Institute of Physiology
Academy of Sciences
of the Czech Republic

Institute of Physiology AS CR
Prague, Czech Republic

1st Faculty of Medicine
Charles University
Prague, Czech Republic

Assembly factors in the biogenesis of mitochondrial ATP synthase

PhD Thesis

Andrea Potocká, MSc.

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CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
ANT	adenine nucleotide translocase
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
ATP10, *ATP11*, *ATP12*, *ATP22*, *ATP23*	ATP synthase assembly proteins
ATPAF1, *ATPAF2*	ATP synthase assembly factors
BAT	brown adipose tissue
BFP	blue fluorescent protein
C I, II, III, IV, V	complexes I, II, III, IV and V involved in oxidative phosphorylation
CATR	carboxyatractyloside
cGPDH	cytosolar glycerophosphate dehydrogenase
Q	coenzyme Q
COX	cytochrome *c* oxidase
cyt	cytochrome
DHAP	dihydroxyacetone phosphate
ETF-OX	electron-transferring flavoprotein oxidoreductase
$FADH_2$	flavin adenine dinucleotide, reduced form
FBSN	familial bilateral striated necrosis
FMC1	formation of mitochondrial complexes 1
GFP	green fluorescent protein
Glu	glutamate
Gly	glycine
G3P	glycerol-3-phosphate
GTP	guanosine triphosphate
Hsp	heat-schock protein
IF_1, Inh1	inhibitory proteins
IM	inner membrane
IMS	intermembrane space
LHON	Leber's hereditary optic neuropathy
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonical epilepsy and ragged red fibers
mGPDH	mitochondrial glycerophosphate dehydrogenase
MILS	maternally inherited Leigh syndrome
MPP	mitochondrial processing peptidase
mtDNA	mitochondrial deoxyribonucleic acid
NADH	nicotinamide adenine dinucleotide, reduced form
NARP	neurogenic muscle weakness, ataxia and retinitis pigmentosa

ncDNA	nuclear deoxyribonucleic acid
NMR	nuclear magnetic resonance
OM	outer membrane
OSCP	oligomycin sensitivity conferral protein
OXPHOS	oxidative phosphorylation
PAM	presequence translocase-associated motor
Pi	inorganic phosphate
PIC	phosphate carrier
RC	respiratory chain
SHR	spontaneously hypertensive rat
siRNA	short interferening ribonucleic acid
Stf1, Stf2, Stf3	stabilizing factors
THM	transmembrane helix
TIM	translocase of the inner (mitochondrial) membrane
TOM	translocase of the outer (mitochondrial) membrane
UCP	uncoupling protein
Val	valine
$\Delta\mu_H+$	electrochemical proton gradient

1. INTRODUCTION

The F_1F_O-ATP synthase, which Boyer once pertinently called „A Splendid Molecular Machine“, represents the central enzyme of energy conversion in living organisms. It couples H^+ or Na^+ transport across the bacterial plasma membrane, thylakoid or mitochondrial membrane to the synthesis or hydrolysis of ATP. The aerobic organisms synthesise vast majority of ATP in the process of oxidative phosphorylation (OXPHOS). The OXPHOS apparatus resides in the mitochondrial inner membrane and consists of respiratory chain (RC) enzymes and ATP synthase (Figure 1). The RC is represented by a set of oxidoreductases which transport electrons from reduced cofactors to molecular oxygen and simultaneously pump protons from the matrix to the inter-membrane space. The resultant electrochemical proton gradient ($\Delta\mu_H^+$) fuels the formation of ATP from ADP and inorganic phosphate by ATP synthase.

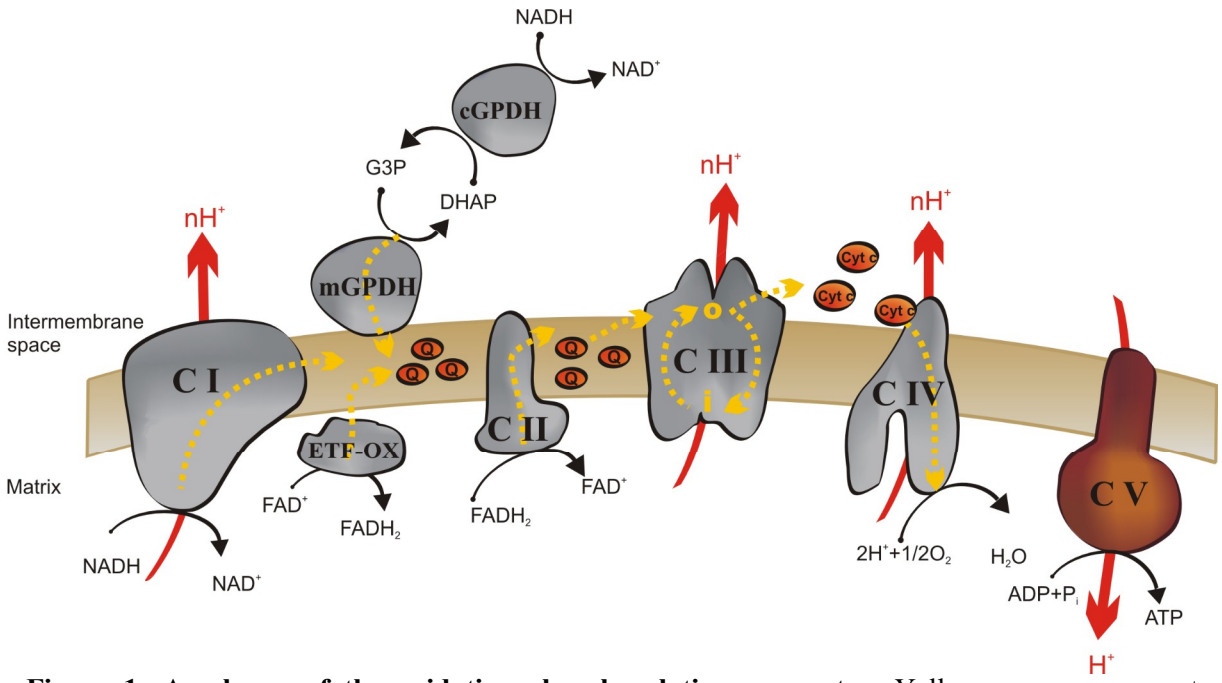

Figure 1: A scheme of the oxidative phosphorylation apparatus. Yellow arrows represent transport of electrons, red arrows the coupled proton transport and the black ones reactions catalysed by the respective enzyme complex. cGPDH/mGPDH – cytosolic/mitochondrial glycerophosphate dehydrogenase; ETF-OX - electron-transferring flavoprotein ubiquinone oxidoreductase; Q – coenzyme Q; cyt *c* - cytochrome *c*; G3P - glycerol-3-phosphate; DHAP - dihydroxyacetone phosphate; CI, II, III and IV - complexes of respiratory chain; CV – ATP synthase. RC complexes are depicted in gray, ATP synthase in redish brown and mobile electron carriers (Q and cyt *c*) in orange. Note that mobile carriers are present in stoichiometric excess to OXPHOS complexes.

The enzyme preserves its fundamental structure, which enables it to fulfil the proper function from prokaryotes to higher eukaryotes. Throughout the course of evolution, the enzyme has acquired additional subunits with putative regulatory roles. Moreover, the ways of its regulation represent a part of the multifarious mechanisms involved in the expression of oxidative phophorylation genes.

1.1. ATP Synthase Structure and Function

The ATP synthase is a multisubunit enzyme, originally described in terms of two sectors based on their biochemical and physical properties: a hydrophilic F_1 part, which can be easily detached from the membrane and still capable of ATP hydrolysis but not ATP synthesis, and a hydrophobic F_O domain possessing the proton-transporting activity. The ATP synthesis is enabled only in case of F_1 coupled to the F_O. Electron microscopy and biochemical studies brought the current view about the structural features of the ATP synthases (reviewed in (Pedersen et al., 2000)). These include a headpiece, a stalk and a basepiece; the tripartite nature of the enzyme arising from the contributions from the 1960s through the 1980s. By the 1990s, other features became evident, such as a peripheral stalk and a cap (Bottcher et al., 1998; Karrasch and Walker, 1999; Wilkens and Capaldi, 1998a; Wilkens and Capaldi, 1998b). The mitochondrial enzyme contains an extra collar above the basepiece (Figure 2, (Karrasch and Walker, 1999)).

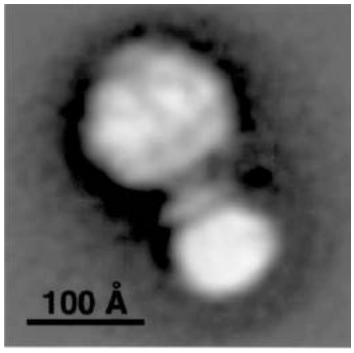

Figure 2: Electron micrograph of bovine ATP synthase. Besides the previously characterised domains F_1, F_O and central stalk, three additional features can be seen: a collar-like structure just above the membrane domain F_O, a stator structure on the right side of the molecule connecting F_1 to the collar structure, and some density on top of F_1 (Karrasch and Walker, 1999).

1.1.1. F_1 Component

The F_1 sector is generally agreed to be made up of five types of subunits in a given stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ (Table I, Figure 3). The crystal structure of bovine heart (Abrahams et al., 1994) and rat liver (Bianchet et al., 1998) F_1 part depicts three α and three β subunits alternatively arranged around the γ subunit. *Escherichia coli* γ subunit extends the full length of the central stalk as documented by the cross-links with the *c* subunit ring (Watts et al., 1995) and interacts also with ε subunit, the equivalent to the mitochondrial subunit δ (Watts et al., 1996). The structure of the mitochondrial inner stalk has recently been obtained by a high-resolution crystallography (Gibbons et al., 2000). All three subunits γδε show extensive interaction in the lower part of the stalk near the membrane domain. The bacterial δ subunit is peripherally located within the second stalk and will be discussed in the following chapter concerning the F_O component.

The activity of mitochondrial enzyme is under the control of a naturally occuring inhibitory protein which inhibits ATP hydrolysis without modification of the synthetic reaction (Pullman and Monroy, 1963). Yeast has two inhibitory peptides Inh1p and Stf1p with different binding affinities (Hashimoto et al., 1990) and two modulators, Stf2p (Yoshida et al., 1990) and Stf3p (Hong and Pedersen, 2002). The mammalian mitochondria contain one protein IF1 (Hong and Pedersen, 2002; Ichikawa et al., 1999; Walker, 1994). Under acidic condition, the mammalian IF1 binds to the ATP synthase in the presence of ATP-Mg^{2+}, the binding is reversible at pH>7.0 (Galante et al., 1981). The functional state of IF1 was proposed to be a dimer (Cabezon et al., 2000).

Table I. Subunits and genes of ATP synthase: comparison among human, yeast and bacterial model organisms.

		H. sapiens		*S. cerevisiae*		*E. coli*	
		subunit	gene	subunit	gene	subunit	gene
F_1	catalysis	α_3	*ATP5A1*	α_3 (1)	*ATP1*	α_3	*uncA*
		β_3	*ATP5B*	β_3 (2)	*ATP2*	β_3	*uncD*
	central stalk	γ	*ATP5C1-2*	γ (3)	*ATP3*	γ	*uncG*
		δ	*ATP5D*	δ	*ATP16*	ε	*uncC*
		ε	*ATP5E*	ε	*ATP15*		
	regulatory protein	IF1	*ATPIF1*	Inh1p	*INH1*		
				Stf1p	*STF1*		
				Stf2p	*STF2*		
				Stf3p	*STF3*		
F_O	proton channel	*a*	*ATP6*[mt]	6	*ATP6/oli2*[mt]	*a*	*uncB*
		$c_{9\text{-}12}$	*ATP5G1-3*	9_{10}	*ATP9/oli1*[mt]	c_{10}	*uncE*
	peripheral stalk	A6L	*ATP8*[mt]	8	*ATP8/aap1*[mt]		
		OSCP	*ATP5O*	OSCP (5)	*ATP5*	δ*	*uncH*
		b	*ATP5F1*	*b* (4)	*ATP4*	b_2	*uncF*
		d	*ATP5H*	*d* (7)	*ATP7*		
		F_6	*ATP5J*	*h*	*ATP14*		
		f	*ATP5J2*	*f*	*ATP17*		
	inter-complex interaction	*e*	*ATP5I*	*e* (Tim11)	*ATP21/TIM11*		
		g	*ATP5L,* *ATP5L2*	*g*	*ATP20*		
				i (*j*)	*ATP18*		
				k	*ATP19*		
	regulatory protein	Factor B (*s*)	*ATP5S*				

The numbers in subscript indicate the subunit stoichiometry. * The *E. coli* δ subunit is a part of the F_1 domain. [mt] Encoded in mtDNA. Data for human ATP synthase come from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene (Maglott et al., 2005). For citations to the yeast and bacterial enzyme see (Ackerman and Tzagoloff, 2005) and (Walker et al., 1984), respectively.

