fully functional despite the incorporation of mutated subunit *ε*.

4. **Knockdown of F_1 epsilon subunit decreases mitochondrial content of ATP synthase and leads to accumulation of subunit *c*.**

   Havlíčková, V., Kaplanová, V., **Nůsková, H.**, Drahota, Z., and Houštěk, J. (2010). Biochim Biophys Acta *1797*, 1124-1129.

   IF 5.132

Subunit *ε* is the smallest and functionally least characterised subunit of the F_1 domain of mammalian ATP synthase. It lacks the N-terminal targeting sequence and there are no known homologues in bacteria and chloroplasts (Xu et al., 2015).

To characterise the function of mammalian subunit *ε*, we knocked down the expression of the respective nuclear gene *ATP5E* in the HEK293 cell line, using the technique of RNA interference. As a consequence, the content and activity of ATP synthase dropped to ∼40 % of controls, which was accompanied by a decrease in the ADP-stimulated respiration and by an increase in the mitochondrial membrane potential.

A more detailed investigation of changes at the protein level revealed that the decrease in subunit *ε* was followed by a decrease in other ATP synthase subunits, except subunit *c*. The accumulated subunit *c* was incorporated into the fully assembled ATP synthase and also into other subcomplexes with the molecular weight of 200–400 kDa that contained neither F_1 subunits (*α*, *β*) nor F_o subunits (*a*, *b*, *d*). Subunit *ε* seems to play an important role in the biosynthesis and assembly of the F_1 domain of ATP synthase and to be involved in the incorporation of hydrophobic subunit *c* to the rotor structure (F_1-*c*-ring) of the mammalian enzyme.

5. **Alteration of structure and function of ATP synthase and cytochrome *c* oxidase by lack of F_o-a and Cox3 subunits caused by mitochondrial DNA 9205delTA mutation.**

   Hejzlarová, K., Kaplanová, V., **Nůsková, H.**, Kovářová, N., Ješina, P., Drahota, Z., Mráček, T., Seneca, S., Houštěk, J. (2015). Biochem J *466*, 601-611.

   IF 4.396 (2014)

Severe mitochondrial disorders are frequently caused by mutations in the *MT-ATP6* gene coding for subunit *a* of ATP synthase. The majority of them are missense mutations (Hejzlarova et al., 2014). In this publication, we studied a unique microdeletion (*m.9205delTA*) in the polycistronic mitochondrial transcript *MT-ATP8*/*MT-ATP6*/*MT-COX3*. This microdeletion removes the stop codon in the *MT-ATP6* gene, which interferes with the processing of this mRNA and negatively affects the cleavage site between *MT-ATP6* and *MT-COX3* and as a result also the translation of both subunit *a* of ATP synthase and subunit Cox3 of cytochrome *c* oxidase (COX) (Jesina et al., 2004).

So far, this rare mutation has been found in two unrelated patients whose clinical phenotypes differed strikingly. While the first patient was characterised by mild transient lactic acidosis (Seneca et al., 1996), the other one suffered from fatal encephalopathy (Jesina et al., 2004). Nevertheless, both patients were reported as homoplasmic. Therefore, we set to compare the cells of both patients to search for another factor that could modulate the outcome of the *m.9205delTA* microdeletion. In the fibroblasts obtained from the first patient with a milder phenotype, a heteroplasmy of this mutation was revealed after a prolonged time of cultivation, most likely due to negative segregation of the mutation. To gain more insight into the effect of *m.9205delTA* heteroplasmy, we prepared transmitochondrial cybrids with a varying mutation load (52–99 %). All parameters that were determined, i.e. the content of subunits *a* and Cox3, ADP-stimulated respiration, mitochondrial ATP production, and COX activity, were found to be strongly dependent on the mutation load with a heteroplasmy threshold at ~90 % mutation. Therefore, the distinct phenotypes of the two reported patients most likely resulted from a different mutation load with a critical threshold for the severity of disease manifestation at a very high heteroplasmy level.