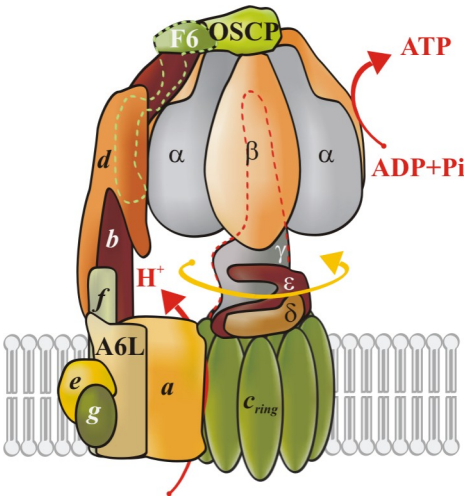

Figure 3: Schematic model of a monomer of mammalian ATP synthase. Red arrows indicate proton translocation through the F_O and ATP synthesis within the $\alpha_3\beta_3$ catalytic unit. Yellow arrow shows the direction of γ subunit rotation.

1.1.2. F_O Component

The composition of the F_O part is more complex and heterogeneous than that of the F_1 domain (Table I, Figure 3). Two hydrophobic proteins, subunits *a* and *c*, form an integral membrane proton channel (ac_n). Electron and atomic force microscopy indicated that subunit *a* as well as subunit *b* abuts the ring of *c* subunits (Birkenhager et al., 1995; Singh et al., 1996). Subunit *a* is known to fold with five transmembrane helices (THMs) (Long et al., 1998; Valiyaveetil and Fillingame, 1998) with THM4 facing subunit *c* (Jiang and Fillingame, 1998). Cross-linking experiments suggest that THM2-5 form a four helix bundle with THM4 at its periphery (Schwem and Fillingame, 2006). *E. coli* subunit *c* spans the membrane as a hairpin of two helices linked by a loop and is packed in a ring with N-termini at the interior and C-termini at the outer side (Girvin et al., 1998; Jones et al., 1998). The number of *c* monomers per ring varies among organisms. 10 monomers were reported for yeast (Stock et al., 1999), termophilic *Bacillus* (Mitome et al., 2004) and most probably also for *E. coli* enzyme (Jiang et al., 2001). Rings in bacterium *Ilyobacter tartaricus* and *Propionigenium modestum* have 11 monomers (Meier et al., 2003). 14 and 15 subunits form *c*-rings in chloroplasts (Seelert et al., 2000) and cyanobacterium *Spirulina platensis* (Pogoryelov et al., 2005), respectively.

A second, outer stalk connects the globular and membrane-embeded portions. The *E. coli* stalk is composed of subunit δ of the F_1 part and two copies of subunit *b* of the F_O

part, while photosynthetic prokaryotes and chloroplasts have instead of subunit *b*-homodimer two different subunits *b* and *b*' (or subunits I and II in chloroplast nomenclature) (Dunn et al., 2000). The δ subunit was cross-linked with the α subunit near the top of the hexagon (Ogilvie et al., 1997). Subunit *b* interacts either with both the α and δ and runs along the full length of the ATP synthase from the top to the membrane (Rodgers and Capaldi, 1998; Rodgers et al., 1997) where it interacts with subunit *a* (Stalz et al., 2003).

The second stalks of mitochondrial ATP synthases are more complex, containing in addition to one subunit *b* and the mitochondrial analog of the prokaryotic δ subunit, called oligomycin sensitivity conferral protein (OSCP), single copies of subunits *d* and F_6 (*h* in yeast nomenclature) (Bateson et al., 1999; Collinson et al., 1996). In a similar vain to the *E. coli* δ subunit, the N-terminal segment of OSCP has been found on the top of the F_1 component in interaction with the N-terminal regions of α subunits (Hundal et al., 1983; Joshi et al., 1996). The C-terminus of OSCP contacts C-terminal domain of subunit *b* (Joshi et al., 1996). The N-terminal region of mitochondrial subunit *b* is probably anchored in the membrane via two α-helices (Walker et al., 1987) in contrast to single transmembrane span of the bacterial counterpart (Dunn et al., 2000). The C-terminus of the yeast ATP synthase subunit *h* points towards the membrane domain (Rubinstein et al., 2005). The understanding of the stator structure has improved by solution of NMR structures of bovine F_6 (Carbajo et al., 2004) and N-terminal portion of OSCP (Carbajo et al., 2005). Recent crystallographic work of a subcomplex of subunits *b, d* and F_6 fragments has confirmed the previous results and improved the present-day knowledge about inter-subunit contacts within the stator stalk (Dickson et al., 2006).

Subunits A6L (yeast subunit 8), *e*, *f* and *g* are components of mitochondrial enzymes and do not have bacterial counterparts, subunits *i* (*j*) and *k* are unique to the yeast ATP synthase. All of these subunits are transmembrane proteins. The A6L or subunit 8 maintains close interactions with subunits *b*, *d*, *f* and *a* (6) (Belogrudov et al., 1995; Belogrudov et al., 1996; Stephens et al., 2003). Cross-links have further been obtained between subunits *b* and *f* (Spannagel et al., 1998b). Subunits *d*, *f*, and 8 have been proposed to fulfill the role of the second bacterial subunit *b* in forming the connection between F_1 and F_0 in yeast ATP synthase (Stephens et al., 2003). In addition, subunit *f* is found in close proximity to subunit *g* (Belogrudov et al., 1996) which also joins subunit *e* (Belogrudov et al., 1996).Yeast subunit *i* (*j*) is located at the periphery of the enzyme and interacts with subunits 6, *f*, *g*, and *d* (Paumard et al., 2000). Subunit *k* has been detected in peripheral

association with inner mitochondrial membrane in yeast, facing the intermembrane space (Arnold et al., 1998).

Factor B (designated previously as subunit *s*) is a peripheral membrane protein required for the coupled activity of the ATP synthase complex and appears to be another regulatory component of the mammalian ATP synthase complex (Belogrudov, 2002; Belogrudov, 2006).

1.1.3. Energy Coupling Mechanism

The electrochemical gradient of protons or sodium ions across the membrane represents a direct energy source for ATP synthesis. The ion channel (ac_n) converts this gradient into subunit rotation (*c*-ring plus central stalk subunits). The mechanical energy of rotation is further converted into chemical bond between ADP and Pi. Either ATP synthesis or hydrolysis occurs at three catalytic sites located primarily on the β subunits at the α/β interfaces (Abrahams et al., 1994; Cross and Nalin, 1982).

Two models have been proposed for ion translocation in the *a*/*c* subunit interface (Dimroth et al., 2006). One for H^+-translocating ATP synthases and the other for Na^+-translocating enzymes. In the mode of ATP synthesis, a specific binding site on the *c* ring captures a coupling ion from the side with high electrochemical potential and releases it to the side with low electrochemical potential. The negative charge of the *c*-subunit binding site after ion release (*E. coli*: *c*D61, *P. modestum*: *c*E65) is shielded by the conserved stator arginine of subunit *a* (*E. coli*: *a*R210, *P. modestum*: *a*R227). As ion binding and release are acomplished at defined positions at the *a*/*c* interface, ion translocation is intimately connected to the rotation of the *c* ring. The two motors differ in the ion release pathway and the energy source that is responsible for unidirectional rotation. While proton entrance and exit are located in a noncoaxial manner in subunit *a*, Na^+ outlet channel is present in the *c* ring. The direction of rotation is determined by pH gradient in H^+ motor or horizontal component of the membrane potential in Na^+ motor.

It was observed directly that F_1 synthesises or hydrolyses three molecules of ATP per revolution, and that direction of rotation during synthesis (clockwise as viewed from the membrane) is opposing to that of hydrolysis (Rondelez et al., 2005). Rotation of the γ subunit during ATP hydrolysis occurs in 120° steps, separated by pauses, most visible at low ATP concentration, during which the enzyme waits for ATP to bind. Each step is further split into 90° and 30° substeps separated by a stationary interval (Yasuda et al., 2001), however, these substeps were revised to be 80° and 40° (Shimabukuro et al., 2003).

Coupling between the rotors of F_1 and F_O was demonstrated by the rotation of the *c* ring during ATP hydrolysis by F_1F_O (Sambongi et al., 1999). As the number of subunit *c* within the ring determines the number of ions transported across the membrane during one cycle, the $H^+(Na^+)$/ATP ratio changes in response to the size of the *c*-ring.

The rotary movement of the central stalk is coupled to sequential changes in the conformation of the catalytic sites, leading to different binding affinities for nucleotides (Boyer, 1997). Binding of substrates and release of products are believed to be energy-requiring processes. According to the model known as "binding change mechanism", three sites go through identical conformations but at any one time all are in different conformation (Boyer, 1997). Formation of ATP is catalysed at the tight site on condition that ADP and Pi are present at a site with low affinity for ATP. ATP is then released from a third site or must be present at this site for hydrolysis of another ATP at the tight site. Therefore, occupancy of two sites is regarded as prerequisite for activation of catalysis.

In contrast to Boyer, other models require occupancy of all three sites for optimal catalysis (Allison, 1998; Menz et al., 2001; Senior et al., 2002). The above models were originally proposed for ATP hydrolysis. Molecular dynamics simulation and kinetic modelling were used in combination with experimental structural, thermodynamic and kinetic data for creating a model for both ATP synthesis and hydrolysis (Gao et al., 2005) whereby synthesis is not the exact opposite of hydrolysis (Senior et al., 2002). The synthesis begins with γ subunit rotation of 30° (40°) changing the open β_E to the "half-closed" β_{HC} (Figure 4). Binding of Pi and ADP to the β_{HC} site occurs prior to 90° (80°) rotation that changes conformation of the catalytic sites. ATP is synthesised during this step with subsequent release from the β_E. Thus, ATP formation is dependent upon energy input in the form of rotation. ATP as a substrate enters the β_E site and the hydrolysis products are released from the β_{HC}. Hydrolytic reaction occurs during transformation of the β_{TP} (a strong binding site for ATP and H_2O) to the β_{DP} (a strong binding site for ADP and Pi). Intermediate states favouring ATP or ADP/Pi are not part of the presented scheme but are included in thermodynamic evaluations (see Figure 3 in (Gao et al., 2005)).