While comparing the control and *m.9205delTA* homoplasmic cybrid lines, we found that a lack of subunit *a* alters the structure but not the content of ATP synthase, which assembles into a labile ~60 kDa smaller complex retaining the ATP-hydrolytic but not ATP-synthesising activity. On the contrary, a lack of Cox3 limits the biosynthesis of COX but does not alter the structure of the enzyme. The reduced content of COX leads to a decrease in the respiratory rates and the total H^+-pumping activity of the RC as evidenced by a reduced mitochondrial membrane potential.

6. **Compensatory upregulation of respiratory chain complexes III and IV in isolated deficiency of ATP synthase due to TMEM70 mutation.**

   Havlíčková Karbanová, V., Čížková Vrbacká, A., Hejzlarová, K., **Nůsková, H.**, Stránecký, V., Potocká, A., Kmoch, S., Houštěk, J. (2012). Biochim Biophys Acta *1817*, 1037-1043.

   IF 4.624

Mutations in *TMEM70* that codes for an ancillary factor of ATP synthase biogenesis are the most frequent genetic cause of isolated ATP synthase deficiencies. Medical symptoms usually fall into the category of early-onset mitochondrial encephalo-cardiomyopathies. As a consequence of decreased content of functional ATP synthase, patient cells are characterised by a lower ATP production and an elevated mitochondrial membrane potential (Cizkova et al., 2008) that often leads to an increase in ROS production. To investigate adaptive mechanisms of patient cells under such metabolic imbalance, we analysed the expression of OXPHOS complexes and intramitochondrial proteases that are involved in their turnover.

We investigated primary fibroblasts derived from skin biopsies of 10 patients with the common homozygous mutation *c.317-2A>G* in *TMEM70.* In patient fibroblasts, the content of fully assembled ATP synthase was reduced to 11 % of controls on average. On the other hand, we found an increase in the content of complex III and IV to 153 % and 184 % of controls, respectively. The absolute content of individual OXPHOS subunits that was analysed under denaturing conditions followed this pattern. The reduced content of fully assembled ATP synthase that was accompanied by a decrease in the content of individual ATP synthase subunits indicated that the synthesised but not assembled ATP synthase subunits are subject to degradation. Therefore, we analysed the protein levels of mitochondrial proteases Lon, paraplegin, and prohibitin 1 and 2, in which we did not find any significant change.

Whole-genome expression profiling revealed generalized up-regulation of transcriptional activity in patient fibroblasts but did not show any consistent changes in mRNA levels of structural subunits or specific assembly factors of OXPHOS complexes that would correspond to the protein data. Therefore, the reported compensatory increase in the RC complexes III and IV (as well as ANT and PiC as reported in Article 1) in response to the ATP synthase deficiency is most likely a result of an adaptive mechanism at the post-transcriptional level.

MY CONTRIBUTION TO THESE PUBLICATIONS:

The presented data resulted from a team effort of all co-authors detailed in the above mentioned publications. Here, my experimental involvement in the individual publications is summarised:

1. Characterisation of structural interactions of ATP synthase, ANT, and PiC using blue-native and multidimensional electrophoreses and immunoprecipitation; quantitative analysis of ATP synthasome components at the protein level using Western blots; preparation of samples for MS analysis; isolation of mitochondria from rat tissues and patient fibroblasts; cell cultivation

2. Detection of structural interactions between ATP synthase and succinate dehydrogenase by means of immunoprecipitation; isolation of mitochondria from rat tissues

3. Evaluation of mitochondrial functions, specifically cell respiration (oxygraph measurements)

4. + 5. Evaluation of mitochondrial functions, specifically mitochondrial membrane potential (TPP^+-selective electrode)

6. Quantitative analysis of the protein content of OXPHOS components using the combination of SDS-PAGE and immunodetection on Western blots; cell cultivation

CHRONOLOGICAL LIST OF MY PUBLICATIONS:

1. **Nuskova, H.**, Mracek, T., Mikulova, T., Vrbacky, M., Kovarova, N., Kovalcikova, J., Pecina, P., and Houstek, J. (2015) Mitochondrial ATP synthasome: Expression and structural interaction of its components. *Biochemical and biophysical research communications* **464**, 787-793