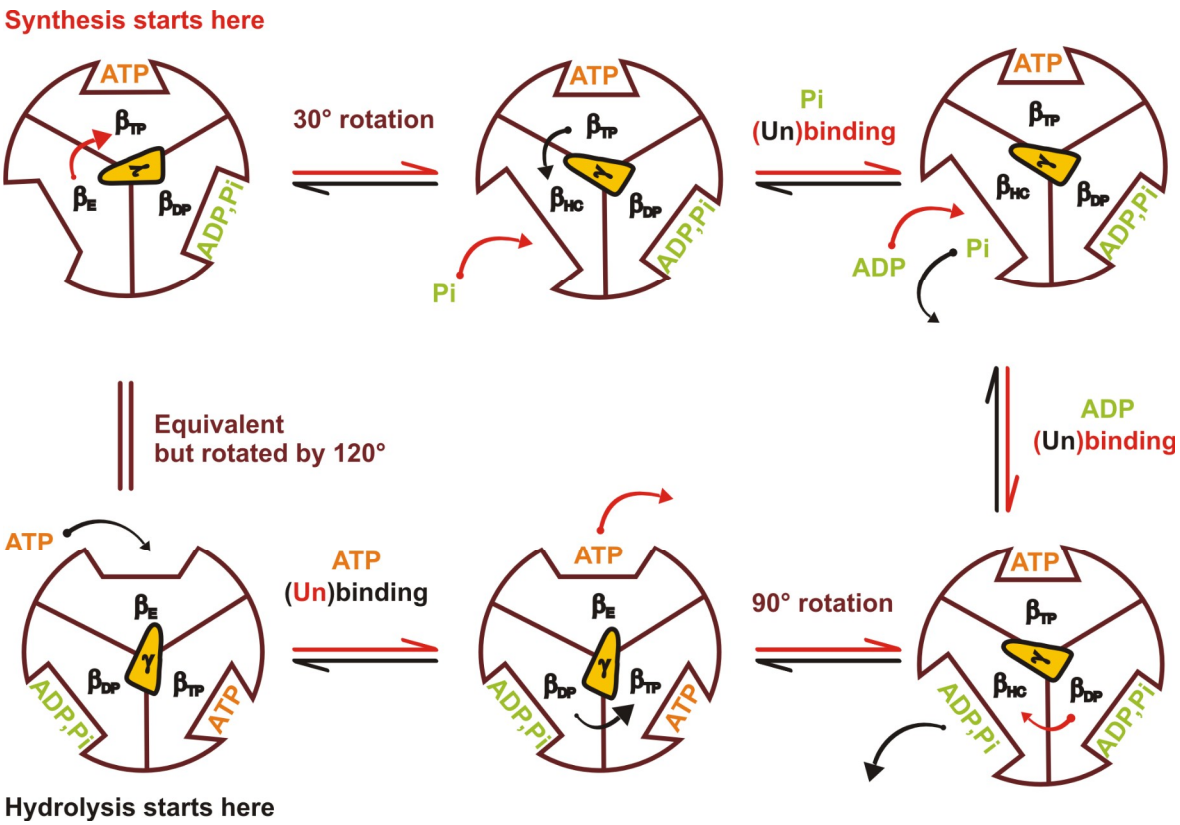

Figure 4: Illustration of ATP synthesis and hydrolysis by F_1. The diagram shows a 120° rotational step of the γ subunit, which corresponds to the synthesis or hydrolysis of one ATP molecule. β_{DP} (a strong binding site for ADP and Pi), β_{TP} (a strong binding site for ATP and H_2O), β_{HC} (half-closed) and β_E (open) mark different conformations of the catalytic sites, which are further depicted in various shapes. ATP is in orange. ADP and Pi are in green. The sequence of ATP synthesis is indicated by red arrows. Black arrows delineate the direction of ATP hydrolysis. Adapted from (Gao et al., 2005).

1.2. ATP Synthase Gene Expression

1.2.1. Gene Organisation

Genes for *E. coli* enzyme are part of a polycistronic *unc* operon and encode subunits in the order of *a*,*c*,*b* of F_O and δ, α, γ, β and ε of F_1 (Walker et al., 1984). Expression of the individual genes is regulated post-transcriptionally by the control of efficiency of translational initiation (McCarthy, 1990) and by mRNA degradation (McCarthy et al., 1991). Contrary to the prokaryotic ATP synthase, the mitochondrial enzyme is encoded in two separate genomes (Attardi and Schatz, 1988). Some of the F_O subunits are expressed from the mitochondrial genome (mtDNA) but majority of the ATP synthase proteins are products of nuclear genes (Table I.). Genes for subunits *a* (6) and A6L (8) are mostly part of the mtDNA whereas *Chlamydomonas reinhardtii* possesses nuclearly-encoded subunits *a* (Funes et al., 2002). Genes for subunit *c* (9) are in the

nucleus of Metazoa (De Grassi et al., 2006) and in mtDNA of some protists (Gray et al., 1998), yeast (Groth et al., 2000) and plant species (Bartoszewski et al., 2004; Dellorto et al., 1993; Dewey et al., 1985; Sugiyama et al., 2005).

1.2.2. Nuclear Genes

The nucleus accomodates structural genes that encode either subunits essential for catalytic activity or components dispensable for synthesis or hydrolysis of ATP including regulatory proteins (Table I.). Genes encoding subunits *e*, *g* and *k* belong to the latter category due to the fact that null yeast mutants remain respiratory competent (Arnold et al., 1998). Other genes that are not part of the mature enzyme define accessory genes that assist different steps in the biogenesis from the coordinate expression of ATP synthase genes through the import of cytosolic precursors into the mitochodria and consequent assembly pathway. A subset of mammalian genes encoding ATP synthase subunits is expressed in tissue-specific manner.

Three separate genes (*ATP5G1-3* or *P1-3*) have been described to code for the subunit *c* of the proton channel in human (Dyer and Walker, 1993; Higuti et al., 1993; Yan et al., 1994), bovine (Dyer et al., 1989; Gay and Walker, 1985), sheep (Medd et al., 1993), mouse (Piko et al., 1994) and rat (Higuti et al., 1993; Li et al., 2001). The genes differ in noncoding regions and regions specifying protein import presequences, however, the resultant mature proteins are identical. The amount of transcripts varies among bovine (Gay and Walker, 1985), rodent (Houstek et al., 1995a; Sangawa et al., 1997) and human (Johnson et al., 1995) tissues and is differentially regulated (Andersson et al., 1997; Houstek et al., 1995a; Sangawa et al., 1997).

Heart and skeletal muscle-specific isoforms of γ subunit are generated by alternative splicing in human, bovine and mouse tissues (Ichida et al., 1998; Matsuda et al., 1993a; Matsuda et al., 1993b). The longer liver-type isoform is expressed in other tissues.

Bioinformatic approach identified possible isoforms of subunit *g* in some metazoan species (Hong and Pedersen, 2004).

1.2.3. Import into Mitochondria

Majority of mitochondrial proteins, with the exception of those encoded by the mitochondrial genome, are synthesised in the cytosolic ribosomes and are transported into their final destination within the organelle in a post-translational or a co-translational manner (Neupert, 1997). The import is initiated by a multiprotein translocase of the outer

membrane, the TOM complex. After that the import routes of the precursors depend on individual or multiple targeting signals within their primary structure.

Precursors with a positively charged amphipathic α-helix at their N-terminus are typically destined for the matrix (Abe et al., 2000). Passing the TOM complex, the preproteins are directed to the translocase of the inner membrane (TIM23 complex) in a membrane potential-dependent manner (Bauer et al., 2000). The ATP-driven presequence translocase-associated motor (PAM) is required for the completion of protein translocation into the matrix and contains the mitochondrial heat-shock protein mtHsp70 as a key component (Bauer et al., 2000). Finally, the mitochondrial processing peptidase (MPP) removes the presequence.

Some proteins of the inner membrane are also synthesised with a presequence. Those precursor proteins use the presequence import pathway with the TOM complex and the TIM23 complex and are guided into the inner membrane (IM) by a hydrophobic sorting sequence that typically follows the positively charged presequence (Stuart, 2002). A group of proteins is completely transported into the matrix from where it is inserted into the IM by Oxa1 translocase or by an Oxa1-independent machinery, both of which interact also with mitochondrial translation products during IM insertion (Stuart, 2002). Polytopic IM proteins such as carrier proteins contain internal cryptic signals and are guided by small Tim proteins through the intermembrane space (IMS) to the membrane-integrated carrier translocase of the inner membrane (TIM22 complex) (Bauer et al., 2000).

Most IMS proteins, e.g. small Tim and Cox17, contain characteristic cysteine motifs. Two essential mitochondrial proteins Erv1 and Mia40 cooperate in the import of these proteins (Mesecke et al., 2005). Mia40 in the oxidised state interacts in a covalent fashion with incoming polypeptides forming mixed disulfides. Oxidation of Mia40 is catalysed by the sulfhydryl oxidase Erv1.

The import of outer membrane (OM) proteins with a single α-helical transmembrane segment can be directly aided by the TOM complex, however, transport of β-barrel proteins such as Tom40 and porin, requires additional steps (Pfanner et al., 2004). The β-barrel proteins are translocated through the TOM complex to the IMS where small Tim proteins guide the precursor proteins to the sorting and assembly machinery (SAM complex) of the OM, which completes their insertion into the OM (Pfanner et al., 2004).

1.3. ATP synthase Assembly

After the proper import into the mitochondria, proteins that are due to become part of the ATP synthase complex are assembled in a step-by-step process which, at least in eukaryotic organisms, is aided by a substrate-specific chaperones. Nevertheless, despite receiving much attention, the process is not well understood and still remains an arduous task.

1.3.1. Consecutive Assembly of the Enzyme Subunits

Studies on Bacterial Enzyme

Escherichia coli served the model organism in many studies. It was shown that F_1 portion containing only the α, β and γ subunits was capable of ATP hydrolysis (Dunn and Futai, 1980; Futai et al., 1980; Sternweis, 1978), however, assembly of all five F_1 subunits were required prior to the reconstitution of F_1-depleted membranes *in vitro* (Dunn and Futai, 1980; Sternweis, 1978) and *in vivo* (Klionsky and Simoni, 1985).

Although not representing the initial step, incorporation of the α and β subunits was proposed to be an essential prerequisite to the formation of a functional F_O part (Cox et al., 1981; Klionsky et al., 1983). Alternatively, independent formation of the F_1 and F_O domains was suggested after the expression of F_1 (Klionsky and Simoni, 1985) and F_O genes from multicopy (Aris et al., 1985; Fillingame et al., 1986) or single-copy plasmids (Brusilow and Monticello, 1994). Nevertheless, F_1 was shown to affect both the function of the assembled F_O component (Brusilow, 1987; Brusilow and Monticello, 1994; Pati and Brusilow, 1989; Pati et al., 1991) and the number of membrane-embedded F_O subunits (Brusilow and Monticello, 1994).

A missense mutation in subunit *b* resulted in the absence of subunit *a* in the bacterial complex (Vik and Simoni, 1987). The interdependence of membrane insertion of the F_O subunits was studied using a number of chromosomal mutants. Subunit *b* and *c* were found to insert into the membrane independently of the other F_O subunits while subunit *a* was not incorporated into membranes that lacked either subunit *b* or subunit *c* (Hermolin and Fillingame, 1995).

Taken together, these studies imply that independent formation of the F_1 and F_O sectors is plausible but assembly of the fully functional proton channel requires the expression of F_1 genes.

Studies on Yeast Enzyme

The assembly of the eukaryotic ATP synthase has received no less attention. Assembled F_1 was characterised by a feeble binding to the inner membrane in *Saccharomyces cerevisiae* mitochondria isolated from the cytoplasmic *petite* mutant or from the cells treated with chloramphenicol, an inhibitor of mitochondrial protein synthesis (Schatz, 1968). On the other hand, the assembly of a functional proton channel of the F_O portion was not observed in mitochondria lacking the β subunit (Takeda et al., 1985).

Ordered assembly of the yeast F_O sector was demonstrated from the investigation of mutants for the mitochondrially-encoded subunits. If a subunit was part of the enzyme complex was judged based on its recovery in the immunoprecipitate with a monoclonal antibody to the β subunit. Thus, a principal role of subunit 9 (*c*) was suggested as in its absence subunits 6 and 8 (A6L) were not associated with the enzyme complex. This proposed a sequential addition of mitochondrially-synthesised subunits that is initiated with the subunit 9 association with the F_1 subcomplex. The following step includes assembly of subunit 8. Subunit 6 appeared to be added as the last mitochondrially-synthesised protein (Hadikusumo et al., 1988). Besides, subunits 6 could not be detected in cells devoid of any of the stator-stalk subunits *b* (Paul et al., 1989), subunit *d* (Norais et al., 1991), OSCP (Prescott et al., 1994), subunit *h* (Arselin et al., 1996), subunit *i/j* (Arnold et al., 1999) and subunit *f* (Spannagel et al., 1997). The assembly of the peripheral stalk subunits *b*, *d* and OSCP was studied after depletion of each subunit expressed from a plasmid under the control of *GAL1* promoter. Such subunits were synthesised during yeast growth in the presence of galactose and subsequent depletion achieved when galactose was omitted. Suppose assembly renders the subunit stability, the subunit will be detectable by immunoblot; otherwise the subunit undergoes degradation and cannot be visualised. Following the experiments, the subunits have been proposed to assemble in the order of *b*, OSCP and subunit *d* (Straffon et al., 1998).

Assembly Intermediates in Human Cells

Electrophoretic assays of subcomplexes of the human ATP synthase strengthened the concept of successive assembly of the enzyme together with the belated incorporation of mitochondrially-encoded subunits. Such conclusions have been inferred mainly from studies on three model situations: cells with mutated subunits *a* due to a point T8993G (Carrozzo et al., 2006; Houstek et al., 1995b; Nijtmans et al., 2001) or T9176G mutation (Carrozzo et al., 2006); cells incapable of mitochondrial translation due to its arrest by

doxycycline (Nijtmans et al., 1995) or absence of mtDNA (Rho^0 cells) (Carrozzo et al., 2006; Garcia et al., 2000); and cells carrying a 2 bp TA deletion (9205ΔTA) in a STOP codon region of the *ATP6* gene, thus displaying diminished synthesis of subunits *a* (Jesina et al., 2004).

Besides minor discrepancies among the published results, F_1 subunits and F_1 plus a ring of subunits *c* represented the two predominant intermediates. Another subcomplex of F_1 with bound IF_1 inhibitor protein has been detected in case of *ATP6* mutations (Carrozzo et al., 2006; Nijtmans et al., 2001) and Rho^0 cells (Carrozzo et al., 2006). However, IF_1 was hardly seen in another study with Rho^0 mitochondrial membranes (Garcia et al., 2000).

Nijtmans and collaborators hypothesized that the newly formed F_1 is attached to the ring of *c* subunits, which is most probably followed by the addition of the outer stalk components (such as subunits *b*, OSCP and F_6) among other nuclearly-encoded subunits. The completion of the membraneous part is achieved after the incorporation of mitochondrially-encoded subunits *a* and A6L ((Nijtmans et al., 1995), Figure 5). Nevertheless, all these analyses of assembly intermediates have identified only a limited number of F_O subunits (subunit *a*, *b*, *c*, *d* and OSCP) and further immunochemical and proteomic studies are needed for better characterisation of their precise composition.

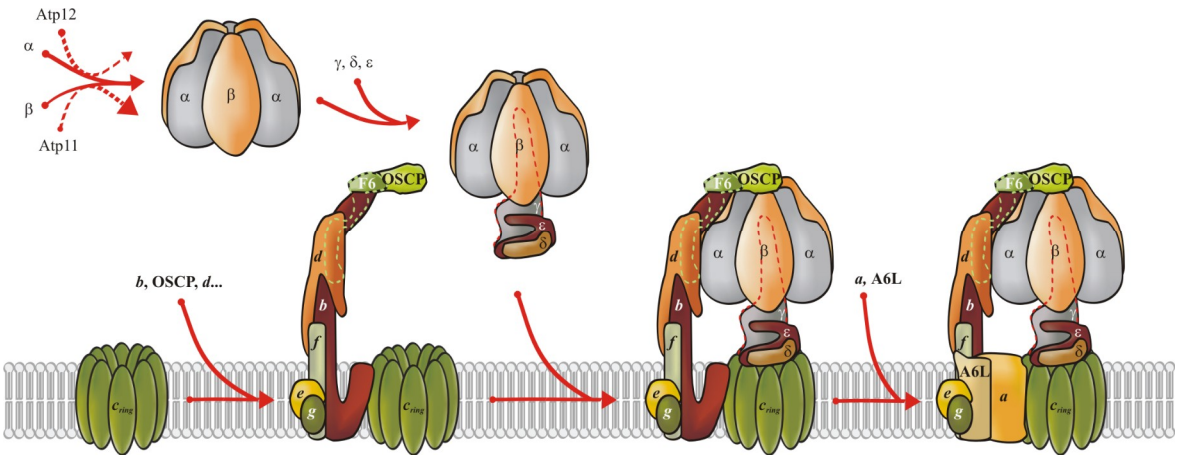

Figure 5: Presumed assembly of the mammalian ATP synthase. Formation of the monomeric enzyme is achieved in several successive steps under the assistance of specific chaperones, Atp11 and Atp12, which are not part of the final complex (see chapter 1.3.2. and 1.3.3.). MtDNA-encoded subunits, *a* and A6L, seem to complete the assembly process.

1.3.2. Molecular Chaperones Specific for the F_1 Assembly

Screens of yeast nuclear-deficient *pet* mutants disclosed the requirement of additional proteins for the F_1 assembly. Phenotypically, mitochondria of these strains displayed severely reduced ATPase activity, consistent with a defect in the F_1 component. The phenotypes were inferred to stem from mutations in genes named *ATP11* and *ATP12* whose products, Atp11p and Atp12p, were excluded from being subunits of the ATP synthase complex (Ackerman and Tzagoloff, 1990b). Furthermore, mitochondria of *atp11* and *atp12* mutants contained near wild-type levels of the mature-size F_1 α and β subunits that, however, accumulated as large aggregates in the matrix compartment. Such altered physical properties hampered the incorporation of the α and β subunits into the enzyme complex and scored positive for a chaperone-type function of Atp11p and Atp12p during the assembly process (Ackerman and Tzagoloff, 1990b). It is noteworthy that absence of the α subunit elicits aggregation of the β subunit and *vice versa* (Ackerman and Tzagoloff, 1990b). The αβ oligomers might also exist due to a deletion in the gene for γ subunit (Paul et al., 1994). Instead, deletions in the genes for δ (Giraud and Velours, 1994) or ε (Guelin et al., 1993) subunits of F_1 leave the α and β subunits in soluble forms.

Deletion of another gene, *FMC1*, leads to aggregation of α and β subunits (Lefebvre-Legendre et al., 2001). However, this phenotype, marked also by a reduced level of Atp12p, is observed at elevated growth temperature (37 °C) and can be cured by overexpression of Atp12p (Lefebvre-Legendre et al., 2001). Although Fmc1p seems to be required for assembly/stability of the yeast ATP synthase at heat stress conditions, its precise function is still unknown.

Substrate Specificity of Atp11p and Atp12p

Atp11p and Atp12p are considered specific-type chaperones that act downstream of the general-type chaperones such as Hsp70p and Hsp60p for the following reasons: deletion of *ATP11* and *ATP12* renders the yeast cells incapable of growth on nonfermentable carbon sources but does not cause lethality (Ackerman and Tzagoloff, 1990b); *atp11* and *atp12* mutants accumulate fully processed proteins, primarily the α and β subunits (Ackerman and Tzagoloff, 1990b), and not precursors that aggregate together with the mature proteins in the case of *hsp70* (Kang et al., 1990) and *hsp60* mutants (Cheng et al., 1989).

The substrate specificity of Atp11p and Atp12p has been further determined by affinity tag precipitation and yeast two-hybrid screen. Under condition in which avidin-

Sepharose beads adsorbed biotinylated form of Atp11p from yeast mitochondrial extracts, the β subunit co-precipitated with the tagged Atp11p (Wang and Ackerman, 2000). In similar experiments, the α subunit was identified as the target of Atp12p as it co-purified selectively with the biotinylated Atp12p from affinity resins (Wang et al., 2000). Yeast two-hybrid screens confirmed the association of Atp11p with the β subunit (Wang and Ackerman, 2000) and Atp12p with the α subunit (Wang et al., 2000). This approach also identified a region of about 200 amino acids in the nucleotide binding domain for each F_1 subunit to which the chaperone binds.

Calculation of interaction energies between the α and β subunits in the $\alpha_3\beta_3$ hexamer suggested that interaction of the Atp12-binding domain of the α subunit with the β subunit will form the noncatalytic site. Atp11p, on the other hand, tends to bind to the β subunit at the surface that contributes to the catalytic site with the adjacent α subunit (Wang et al., 2000). Thus, it was hypothesised that assembly of the $\alpha_3\beta_3$ hexamer results from the sequence of interactions in which the chaperones shielding the unassembled subunits are replaced by the neighbouring subunit. Namely, Atp11p structurally mimics the α subunit when bound to the unassembled β subunit and Atp12p provides the free α subunit a structural substitution of the β subunit (Figure 6).

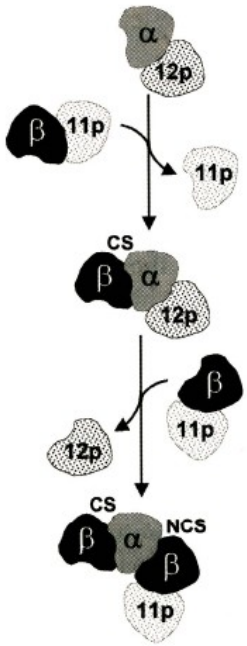

Figure 6: Model of the Atp11p and Atp12p action. Atp11p and Atp12p portray structural mimics of the α and β subunits, respectively, that are displaced during the sequence of interactions leading to the $\alpha_3\beta_3$ hexamer. CS - catalytic site; NCS – noncatalytic site (Ackerman and Tzagoloff, 2005).

Structural and Physical Properties of Atp11p and Atp12p

The primary translation products of *ATP11* (Ackerman et al., 1992) and *ATP12* (Bowman et al., 1991) genes are transported via N-terminal leader sequence to the protease-protected compartment of mitochondria in a membrane potential-dependent manner. The N-termini of mature Atp11p (cca 32 kDa) and Atp12p (cca 33 kDa) are predicted to begin with Glu-40 and Gly-31, respectively. Deletion studies were used to map the active domain of Atp11p between Asp-112 and Arg-183 (Wang and Ackerman, 1996). The functional domain of Atp12p lies within the region between Asn-181 and Val-306 (Wang and Ackerman, 1998).

The C-terminal part of Atp12p were assigned to a heterooligomerisation domain (Wang and Ackerman, 1998). The composition of the heterooligomer is not known. It might consist of Atp12p and the α subunit (Wang et al., 2000) or Atp12p may associate with another protein in the presence of the α subunit (Ackerman and Tzagoloff, 2005). On the other hand, Atp11p behaved as a monomer during sedimentation analysis of mitochondrial crude extracts (White and Ackerman, 1995).

Orthologues of Atp11p and Atp12p

Proteins homologous to Atp11p (*Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Drosophila yakuba* and *Homo sapiens*; (Wang et al., 1999; Wang et al., 2001)) and Atp12p (*Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Drosophila yakuba*, *Homo sapiens, Caenorhabditis elegans and Rhodobacter capsulatus*; (Wang et al., 2001)) suggest that both proteins can be involved in assembly of ATP synthases from variety of organisms. Approvingly, results from complementation analyses and/or two-hybrid screens showed that products of *D. yakuba* gene 2A5 (Wang et al., 1999) and *H. sapiens* genes *ATPAF1* and *ATPAF2* (Wang et al., 2001) are functional counterparts of yeast factors. Based on a sequence similarity, we further showed that both proteins are present in majority of eukaryotic lineages, which corresponds to the conservation of catalytic $\alpha_3\beta_3$ hexamer (Pickova et al., 2005).

1.3.3. Molecular Chaperones Specific for the F_O Assembly

Mutations in a yeast nuclear gene *ATP10* are responsible for very poor inhibition of ATPase by rutamycin or oligomycin due to incomplete assembly of F_O (Ackerman and Tzagoloff, 1990a; Paul et al., 2000). Partial recovery of wild-type phenotype by a mutation

in the *ATP6* gene implied functional interaction between the subunit 6 (*a*) and Atp10p (Paul et al., 2000). This mutation lay within the C-terminus, which together with the presence of mature subunit 6 and the results of mass measurements opposed the involvement of Atp10p in post-translational modification (Paul et al., 2000). Atp10p is rather considered the subunit 6 chaperone. This is supported by cross-links between Atp10p and newly synthesised subunit 6 (Paul et al., 2000). Furthermore, pulse labelling and chase of mitochondrial translation products *in vivo* in *atp10* mutant diclosed the presence of only one subcomplex, most probably referring to the subunit *9* (*c*) oligomer (Paul et al., 2000).