2. Hermanova, I., Arruabarrena-Aristorena, A., Valis, K., **Nuskova, H.**, Jorda, M. A., Fiser, K., Fernandez-Ruiz, S., Kavan, D., Pecinova, A., Niso-Santano, M., Zaliova, M., Novak, P., Houstek, J., Mracek, T., Kroemer, G., Carracedo, A., Trka, J., and Starkova, J. (2015) Pharmacological inhibition of fatty-acid oxidation synergistically enhances the effect of l-asparaginase in childhood ALL cells. *Leukemia* (in press)

3. Hejzlarova, K., Kaplanova, V., **Nuskova, H.**, Kovarova, N., Jesina, P., Drahota, Z., Mracek, T., Seneca, S., and Houstek, J. (2015) Alteration of structure and function of ATP synthase and cytochrome c oxidase by lack of Fo-a and Cox3 subunits caused by mitochondrial DNA 9205delTA mutation. *The Biochemical journal* **466**, 601-611

4. Pecina, P., Houstkova, H., Mracek, T., Pecinova, A., **Nuskova, H.**, Tesarova, M., Hansikova, H., Janota, J., Zeman, J., and Houstek, J. (2014) Noninvasive diagnostics of mitochondrial disorders in isolated lymphocytes with high resolution respirometry. *BBA Clinical* **2**, 62-71

5. Hejzlarova, K., Mracek, T., Vrbacky, M., Kaplanova, V., Karbanova, V., **Nuskova, H.**, Pecina, P., and Houstek, J. (2014) Nuclear genetic defects of mitochondrial ATP synthase. *Physiological research / Academia Scientiarum Bohemoslovaca* **63 Suppl 1**, S57-71

6. Kovarova, N., Mracek, T., **Nuskova, H.**, Holzerova, E., Vrbacky, M., Pecina, P., Hejzlarova, K., Kluckova, K., Rohlena, J., Neuzil, J., and Houstek, J. (2013) High molecular weight forms of mammalian respiratory chain complex II. *PloS one* **8**, e71869

7. Folbergrova, J., Jesina, P., **Nuskova, H.**, and Houstek, J. (2013) Antioxidant enzymes in cerebral cortex of immature rats following experimentally-induced seizures: up-regulation of mitochondrial MnSOD (SOD2). *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience* **31**, 123-130

8. Havlickova Karbanova, V., Cizkova Vrbacka, A., Hejzlarova, K., **Nuskova, H.**, Stranecky, V., Potocka, A., Kmoch, S., and Houstek, J. (2012) Compensatory upregulation of respiratory chain complexes III and IV in isolated deficiency of ATP synthase due to TMEM70 mutation. *Biochimica et biophysica acta* **1817**, 1037-1043

9. Pecinova, A., Drahota, Z., **Nuskova, H.**, Pecina, P., and Houstek, J. (2011) Evaluation of basic mitochondrial functions using rat tissue homogenates. *Mitochondrion* **11**, 722-728

10. **Nuskova, H.**, Vrbacky, M., Drahota, Z., and Houstek, J. (2010) Cyanide inhibition and pyruvate-induced recovery of cytochrome c oxidase. *Journal of bioenergetics and biomembranes* **42**, 395-403

11. Mayr, J. A., Havlickova, V., Zimmermann, F., Magler, I., Kaplanova, V., Jesina, P., Pecinova, A., **Nuskova, H.**, Koch, J., Sperl, W., and Houstek, J. (2010) Mitochondrial ATP synthase deficiency due to a mutation in the ATP5E gene for the F1 epsilon subunit. *Human molecular genetics* **19**, 3430-3439

12. Havlickova, V., Kaplanova, V., **Nuskova, H.**, Drahota, Z., and Houstek, J. (2010) Knockdown of F1 epsilon subunit decreases mitochondrial content of ATP synthase and leads to accumulation of subunit c. *Biochimica et biophysica acta* **1797**, 1124-1129

13. Drahota, Z., Vrbacky, M., **Nuskova, H.**, Kazdova, L., Zidek, V., Landa, V., Pravenec, M., and Houstek, J. (2010) Succinimidyl oleate, established inhibitor of CD36/FAT translocase inhibits complex III of mitochondrial respiratory chain. *Biochemical and biophysical research communications* **391**, 1348-1351

14. Endlicher, R., Krivakova, P., Rauchova, H., **Nuskova, H.**, Cervinkova, Z., and Drahota, Z. (2009) Peroxidative damage of mitochondrial respiration is substrate-dependent. *Physiological research / Academia Scientiarum Bohemoslovaca* **58**, 685-692