Product of another nuclear gene *ATP23* has recently been identified to exert dual function during assembly of the yeast enzyme (Osman et al., 2006; Zeng et al., 2006). It mediates the maturation of a newly synthesised subunit 6 precursor and, independent of its proteolytic activity, promotes incorporation of the mature subunit 6 into the F_O channel. Atp23p affects assembly of the ATP synthase in similar step as Atp10p for the following reasons: overexpression of *ATP23* suppress the phenotype of *atp10* mutant (Zeng et al., 2006) and the absence of Atp10p or Atp23p leads to accumulation of intermediate assemblies containing at least subunits α and 9 (Osman et al., 2006). Nevertheless, while Atp10p is an inner membrane protein exposing a domain to the matrix (Paul et al., 2000), Atp23p seems to be associated with the inner membrane with its C-terminus facing the intermembrane space (Osman et al., 2006; Zeng et al., 2006). Thus, the topology of Atp10p and Atp23p suggests that subunit 6 precursor interacts first with Atp10p before it is bound by Atp23p. This scenario does not seem to be valid in general because the distribution of Atp10 (Pickova et al., 2005) does not correspond to the presence of Atp23p orthologues (Osman et al., 2006; Zeng et al., 2006). Interestingly, mammalian orthologue of subunit 6 is not synthesised with as a precursor with cleavable N-terminal extension. Therefore, Atp23 could function only in assembly process but not as subunit 6 (subunit *a*) maturase in mammalian mitochondria.

Function of Atp22p has also been connected with a post-translational stage in F_O assembly (Helfenbein et al., 2003). Localisation study indicated that Atp22p is an intrinsic component of mitochondrial inner membrane facing its matrix side (Helfenbein et al., 2003).

1.4. Supramolecular Organisation of ATP Synthase

Interaction of ATP synthase with mitochondrial translocases favours tight coordination between the final steps in oxidative phosphorylation. Individual ATP synthases are also in physical contacts with each other in the mitochondrial inner membrane and there is mounting evidence that the supramolecular structures of ATP synthase are involved in maintaining the integrity of the mitochondrial cristae, which traverse the interior of the organelle.

1.4.1. Phosphorylating Assembly

Soon after the isolation of ATP synthase in a supercomplex with both adenine nucleotide translocase (ANT) and phosphate carrier (PIC) (Ko et al., 2003), three-dimensional structure of the so-called "ATP synthasome" (ATP synthase, ANT and PIC in a ratio of 1:1:1) was determined at a resolution of 23 Å (Chen et al., 2004). Near location of the proteins has been hoped-for since both carriers replenish ATP synthase with its substrates, ADP and inorganic phospate, and ANT delivers ATP to the cytosol to energise numerous metabolic processes. Sure enough, ANT had been a frequent contaminant in ATP synthase preparations, e.g. (Freisleben and Zimmer, 1986; Kiehl, 1980). Recent high-resolution crystallography data showed a monomeric bovine ANT in the presence of its inhibitor carboxyatractyloside (CATR) (Pebay-Peyroula et al., 2003). This was puzzling since the calculated binding stoichiometry is 1CATR:2ANT (Block et al., 1982; Hackenberg and Klingenberg, 1980; Klingenberg et al., 1978; Riccio et al., 1975). Dimerisation was also mandatory for PIC activity in reconstitution experiments (Schroers et al., 1998). A group studying covalent tandem dimers of wild type and inactive ANT together with ANT-PIC chimeras confirmed the former proposals of a dimer as a functional unit of both ANT and PIC (Postis et al., 2005). Additionally, a recent crystalographic work presents interactions between two monomers mediated by endogenous cardiolipins (Nury et al., 2005) where the protein-protein interface finds support in several other experimental data (see Refs in (Nury et al., 2005)). Thus, although we are still not absolutely convinced about the oligomerisation state of ANT, most of the data are in favour of the dimeric nature of the translocase. Besides the ATP synthasome, ATP synthase, ANT and PIC together with the mitochondrial ATP-binding cassette protein 1 have been found to associate with succinate dehydrogenase to form a macromolecular complex that confers mitochondrial ATP-sensitive K^+ channel activity (Ardehali et al., 2004). As there are two or three diferentially expressed isoformes of ANT

in mammalian mitochondria (Dorner et al., 1999; Levy et al., 2000; Stepien et al., 1992), it would be interesting to know which of them constitute these supercomplexes.

1.4.2. Oligomerisation of ATP synthase

Contrary to the ATP synthase complexes with ANT and PIC, much has been resolved in the field of multimeric forms of the ATP synthase itself. The inspection of the mitochondrial inner membrane by electron tomography revealed two contiguous but distinct components, the inner boundary membrane abutted on the outer membrane and the tubule- or lamellae-shaped cristae membrane that projects into the mitochondrial interior (Frey et al., 2002). Recent immunogold labelling experiments showed a more than a 2-fold enrichment of complexes III and V in the cristae versus the inner boundary membrane of bovine heart mitochondria (Gilkerson et al., 2003). Based on electron microscopy observations of *Paramecium multimicronucleatum* mitochondria, the ATP synthases are arranged in a non-random fashion interacting with other protein complexes, and the number of such clusters was put into relation with cristae morphology (Allen et al., 1989). In particular, the F_1 particles were seen to lie as a zipper in a twin row along the full length of the helical-shaped tubular cristae. Allen proposed that F_1F_O dimers could induce a distorsion of planar inner membrane to form tubules upon linking with adjacent complexes (Allen, 1995).

Soon after that mitochondrial ATP synthase dimers were recovered from yeast mitochondria by a mild detergent lysis with subsequent analysis by blue native polyacrylamide gel electrophoresis (Arnold et al., 1998; Schagger and Pfeiffer, 2000). The dimeric enzyme contained three additional subunits (subunit *e*, *g* and *k*) associated with the F_O sector. The dimeric ATP synthases could not be isolated from mitochondria lacking either the *e* or *g* subunit, suggesting their essentiality in the dimerisation (Arnold et al., 1998). Similar procedure has led to the identification of ATP synthase dimers from bovine heart mitochondria (Schagger and Pfeiffer, 2000), mitochondria and chloroplast of algae *Chlamydomonas reinhardtii* (Rexroth et al., 2004; van Lis et al., 2003) and *Arabidopsis thaliana* mitochondria (Eubel et al., 2004). Correspondingly, isolation of dimers and even higher oligomers were seen in yeast (Paumard et al., 2002b). Both blue native and colourless native electrophoresis showed presence of V_1-V_4 complexes in mitochondria isolated from five different bovine and rat tissues (Krause et al., 2005). Disulfide bridges formation between two subunits *b* (Paumard et al., 2002b; Spannagel et al., 1998a) or two subunits *h* (Fronzes et al., 2003; Fronzes et al., 2006) and cross-linking experiments

between two subunits *i* (Giraud et al., 2002; Paumard et al., 2002a) provided another evidence of inter-enzyme interactions. ATP synthase associations in respiring cells were observed by fluorescence resonance energy transfer between subunit *b*-BFP donor and *b*-GFP acceptor (Gavin et al., 2005). Finally, structure of the dimeric ATP synthase from bovine heart was resolved by transmission electron microscopy (Minauro-Sanmiguel et al., 2005). In parallel, structural analysis of ATP synthase dimers was achieved after their purification from non-green alga *Polytomella* (Dudkina et al., 2005).

1.4.3. Oligomeric ATP Synthase and Mitochondrial Biogenesis

The physiological role of the ATP synthase oligomerisation is still a matter of debate. Dimerisation has been proposed to enhance stability to the complex as well as to modulate the activity or level of the enzyme in the membrane (Arnold et al., 1998). In line with this hypothesis, dimeric state of ATP synthase was regarded as a stable inactive structure which could serve to accommodate more protein in the membrane (Tomasetig et al., 2002). At odds with the observation of active monomer versus inactive dimer, the oligomerisation was considered a constitutive, enzymatically functional organisation of the mitochondrial ATP synthase (Krause et al., 2005). Similarly, dimerisation of the chloroplast enzyme was seen rather as an advantage in reducing the futile rotational movement of the stator relative to the rotor during ATP synthesis (Rexroth et al., 2004).

Currently the central issue is an interconnection of ATP synthase oligomerisation with cristae formation (Allen et al., 1989). Consistent with this idea, yeast cells devoid of subunits *e*, *g* and *b* displayed aberrant mitochondrial morphologies characterised by numerous digitations and onion-like structures. Such alterations to mitochondria were interpreted as an uncontrolled biogenesis of the inner membrane thanks to the abolished dimerisation/oligomerisation of ATP synthase (Paumard et al., 2002b). Correct arrangement of mitochondrial ATP synthase as a prerequisite for the genesis and/or maintenance of cristae was demonstrated by an alternative approach exploiting the ability of the fluorescent protein DsRed to anchor multiple complexes. Cells containing trapped ATP synthase oligomers displayed altered mitochondrial morphology and an absence of cristae (Gavin et al., 2004). Remarkably, not only cells deficient in F_O subunits but also mutants deficient in F_1-α or F_1-β subunits or proteins that mediate F_1 assembly (*i.e.* Atp11p, Atp12p, or Fmc1p) show loss of cristae (Lefebvre-Legendre et al., 2005).

1.4.4. Basis of Interactions between Monomers

Despite extensive work, little is known about the nature of physical interactions of ATP synthase leading to its supramolecular organisation. Even though numerous subunits are involved, differences are apparent between evolutionary distinct ATP synthases. Electron microscopy images of the ATP synthase dimers from bovine heart mitochondria (Minauro-Sanmiguel et al., 2005) and from mitochondria of the algae *Polytomella* (Dudkina et al., 2005) revealed contact sites on the F_O parts. A protein bridge between the F_1 domains was reported only for the dimeric ATP synthase from bovine heart (Minauro-Sanmiguel et al., 2005). It is also worth noticing that the membrane-bound parts of the *Polytomella* enzyme, coupled to the catalytic units via robust stalks, formed an angle of 70°, in contrast to the angle of 40° between the bovine F_O parts (Dudkina et al., 2005).

Based on the fact that the absence of subunits *e* and *g* destabilised the oligomeric network of ATP synthases and damaged the mitochondrial morphology, a model was presented whereby ATP synthase monomers interact via a subunit *e*/*g* interface (Paumard et al., 2002b). A second interface mediated by subunit *b* was proposed since the subunit *b* dimers were formed either in the presence or in the absence of subunits *e* and *g* (Paumard et al., 2002b). Nevertheless, the interdependence of the two interfaces is likely as the removal of the first membrane-spanning segment of subunit *b* resulted in the loss of subunit *g*, as well as the supramolecular organisation of ATP synthase (Soubannier et al., 2002).

Disulfide bond formation promoted either homodimerisation or heterodimerisation of subunits *e* and *g* (Arselin et al., 2003; Bustos and Velours, 2005; Everard-Gigot et al., 2005; Saddar and Stuart, 2005). Upon cross-linking, the *e* + *e* and *g* + *g* homodimers were associated with ATP synthase oligomers, probably tetramers, even after during digitonin extraction (Arselin et al., 2003; Bustos and Velours, 2005). The *e* + *g* heterodimers seemed more stable in digitonin extracts and corresponded mainly to the dimeric enzyme (Bustos and Velours, 2005).

Quite recently, chemical oxidation stabilised homodimers of subunits *h* and *i* that could be extracted from mitochondria lacking the *e* and *g* subunits using digitonin and related to the dimeric ATP synthase (Fronzes et al., 2006). Thus in the absence of subunit *e* and/or *g*, the contact between two complexes can occur but is more susceptible to detergent treatment as also indicated in the studies on subunit *b* dimerisation (Gavin et al., 2005; Paumard et al., 2002b). According to the listed results, a revised model of supramolecular organisation of the ATP synthase has been presented (Fronzes et al., 2006): a dimerisation

interface includes not only membraneous subunits of the F_O part but also the matrix portion of the peripheral stalk, such as subunit *h*. Subunits *e* and *g* play a stabilisation role at the periphery of the dimerisation contact site and form the second interface facilitating the oligomeric arrangement.

The presence of subunit *e* is required for the stability of subunit *g* whereas the absence of subunit *g* does not preclude the presence of subunit *e* (Arselin et al., 2003; Arselin et al., 2004; Everard-Gigot et al., 2005). The significance of the dimerisation-prone GXXXG motif within the transmembrane segment of subunit *e* and/or *g* and the C-terminal coiled-coil region of subunit *e* in the intra- and inter-enzyme association has been examined (Arselin et al., 2003; Bustos and Velours, 2005; Everard-Gigot et al., 2005; Saddar and Stuart, 2005). Another group of authors put the ATP synthase assembly into a wider context whereby the subunit *e* stability, complex assembly and inner membrane morphology are subjected to an upstream regulatory function of Mgm1p, a dynamin-related GTPase that participates in mitochondrial fusion (Amutha et al., 2004).

1.5. Extramitochondrial ATP Synthase and its Function

There are several examples that mitochondria may accomodate proteins that fulfil dual function withing the organelle. Plant Core proteins, the noncatalytic subunits of the cytochrome bc_1 complex, are identical to subunits of the mitochondrial processing peptidase (MPP) (Glaser and Dessi, 1999). The MPP activity has also been reconstituted from the purified Core subunits of the bovine heart complex (Deng et al., 2001). Nevertheless, it is of question whether the mammalian Core proteins exert the peptidase function *in vivo*, especially when the mammalian MPP localises to the matrix. Besides the transport of ADP and ATP across the mitochondrial inner membrane, ANT is a component of the mitochondrial permeability transition pore, a nonspecific pore complex that mediates the necrotic and apoptotic cell death (Halestrap and Brenner, 2003). In case of ATP synthase, subunit α was related to the hsp60 protein family (Luis et al., 1990) and was proposed to influence protein import into mitochondria (Yuan and Douglas, 1992). Unexpectedly, within the last decade many new roles have been ascribed to the ATP synthase and its subunits in connection with the extramitochondrial localisation (Table II).

Table II. Extramitochondrial function of ATP synthase and/or its subunits.

Ectopic function	ATP synthase subunit
angiostatin receptor	α/β
high density lipoprotein receptor	β
enterostatin receptor	β
insulin secretion	β?
vasoconstriction	F6 binds to β as a receptor

Immunological localisation of the α subunit in rat peroxisomes promoted speculations about its relevance to the biogenesis of mitochondria and peroxisomes (Cuezva et al., 1990). In 1994 Das presented data concerning the location of a protein identical to the β subunit of mitochondrial ATP synthase on the plasma membrane of human tumor cell lines (Das et al., 1994). Since, a growing number of observations on the cell-surfaced association of ATP synthase subunits have been published. ATP synthase has been detected on the surface of endothelial cells (McMahon et al., 2006; Moser et al., 1999), keratinocytes (Burrell et al., 2005), fibroblasts (McMahon et al., 2006), hepatocytes (Bae et al., 2004; Martinez et al., 2003; Park et al., 2004), brain cells (Park et al., 2004), pancreatic β-cells (Yang et al., 2004), adipocytes (Kim et al., 2004) and lymphocytes (Man et al., 2005; McMahon et al., 2006). Evidence ranges from immunodetection in non-permeabilised cells (e.g. (Kim et al., 2004; Martinez et al., 2003; Moser et al., 1999)) through proteomic studies in lipid domains isolated from the plasma membranes (e.g. (Bae et al., 2004; Man et al., 2005; McMahon et al., 2006)) to functional studies. The ectopic enzyme may be involved in extracellular ATP synthesis that can be suspended by oligomycin (Burrell et al., 2005; Kim et al., 2004) or ATP hydrolysis (Martinez et al., 2003; Osanai et al., 2005). Two groups have shown recently that the hydrolytic but not the synthetic reaction is inhibited by a natural inhibitor protein IF1, which is also present on the plasma membrane of endothelial cells (Burwick et al., 2005; Cortes-Hernandez et al., 2005).

Angiogenesis

In endothelial cells, ATP synthase has been found to serve as a receptor for angiostatin, an endogenous antagonist of angiogenesis and an inhibitor of endothelial cell proliferation and migration. Namely, the α/β subunits form the angiostatin binding site (Moser et al., 1999). Angiostatin is capable of inhibiting ATP synthesis as well as ATP

hydrolysis (Moser et al., 2001). Furthermore it competes with IF1 binding to ATP synthase, thus abolishing IF1 ability to conserve ATP in low pH. Low pH and low oxygen are characteristic of tumor microenvironment in which such angiostatin action would be devastating to the growing tumor (Burwick et al., 2005). Induction of endothelial cell apoptosis by a plasminogen fragment K1-5 involved not only the interaction with endothelial ATP synthase but also a sequential activation of caspases-8,-9 and -3. Caspase-mediated endothelial apoptosis has also been proposed to be essential for angiostatin function (Veitonmaki et al., 2004).

Lipid Metabolism

On the surface of hepatocytes, the β subunit of the ATP synthase was identified as a high-affinity receptor for apolipoprotein A-I, and the hydrolytic activity of the ectopic enzyme was shown to be vital to the lipid transport (Martinez et al., 2003). The F_1-β subunit might be further involved in suppression of fat intake by acting as the receptor for enterostatin, a peptide released from procolipase during lipid digestion. The enterostatin agonist β-casomorphin$_{1-7}$ is likely to convey its message through a distinct binding site on the β protein (Park et al., 2004).

Insulin Secretion

Leucine induced up-regulation of the β subunit in a culture of rat pancreatic β-cells, increased ATP production, followed by elevated calcium level and increased insulin secretion in the presence of glucose. Down-regulation of the β subunit by siRNA had the opposite effect, suggesting an essential role of the ATP synthase in insulin secretion (Yang et al., 2004). Quite recently, up-regulation of glucokinase was demonstrated to precede the increased expression of the β subunit in leucine culture of rat islets and also in type-2 diabetic human islets (Yang et al., 2006).

Vasoconstriction

A quite unexpected role for the F_6 subunit, known as the outer stalk component of the mitochondrial ATP synthase, has also been postulated. F_6 was identified as an inhibitor of prostacyclin synthesis in endothelial cells of spontaneously hypertensive rats (SHR) that functions via suppression of Ca^{2+}-dependent cytosolic phospholipase A_2 (Osanai et al., 1998). Later high plasma level of F_6 was shown in human hypertension (Osanai et al., 2003) and acute myocardial infarction (Ding et al., 2004). F_6 was found in the vascular

system of SHR where it functioned as a vasoconstrictor in the fashion of a circulating hormone (Osanai et al., 2001b). F_6 release from the surface of vascular endothelial cells to the circulation was promoted by a shear stress (Osanai et al., 2001a) and tumor necrosis factor α through the activation of NF-κB signaling pathway (Osanai et al., 2001a). The vasoconstrictor effect of F_6 is mediated by binding to the β subunit of the extracellular ATP synthase and subsequent alteration of cytosolic pH due to the ATP hydrolysis (Osanai et al., 2005).

The Puzzle over the Origin and Structure of Ectopic ATP synthase

Although there is a strong experimental support for the extramitochondrial localisation and function of ATP synthase, the principal questions, considering the enzyme biogenesis, are awaiting their answers: what is the composition of the ectopic ATP synthase versus the known structure of the mitochondrial enzyme and which mechanism leads to its appearance outside the mitochondria? Since we lack any proof of the protein export from mitochondria and subsequent assembly of the holoenzyme in cytosol, the complex might be translocated to its final destination after its formation within mitochondria, presumbly via membrane fusion. There are plenty of other mitochondrial proteins that possess previously unsuspected roles within the cell, e.g. minor histocompatibility antigens, aspartate aminotransferase, fumarase, P32 protein, cytochrome *c*, etc. ((Soltys et al., 2001); for reviews see (Soltys and Gupta, 1999; Soltys and Gupta, 2000)). Several plausible mechanisms dealing with transport of mitochondrial proteins to specific extramitochondrial sites have been discussed including vesicle-mediated export, transient fusion of membranes, protein passage through membrane channels etc. (Soltys and Gupta, 1999; Soltys and Gupta, 2000). Interestingly, close contact between mitochondria and endoplasmic or sarcoplasmic reticulum was demonstrated in Ca^{2+} signalling studies (reviewed in e.g. (Bianchi et al., 2004)). Spatial localisation of mitochondria within the cell seems also to be crutial for the communication with plasma membrane (Park et al., 2001; Thomas et al., 2006), nucleus (Park et al., 2001) and Golgi apparatus (Dolman et al., 2005).

1.6. Regulation of Mammalian ATP Synthase Biogenesis

The mitochondrial ATP production responses to various energy demands during ontogenetic development, hormonal stimuli, oxygen availability and workload etc. The biogenesis of ATP synthase as well as other OXPHOS enzymes is regulated at different levels of gene expression, which can be seen even for genes coding for subunits of the same complex.

Development

Fetal **liver** has low content of mitochondria compared to the adult rat tissue. Birth promotes an increase in the cellular number of mitochondria, i.e. proliferation and an increase in the functional capability of pre-existing mitochondria, i.e. differentiation of the organelles that is accompanied by increased rate of mitochondrial protein synthesis (Izquierdo et al., 1995; Luis et al., 1993; Valcarce et al., 1988). Whereas proliferation of mitochondria is a continuous and subtle process that spans the whole developmental period, differentiation of mitochondria is a very rapid process occurring during the first hour of life. Expression of *ATP5B* gene encoding F_1 β subunit seems to be governed at the level of transcription during the mitochondrial proliferation (Izquierdo et al., 1995). In contrast, during the differentiation process, expression of OXPHOS genes including ATP synthase genes (*ATP5B*, *ATP6* and *ATP8*), is regulated at post-transcriptional level (Ostronoff et al., 1995; Ostronoff et al., 1996) by the control of mRNA stability (Izquierdo et al., 1995) and translational efficiency (Izquierdo and Cuezva, 1997; Luis et al., 1993) as previously demonstrated for the β subunit transcript. This mRNA is present in granules associated with mitochodrial outer membrane (Egea et al., 1997). During postnatal differentiation, the β mRNA is mobilised into cytoplasmic polyribosomes and its translational efficiency increases (Izquierdo et al., 1995; Luis et al., 1993).

Brown adipose tissue (BAT) is responsible for non-shivering thermogenesis in mammals (Nicholls et al., 1986). This function in heat production is determined by a large number of mitochondria that are naturally uncoupled owing to the dissipation of proton gradient by the uncoupling protein 1 (UCP1). Differentiation of brown adipocytes is accompanied by lipid accumulation, increase in the content of mitochondria and induced expression of OXPHOS genes including UCP1 and F_1-ATP synthase, thus the early prenatal BAT contains nonthermogenic mitochondria lacking UCP1 that are transformed to the thermogenic ones during the late fetal development of mouse and rat (Houstek et al.,

1988; Villena et al., 1998). The thermogenic function of the hamster BAT develops after the first postnatal week with concomitant increase in UCP1 level, reaching maximum at day 17, but decrase in ATP synthase (Houstek et al., 1990).

Increase in both the activity and content of ATP synthase has been documented during perinatal maturation of rat **kidney** and within the first day after birth (Prieur et al., 1995).

ATP synthase content and activity declined due to the oxidative damage in **heart and skeletal muscle** of senescent mice (Yarian et al., 2005).

Tissue Specificity - Brown Adipose Tissue

The biogenesis of ATP synthase in BAT is regulated at transcriptional and post-transcriptional level. Selective decrease in the expression of *P1* gene in BAT of rodents was observed and, in contrast to high mRNA levels for other ATP synthase subunits, corresponded to low ATP synthase amount in this tissue (Houstek et al., 1995a). Further, high *P1* gene expression mirrored high enzyme content in brown adipocytes from newborn lamb (Houstek et al., 1995a), which, unlike rodent cells, are rich in ATP syntase content (Cannon and Vogel, 1977). In analogy, correlation between the *P1* expression and the amount of mitochondrial ATP synthase was documented during the ontogenic development of brown adipose tissue in hamster (Houstek et al., 1995a).

Following studies reported differentially regulated expression of *P1* and *P2* genes in mammalian tissues (Andersson et al., 1997; Sangawa et al., 1997). While *P1* expression reflected the need for ATP synthase capacity arising from miscellaneous physiological stimuli (cold aclimation, cell differentiation and hormonal treatment), expression of the *P2* gene remained constitutive. Thus, key role of the *P1* gene in the biogenesis of mammalian ATP synthase was suggested (Andersson et al., 1997; Houstek et al., 1995a).

Muscle Adaptation to Endurance Excercise

Regular exercise consisting of repeated muscle contractions for 30 min or more per day leads to increases in the fractional volume of mitochondria and enzymatic capacity for oxidative metabolism (Holloszy and Coyle, 1984). The physiological benefits of mitochondrial adaptations in muscle are an alteration in metabolic preference with a greater reliance on lipid, rather than carbohydrate, metabolism. This reduces the formation of lactic acid, attenuates the loss of glycogen, reduces high-energy phosphate utilisation and reduces muscle fatigue. This mitochondrial biogenesis can be replicated using artificial models of

exercise such as chronic contractile activity produced by electrical stimulation of the motor nerve. This model has the advantage of producing relatively larger changes in mitochondrial biogenesis that can occur in a shorter time frame. Long term electrical stimulation of rabbit muscle upregulates ATP synthase β subunit (Williams et al., 1987). Similarly, ATP synthase expression increases during skeletal and heart muscle contraction and is regulated via calcium signalling (Das, 2003; Freyssenet et al., 2004).