DISCUSSION

Cell lines obtained from ATP synthase-deficient patients represent a valuable model to study the biogenesis, function and regulation of ATP synthase as well as of the whole ADP-phosphorylating apparatus. Specifically, we studied nuclear mutations in *ATP5E* (**Article 3** and **4**) and *TMEM70* (**Article 6**) that affect subunit *ε* of ATP synthase and the ancillary factor of its biogenesis TMEM70, respectively. These genetic defects of ATP synthase result in a markedly reduced content of fully assembled and functional ATP synthase. On the other hand, a normal amount of incomplete and non-functional ATP synthase complex lacking subunit *α* is a result of the unique mitochondrial microdeletion *m.9205delTA* that impairs the translation of both subunit a of ATP synthase and subunit Cox3 of cytochrome *c* oxidase (**Article 5**). Unlike the nuclear mutations, the pathological outcome of this mutation depends on the level of heteroplasmy (mutational load).

When we described a pathogenic homozygous mutation in the *ATP5E* gene in 2010 (**Article 3**), it was the very first reported patient with a mutation in a nuclear encoded structural subunit of ATP synthase (Mayr et al., 2010). Three years later, a mutation in *ATP5A1* coding for subunit *α* was reported in two siblings (Jonckheere et al., 2013). Currently, no other mutations in the nuclear genes encoding ATP synthase subunits have been described. The two cases that were published indicate that pathogenic nuclear mutations are usually recessive and likely very rare. As a consequence, the frequency of ATP synthase defects of this kind is extremely low. Furthermore, the functional outcomes of mutations in the structural subunits of ATP synthase may be so severe that they are embryonically lethal for their homozygous carriers, which would also contribute to the fact that more cases are not recorded. Therefore, the prevalence of all pathogenic mutations in mitochondrial components may be significantly underestimated (Seifert et al., 2015).

The yeast subunit *ε* is required for the functional coupling of proton translocation and ATP synthesis (Tetaud et al., 2014). We have shown in the patient with a mutation in *ATP5E* (Mayr et al., 2010) (**Article 3**) and also in our model of *ATP5E* knock-down (Havlickova et al., 2010) (**Article 4**) that the mammalian and yeast ATP synthase differs in this regard. Whereas a reduction in the content of subunit *ε* results in uncoupling of proton translocation from ATP synthesis in yeast, it leads to a decrease in the total amount of ATP synthase that is otherwise fully functional and coupled in humans. As a consequence, the mitochondrial membrane potential is partially depleted in yeast but increased in humans due to a decreased capacity of ATP synthase to consume it. The pathogenic mutation p.Tyr12Cys in *ATP5E* that we had reported was later studied in detail in *Saccharomyces cerevisiae* (Sardin et al., 2015). A yeast equivalent of the reported mutation did not affect the assembly or stability

of ATP synthase complex, as opposed to the patient, which suggests that the biogenesis of ATP synthase differs substantially between humans and yeast.

Another example of this difference is the importance of TMEM70, an ancillary factor of ATP synthase biogenesis in mammals, whose homologue has not been found in yeast. We found a compensatory up-regulation of CoIII and CoIV in patient fibroblasts (Havlickova Karbanova et al., 2012) (**Article 6**) and the same was later observed also by Torraco et al. (2012). A similar adaptive response was found in tissues (Mayr et al., 2004) and fibroblasts of the *ATP5E* patient (Mayr et al., 2010) (**Article 3**). However, these changes obviously cannot compensate for the energetic dysfunction originating from the lack of functional ATP synthase. The same applies for the compensatory increase in the content of other components of mitochondrial phosphorylation apparatus, ANT and PiC, that we observed in *TMEM70*, *ATP5E*, and *MT-ATP6* patients (Nuskova et al., 2015) (**Article 1**). A compensatory up-regulation in the total mitochondrial content, another adaptive mechanism reported in mice lacking PiC (Kwong et al., 2014) and also in hearts of patients lacking PiC-A (Mayr et al., 2011), was not associated with any of the studied mutations. Since the transcript levels of up-regulated proteins were not changed, a post-transcriptional adaptive mechanism is likely involved. Under physiological conditions in rat tissues, in contrast, the protein content of ATP synthase, ANT, and PiC appears to be co-regulated at the level of transcription.