1.7. Genetic Defects of ATP synthase

Until relatively recently, mitochondrial disorders were considered to be exceptionally rare diseases. However, epidemiological studies demonstrate that mitochondrial disorders are amongst the most common inherited human diseases (Schaefer et al., 2004).

Mitochondrial biogenesis depends on the expression of nuclear (ncDNA) and mitochondrial genome (mtDNA). As a consequence, genetic defects in both genomes can be responsible for mitochondrial diseases. Both types of genetic defects may result in ATP synthase disorder (Houstek et al., 2006). At the biochemical level, mitochondrial disorders are associated with isolated or combined OXPHOS deficiency (DiMauro and Hirano, 2005), disorders of ATP synthase are typically isolated deficiencies affecting all tissues. Clinicaly, the OXPHOS disorders manifest at any age, affecting only single organ or resulting in a multisystem disorder. The variability of phenotypic presentation of mitochondrial genetic defects is even more complex. The same clinical sings can be caused by different mutations and, conversely, the same genetic defect can present with divers phenotype between different patients or tissues of a given individual (Rossignol et al., 2003).

Such variability can be partially explained by the characteristics of mitochondrial genetics (DiMauro and Hirano, 2005). In contrast to the two copies of the nuclear genes, each cell containts hundreds or thousands of mtDNA molecules (polyplasmy). The situation in which all mtDNA molecules are identical is called homoplasmy. Nevertheless, dual population of mutant and wild-type mtDNA can co-exist within the same cell. Their ratio is termed heteroplasmy and can vary between cells, tissues or individuals.

A minimum critical number of mutant mtDNAs is required to cause mitochondrial dysfunction in a particular organ or tissue and mitochondrial disease in an individual (phenotypic threshold effect) (Rossignol et al., 2003). The cell or the organism possesses safety mechanisms against mutations in mtDNA and also ncDNA. The compensation can

occur at several levels of mitochondrial gene expression: transcription, translation, enzyme assembly and activity, mitochondrial respiration and ATP synthesis, as well as cell activity and organ function.

The percentage of mutated mtDNA can change at cell division, including oogenesis (DiMauro and Hirano, 2005). This phenomenon, known as mitotic segregation, can explain how certain patients with mtDNA-related disorders may actually shift from one clinical phenotype to another as they grow older.

At fertilisation, mtDNA of the zygote comes from the oocyte. Therefore, the mode of mtDNA transmision differs from Mendelian inheritance and point mutations in maternal mtDNA are transmitted to the progeny.

Insertions and deletions are usually sporadic or of nuclear origin. In addition, defective mtDNA replication or translation due to mutations in ncDNA are responsible for multiple mtDNA deletions or decrease in mtDNA content (mtDNA depletion). Thus mtDNA copy number also play a role in determining the phenotypic presentation of a mtDNA mutation.

Other factors may influence the phenotypic expression of mtDNA mutation including nuclear background, mtDNA haplotype (set of polymorphic changes that has been fixed in a certain population or a group of individual) and environmental factors.

1.7.1. ATP synthase Defects due to Mutations in mtDNA

As mentioned above, defects of ATP synthase as well as of other OXPHOS enzymes may originate from a mutation either in mtDNA or in ncDNA. Isolated deficiencies of ATP synthase are associated with severe and often fatal clinical phenotypes with early onset during neonatal age or infancy. Brain and heart muscle are among the most affected ones. Despite differencies in phenotypic manifestation, decreased ATP production and increased mitochondrial membrane potential and oxidative stress are common to both types of ATP synthase defects (Houstek et al., 2006).

So far, maternally transmitted mutations leading to isolated deficiency of ATP synthase have been located within the *ATP6* gene. A point mutation at position 8993 was described among the first pathogenic mutations in mtDNA (Holt et al., 1990). Most patients harbour T8993G or T8993C transversion (de Vries et al., 1993; Holt et al., 1990). Clinical symptoms are proportional to the degree of heteroplasmy. At levels below 90-95 % the most frequent T8993G mutation manifests as neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) which is characterised by retinitis pigmentosa, proximal

neurogenic muscle weakness, seizures, dementia, ataxia, developmental delay, and sensory neuropathy. At high mutation loads close to homoplasmy (above 90-95 %) there develops degenerative encephalopathy - a maternally inherited Leigh syndrome (MILS). The NARP/MILS presentations have also been identified with two other mutations in *ATP6* gene, T9176G and T9176C (Schon et al., 2001). At low mutation loads (cca 70 %) they cause NARP or familial bilateral striated necrosis (FBSN), above 90% mutation they manifest as MILS. In both sets of mutations, the T→G (i.e. conserved Leu→Arg) mutation is clinically far more severe than the T→C (i.e. conserved Leu→Pro) mutation. A single case of FBSN was also caused by a nearly homoplasmic T8851C (i.e. conserved Trp→Arg) mutation.

While ATP hydrolysis is normal, ATP synthase activity is impaired in the cells with T8993G mtDNA mutation and correlates with the level of heteroplasmy with no evidence of a biochemical threshold, however, a 60–75% mutant mtDNA load is required for obvious clinical expression of central-nervous-system symptoms (Carelli et al., 2002). As the mutated membrane channel can translocate protons from the cytoplasm to the matrix, it is likely that the enzyme is unable to couple phosphorylation of ADP to proton translocation down the membrane proton gradient (Sgarbi et al., 2006). Moreover, mitochondria bearing the T8993G mutation are hyperpolarised (Carrozzo et al., 2004; Mattiazzi et al., 2004; Vojtiskova et al., 2004) and have increased free radical production (Carrozzo et al., 2004; Geromel et al., 2001; Mattiazzi et al., 2004). Despite functional inhibition, the amount of ATP synthase is unchanged (Houstek et al., 1995b) and so is the capability of oligomerisation (Cortes-Hernandez et al., 2007).

Another point mutation, T9101C transition, manifests as Leber's hereditary optic neuropathy (LHON, (Lamminen et al., 1995), a disease characterised by loss of vision in young adults, more frequently males, caused by degeneration of the optic nerve but associated mainly with mtDNA mutations in genes encoding complex I subunits.

An ATP synthase deficiency associated with a two-base microdeletion at position 9205-6 (9205ΔTA) has only been detected in two cases that differ in biochemical and clinical symptoms. 9205ΔTA disturbs the termination codon of *ATP6* gene and sets the adjacent *COX3* immediately in frame. The first case presents with mild psychomotor retardation, normal biosynthesis of subunit *a* and unchanged mitochodrial ATP production (Seneca et al., 1996). On the contrary, the second case shows severe encephalopathy with decreased ATP synthesis and COX activity but normal ATP hydrolysis (Jesina et al., 2004). Here the subdued synthesis of subunit *a* impared assembly of ATP synthase

complex. The cause of such a pronounce difference in the manifestation of 9205ΔTA is currently unclear and seems to reside in defective post-transcriptional regulation.

1.7.2. ATP synthase Defects due to Mutations in ncDNA

In 1992, ATP synthase deficiency was reported in a distinct fatal mitochondrial disorder with 3-methylglutaconic aciduria, severe lactic acidosis and cardiomyopathy (Holme et al., 1992). Despite psychomotor retardation, there were no other neurologic signs. Deletions in mtDNA and mutations in *ATP6* and *ATP8* genes were excluded.

A case with severe fatal lactic acidosis due to isolated ATP synthase deficiency was published in 1999 (Houstek et al., 1999). The patient possessed generalised and selectively reduced activity and content of the mitochondrial ATP synthase relative to other OXPHOS enzymes (Houstek et al., 1999). Pulse-chase labelling and electrophoretic experiments indicated that the biosynthesis of the ATP synthase was most probably suppressed at the level of F_1 formation. Nevertheless, the enzyme size and composition was normal. Analysis of mtDNA, patient fibroblasts and derived transmitochondrial cybrids proved nuclear origin of this defect. The exact genetic cause of this disorder is uknown.

Until know, 14 patients with selective deficiency of ATP synthase have been documented (Sperl et al., 2006). Their phenotypes have surprisingly common features with early neonatal onset, severe and often fatal hyperlactacidemia and 3-methylglutaconic aciduria and hypertrophic cardiomyopathy. Most of the newborns had low birth weight. Half of the patients died and only two of the surviving patients are older than 10 years. Nevertheless, the length of survival has no correlation with the severenity of the enzyme defect. No mutation in ATP synthase structural genes could be detected in the patients tested. Mutations in F_1-assembly genes were also excluded and the level of expression remains unaffected. The only exception is a patient harbouring a missence mutation in *ATPAF2* gene encoding Atp12 assembly protein, the only known pathogenic mutation of nuclear origin resulting in the selective reduction in ATP synthase (De Meirleir et al., 2004). The phenotype contrasts with prominent heart involvement in the previous cases and is marked by dysmorphic features, progressive encephalopathy, lactic acidosis and 3-methylglutaconic aciduria. One of the longer surviving patients developed severe peripheral neuropathy (Sperl et al., 2006). These examples show that different phenotypes, relating to different genotypes, can be associated with quantitative deficiency of ATP synthase.

In analogy with mtDNA-based defects, the cells from patients with ATP synthase defects of nuclear origin display increased mitochondrial membrane potential (Houstek et al., 1999; Mracek et al., 2006) and generation of reactive oxygen species (Mracek et al., 2006).

2. AIMS OF THE THESIS

The biogenesis of mitochondrial ATP synthase was studied with respect to its assembly from individual subunits under the assistance of specific proteins with chaperone-type function and with respect to several similar cases of mitochondrial disorders due to selective deficiency of this enzyme. The first part of the work focused on the distribution and structural features of potential assembly factors in various genomes. The second part was devoted to the expression pattern of specific mammalian chaperones. The particular aims were as follows:

1) To characterise the occurence of orthologues of the yeast ATP synthase assembly factors

- To find orthologues of the known yeast ATP synthase assembly factors based on sequence similarity across genomes
- To analyse structural features of the orthologues (and compare them with the results of mutagenesis studies on yeast counterparts)

2) To analyse expression of the mammalian ATP synthase assembly factors

- To evaluate the expression of assembly factors in mouse tissues
- To study the expression of assembly factors in five patients with selective deficiency of ATP synthase of nuclear origin

3. SUMMARY OF THE RESULTS

The thesis consists of four publications. Two of them concentrate on the genome distribution of genes coding for factors specific for assembly of the ATP synthase and differential expression of mammalian assembly genes.

The other studies compare phenotypic features of selective ATP synthase deficiencies with respect to the genetic cause of the diseases. They also stress that the inability of mitochondria to supply sufficient ATP is not the only consequence of OXPHOS failure.

Differential expression of ATPAF1 and ATPAF2 genes encoding F(1)-ATPase assembly proteins in mouse tissues. Pickova A, Paul J, Petruzzella V, Houstek J. (2003) FEBS Lett.;551(1-3):42-6.

This is the first article showing tissue-differential expression of ATP synthase assembly factors. Only two genes (*ATPAF1* and *ATPAF2*) have been found in mammalian cells to code for Atp11 and Atp12 chaperones specific to the F_1 assembly. Quantitative reverse-transcription polymerase chain reaction was used in order to assess the transcript content in several tissues (brown adipose tissue, kidney, liver, heart, brain and skeletal muscle) from adult (2-month-old) and young (5-day-old) mice. The nearly constant *ATPAF1* mRNA levels contrasts with highly variable expression profile of *ATPAF2*, which resembles expression profiles of F_1 α and β genes. The most striking feature is a strongly upregulated *ATPAF2* in ATP synthase-deficient brown adipocytes. Further search for *cis*-acting elements in putative promoter region of human, mouse and rat genes revealed regulatory sites, commonly modulating the biogenesis of mitochondria-related genes including ATP synthase genes, within *ATPAF2* but not *ATPAF1* gene. Thus *ATPAF2* seems to be highly regulated gene, while *ATPAF1* may provide with constant level of chaperone transcipt.