The supercomplex of ATP synthasome is generally accepted although the interaction of its components has not been studied as thoroughly as other mitochondrial higher molecular structures. Two key publications from Pedersen's laboratory that established ATP synthasome as a mitochondrial supercomplex are based on the fact that all its components co-localized in vesicles of enriched mitochondrial cristae membrane from rat liver (Ko et al., 2003; Chen et al., 2004). The evidence for its existence is therefore lacking and the functional advantages that it would provide for ADP phosphorylation are also questionable. We were able to detect this supercomplex in rat heart mitochondria by BNE and immunoprecipitation experiments but its prevalence was strikingly low (Nuskova et al., 2015) (**Article 1**). Reasons for this observation may be found in the recent plasticity model of protein organisation in the IMM (Enriquez & Lenaz, 2014). The ratio between ATP synthasome and its separate components may be indeed very low. ATP synthasome could also represent an intermolecular association that is extremely short-lived and thus difficult to capture by biochemical methods. Nevertheless, its functional significance still remains elusive.

While we were studying the putative supercomplex of ATP synthasome, we found an association of ATP synthase with another mitochondrial protein – succinate dehydrogenase (Kovarova et al., 2013) (**Article 2**). The interaction of the entire ATP synthasome with succinate dehydrogenase had been

reported and assigned the function of mitoK$_{ATP}$ channel (see chapter 3.2.4.). Despite all controversies, strong evidence links the functional properties of mitoK$_{ATP}$ channel with succinate dehydrogenase (Garlid & Halestrap, 2012). Diazoxide, an inhibitor of succinate dehydrogenase, has been used by many as an opener of mitoK$_{ATP}$ channel, which is proposed to mediate its cardioprotective effects (O'Rourke, 2004). Taking into account recently published findings (Chouchani et al., 2014), the cardioprotective effect might result not from the mitoK$_{ATP}$ channel opening but from a reduction of ROS production in the ischemic heart. After reperfusion, rapid oxidation of succinate accumulated under hypoxia saturates the RC with electrons, which leads to reverse electron flow to complex I. ROS production by this enzyme is then responsible for the ischemia/reperfusion injury. The complex II inhibitors might therefore protect the heart from oxidative damage without any involvement of mitoK$_{ATP}$ channel.

In conclusion, my thesis shows that studying the phenotype of patients and molecular and biochemical processes in their cells can not only shed light on the pathogenic mechanism of their disease but also contribute to elucidating the function of the affected gene and its protein product. In a broader picture, we can learn more about associations of the affected protein with other partners and the interdependence of their regulation.

CONCLUSIONS

- Structural analyses indicate presence of ATP synthasome in rat heart mitochondria. However, the majority of PiC, ANT, and ATP synthase exist as separate entities. In addition, ATP synthase was found to interact with succinate dehydrogenase. This association had been suggested to confer the activity of mitochondrial ATP-sensitive K^+ channel.

- In rat tissues, levels of ATP synthase correlate with those of ANT and PiC. On the other hand, human ATP synthase deficiencies lead to a compensatory increase in the content of ANT and PiC, likely due to a post-transcriptional adaptive mechanism. Similarly, complex III and IV are also up-regulated in the patients harbouring a mutation in *TMEM70*.

- The *ATP5E* gene was identified as the first nuclear gene coding for a structural subunit of ATP synthase responsible for deficiency of this enzyme in human patients. The respective subunit ε is required for assembly and/or stability of the F_1 catalytic domain of the mammalian ATP synthase and plays a role in the incorporation of the hydrophobic subunit *c* into the F_1-*c* oligomer during the process of ATP synthase assembly.

- The unique microdeletion *m.9205delTA* in the mitochondrial gene *MT-ATP6*, affecting the function of both ATP synthase and cytochrome *c* oxidase, only leads to a mitochondrial disease phenotype when heteroplasmy is over 90 %. The lack of subunit *a* does not affect the amount of ATP synthase. The incomplete enzyme is, however, unstable and unable to produce ATP.

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