Assembly factors of F_1F_O-ATP synthase across genomes. Pickova A, Potocky M, Houstek J. (2005) Proteins;59(3):393-402.

The studies about assembly of the yeast ATP synthase had promted us to search available genomic data for orthologues of the known yeast chaperones. Atp11 and Atp12 appear to be ubiquitous proteins and are the only proteins found in metazoan species. Nevertheless, only Atp12 was identified within bacterial genomes, namely in the α-

proteobacterial species. Atp10 was detected in a limited number of species in contrast to the distribution of its interaction partner, the subunit *a*. Surprisingly, the primary sequence of subunit *a* differed according to the presence or absence of *ATP10* gene. In organisms possessing Atp10p orthologue, the C-terminus of subunit *a* contains sonserved proline residue and the two last amino acids are hydrophobic, represented mainly by leucine or histidine. Fmc1 distribution is quite sporadic and is confined to fungal species. Atp22 was detected only in selected members of Saccharomycetales. Atp10p, Atp11p and Atp12p orthologues were further characterised with respect to primary and secondary structural features, which may serve as a basis for in-depth analysis and functional studies.

To conclude, the distribution of assembly genes strengthened the structural and functional differences between the F_1 and F_O domains in eukaryotes. The F_1-assembly mechanism seems to be preserved among species. In contrast, F_O structure and biogenesis shows higher variability.

Deficiency of mitochondrial ATP synthase of nuclear genetic origin. Sperl W, Jesina P, Zeman J, Mayr JA, Demeirleir L, Vancoster R, Pickova A, Hansikova H, Houst'kova H, Krejcik Z, Koch J, Smet J, Muss W, Holme E, Houstek J. (2006) Neuromuscul Disord.;16(12):821-9.

Here we compared and analysed clinical and biochemical data on 14 patients with isolated ATP synthase deficiency of nuclear origin characterised by a markedly decreased content of an otherwise fully assembled and active enzyme. Half of the patients is reported for the first time. The exact genetic cause of the defects is unknown. No mutated structural genes have been found in the cases tested. There is only one case in which mutation was localised to the *ATPAF2* gene, encoding F_1-α chaperone. Most of the patients present with remarkably uniform phenotype with onset in early neonatal age, hypertrophic cardiomyopathy, pronounce often fatal metabolic acidosis and high lactate levels. Some patients had elevated 3-methylglutaconic acid in urine. All surviving patiens had some degree of psychomotor retardation. Lack of striatal brain involvement and decreased amount of structurally normal ATP synthase distinguish these defects from those caused by mutations in *ATP6* gene. In conclusion, the ncDNA-based defects represent a spefic type of mitochondrial diseases that can be recognised at both clinical and biochemical level.

Mitochondrial diseases and genetic defects of ATP synthase. Houstek J, Pickova A, Vojtiskova A, Mracek T, Pecina P, Jesina P. (2006) Biochim Biophys Acta.;1757(9-10):1400-5.

Defective biogenesis of ATP synthase complex leads to severe mitochondrial diseases. Here we review current knowledge on the ATP synthase defects and show that there are two types of isolated deficiency of ATP synthase with respect to the structural and functional features of ATP synthase as well as the consequences of the enzyme failure. The defects of mtDNA-origin are qualitative, i.e. the enzyme structure is altered and its function impaired. Known defects due tu mutations in nuclear genome are quantitative where the amount of correctly assembled enzyme is significantly lowered. Most of the current patients from the second group do not bear currently known mutations, however, there was no indication of abnormal expression of F_1-assembly genes and no mutation has been located within their coding region. Common to both types of defects is the energy deprivation and increased oxidative stress.

4. COMMENTARY ON THE RESULTS

There is often tendency to regard a certain gene as a generally conserved in eukaryotes based on its presence in Metazoa and Fungi (Hall and Russell, 2004; Wang et al., 2001). Unless members of other lineages are documented, this may lead to a false conclusion, as these phyla are closely related and belong to group of opisthokonta that represents only a small part of the whole eukaryotic kingdom (Simpson and Roger, 2004). At the same time, it is worth considering the possibly negative effect of a given selection of species on the results of sequential analysis. We have been therefore tempted to search for ATP synthase assembly factors in 55 complete or nearly finished genomes to cover the diversity of eukaryotic organisms. Data from five of six major groups were available at the time of our study. Our analysis was extended to all sequenced mitochondrial genomes and to complete prokaryotic genomic sequences. The widespread presence of Atp11p and Atp12p orthologues makes us believe in their indispensable function during the biogenesis of the catalytic sector of ATP synthase (Pickova et al., 2005). Our hypothesis is backed by the subtle changes within the composition of the F_1 domain.

Completely different picture can be drawn in case of the biogenesis and the structure of the membrane-spanning part. The eukaryotic F_O sector is composed of more then three times more subunits with respect to the structure of the bacterial F_O part. Nevertheless, the composition of the F_O domain is not uniform even within the eukaryotic enzymes ((Ackerman and Tzagoloff, 2005; Pickova et al., 2005; van Lis et al., 2007; Vazquez-Acevedo et al., 2006); and our unpublished data). Additionally, the presence of the F_O-*a* chaperone Atp10 shows considerable variation within the eukaryotic kingdom and does not even follow the distribution of the F_O-*a* subunit (Pickova et al., 2005). The most surprising finding is that its function seems to be conditional to the specific structure of the C-terminus of the subunit *a*. In accordance, mutation of a single amino acid within the C-terminal part of subunit 6 (*a*) conferred the yeast cells ability to grow on rich glycerol medium (and to display oligomycin-sensitive ATPase activity) in the absence of Atp10p (Paul et al., 2000).

A rather rare occurrence of Fmc1 and Atp22 orthologues promted us to believe in factors unique to a certain closely related group of eukaryotes (Pickova et al., 2005). Fmc1p action has been linked to the biogenesis of the catalytic portion under heat stress condition and its deficiency could be overcome by overexpression of Atp12p (Lefebvre-

Legendre et al., 2001). Atp22p is most probably involved in formation of the membrane part (Helfenbein et al., 2003).

In recent studies, a new ancillary protein has been identified to function both as a maturase and a specific chaperone of the yeast subunit 6 (*a*) (Osman et al., 2006; Zeng et al., 2006). Our unpublished data favour ubiquitous distribution of Atp23p orthologues concerning the eukaryotic organisms. The question is, how are its proteolytic and chaperone activities preserved in other than yeast species. The mammalian subunit *a* is encoded in mtDNA as is the yeast counterpart, however, it lacks the N-terminal presequence. The sequentional difference stems from the absence of untranslated regions along with introns in the mammalian mtDNA (Anderson et al., 1981); hence Atp23 might serve solely during the incorporation of subunit *a*. This situation, nevertheless, does not preclude the exertion of Atp23 proteolytic function on other substrates. A similar shift in protein function was discussed above for Atp10. Another intriguing example provides the biogenesis of COX. Yeast Pet309p regulates processing and translational activation of mtDNA-encoded COX transcripts by binding to their untranslated regions. Its human homologue LRPPRC is a polyA-mRNA binding protein that was reported to govern the transcription of genes encoding mitochondrial proteins from his post in mitochodria and also in nucleus (Mootha et al., 2003).

As the Atp11 and Atp12 chaperone activities seem to be crucial also for the formation of the mammalian F_1 domain, we studied expression of the corresponding genes, *ATPAF1* and *ATPAF2,* together with the expression of genes coding for their respective interaction partners, the β and α subunits. The expression results could further give an implication for the role of the assembly genes in regulation of the enzyme biogenesis under physiological and pathophysiological states.

The cellular amount of mammalian ATP synthase is relatively stable in many tissues when compared to the content of respiratory chain complexes. Regulation of the enzyme biogenesis includes regulatory mechanisms common to other OXPHOS complexes and is exerted at transcriptional and post-transcriptional levels (Izquierdo et al., 1995). Transcription of the subunit *c* gene *P1* appears to be a key determinant of the content of the ATP synthase complex in brown adipocytes and most probably in other tissues (Andersson et al., 1997; Houstek et al., 1995a; Sangawa et al., 1997). Brown adipose tissue, as a thermogenic organ, is known for its physiologicaly lowered amount of ATP synthase, up to a tenfold compared to RC enzymes.

The data on expression of *ATPAF1* and *ATPAF2* indicated a two-orders or one-order of magnitude lower expression of the assembly genes with respect to the expression of F_1 structural genes in mouse and human cells, respectively (Pickova et al., 2003). Nearly constant levels of *ATPAF1* mRNA contrast with a highly variable amount of *ATPAF2* transcripts in mouse tissues. The difference in expression of the assembly genes is underscored by a strong correlation between the expression profiles of *ATPAF2* gene and the α and β subunit genes, showing the highest transcript levels in brown adipose cells. Moreover, promoter of *ATPAF2* but not *ATPAF1* gene contains sequences for transcriptional regulators common to OXPHOS biogenesis (Pickova et al., 2003). The amount of β protein did not paralleled the mRNA level in brown adipocytes as was reported for subunit *c* (Houstek et al., 1995a). The expression of β subunit gene was therefore not considered to determine the content of ATP synthase. From our results, we might hypothesize that the expression of the assembly genes is not probably involved in the regulation of ATP synthase content. Unfortunately, we currently lack data on protein levels, which would bring more light to this problem.

The selective decrease in the content of ATP synthase in brown adipose tissue resembles the situation in patients with ATP synthase deficiency of nuclear origin. To our disappointment, neither the transcript levels of the F_1 structural genes nor the expression of the F_1 assembly genes were markedly different from the control values ((Houstek et al., 2006) and our unpublished results). The situation in human ATP synthase defects of nuclear origin opposes the upregulated levels of *ATPAF2* and α and β subunit genes. To our knowledge, besides the one and only case with mutated *ATPAF2* gene, no mutation in assembly or structural genes has been found (Sperl et al., 2006). Which nuclear gene is primarily responsible for this type of defect is unfortunately unknown so far. A mutation in a yet unidentified accessory gene may be linked to this type of ATP synthase disorder. One might find a nice example in case of the identification of the *LRPPRC* as a COX disease gene employing three sources of information: human genomic sequences, mRNA expression profiles from a variety of cells and tissues and tandem mass spectra from a mitochondrial proteomics (Mootha et al., 2003).

5. CONCLUSIONS

The subsequent conclusions stem from our bioinformatic studies:

- We have performed a comprehensive survey of orthologues to Atp11p, Atp12p, Fmc1p, Atp10p and Atp22p from all available genomes.
- The distribution of the orthologues mirrors the structural and functional difference between the F_1 and F_O moieties: assembly of the catalytic domain depends on action of at least two specific factors, Atp11 and Atp12, with the exception of Nematoda whose genome bears homology with *ATP12* only.
- The presence of Atp12p orthologue in α-proteobacteria points to eubacterial origin of *ATP12*
- The membranous F_O part seems to be more versatile in terms of both structure and biogenesis: the presence of Atp10p orthologue matches a specific structure of the C-terminal part of subunit *a*.
- The finite occurence of Fmc1 and Atp22 may reflect specific requirements of some lineages.
- Sequence analysis of Atp11p, Atp12p and Atp10p orthologues provides basis for detailed molecular studies of these chaperones.

The expression studies have resulted in the following outcome:

- *ATPAF1* and *ATPAF2* are genes with low or moderate level of expression.
- The tissue distribution of *ATPAF2* correlates with the expression profiles of the α and β subunits and peaks in brown adipose tissue, which is marked by a diminished content of ATP synthase.
- *ATPAF1* manifested a constant expression in adult and 5-day-old mouse tissues.
- Nowadays, the primary cause of the 13 from the 14 published nuclear genetic defects remains enigmatic. The clinical and biochemical data, however, do not exclude mutation in different nuclear genes, which is also indicated by our results.
- Concerning the F_1-assembly genes, we have not found abberant transcript levels in five patients with selective ATP synthase deficiency. Similarly, the amount of Atp11 did not vary significantly among controls and patiens with ATP synthase defect. We also have not detected any mutation in the enzyme assembly or structural genes (collaboration with a research group of Prof. V. Pačes, unpublished data). Nevertheless, altered biogenesis of another currently unknown ancillary protein may lead to this type of defect.

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