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F_0F_1 ATP Synthase: A Fascinating Challenge for Proteomics

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1. Introduction

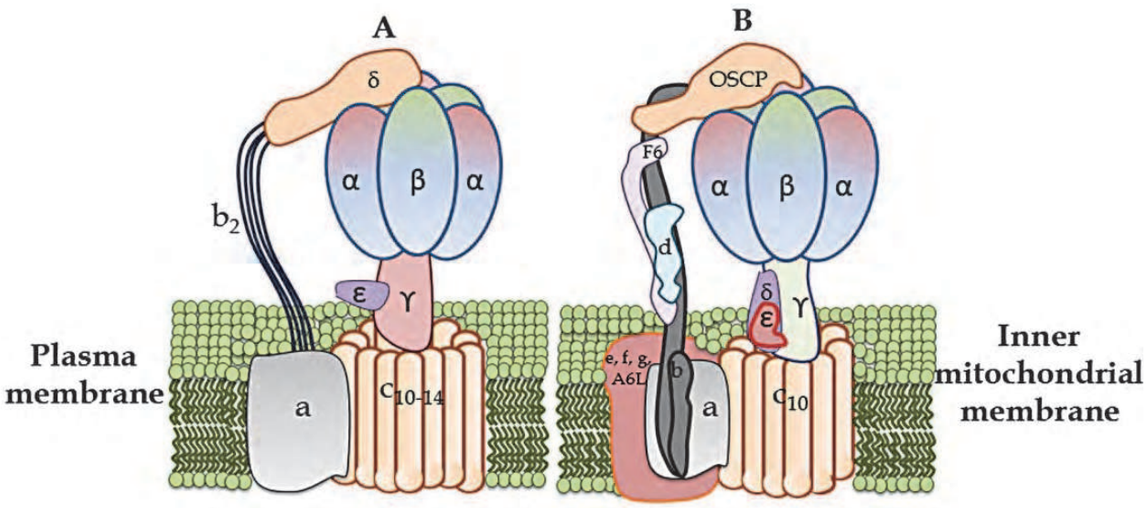
The aim of this review is to provide insight and encouragement into the development of new proteomic approaches aimed at analyzing the relationship between structure and function of ATP synthase in different organisms and under different metabolic conditions. Particular attention will be paid to the preparation of the sample for the mass spectrometry (MS) analyses, which is also a critical step and requires a specific competence.

2. The nano-motor enzyme F_0F_1ATP synthase

F_0F_1ATP synthase is the terminal enzyme of the oxidative phosphorylation pathway (named complex V of the OXPHOS system) that is responsible for the majority of ATP synthesis in all living cells (Boyer, 1997). It is an exceptionally complicated protein complex, whose molecular mass varies from 540 to 585 kDa depending on the source, which is organized into a globular catalytic part (F_1) and a membranous moiety (F_0) linked by central and peripheral stalks.

The enzyme is present in bacterial plasma membranes and chloroplast thylakoids, where it contains 8 and 9 subunits, respectively (Borghese et al,. 1998; Richter et al,. 2000), and in mitochondria, where it is located in the inner membrane and consists of at least 15 and 17 different subunits in mammals and yeasts, respectively (Wittig and Schägger, 2008). The F_1 sector always consists of five subunits $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ and the α- and β-subunits are arranged alternatively, forming a hexagonal cylinder around the coiled-coil structure of the γ subunit. The F_0 part, which is responsible for ion translocation across the membrane, has instead a variable composition. The simplest form is present in bacteria and consists of the subunits $ac_{10\text{-}14}$. The c subunits form a ring structure with variable stoichiometry among species connected to F_1 by the central stalk, constituted by the subunits γ and ε, latter being homologous to subunit δ of the mitochondrial enzyme (Vignais and Satre, 1984) and able to modulate the catalysis (Suzuki et al,. 2011). The a subunit associates with the c-ring peripherally and with the lateral stalk, which is formed by the homo-dimer of subunits b and by subunit δ, present in single copy and located at the top of F_1(Walker and Dickson, 2006)(Fig.1A).

In mitochondria the additional subunits d, e, f, g, A6L, F6 and OSCP are associated to the complex, of which the subunits b, d, F6 and OSCP form the lateral stalk in single copies, OSCP homologous to prokaryotic subunit δ (Walker and Dickson, 2006). The so-called



(A) Bacterial (*E.coli*) enzyme is assembled with a stiochiometry of α (3), β (3), γ, δ, ε, b (2), a, c (10-14). The central stalk is composed of γ and ε subunits, the peripheral one is composed of subunits b and δ, the proton channel is formed by the subunits $ac_{10\text{-}14}$.
(B) In the eukaryotic enzyme (*Bos taurus*) the central stalk is formed by subunits γ, δ and ε, the peripheral stalk is composed of subunits OSCP, b, d and F6 and the additional subunits A6L, e, f, g are associated to the proton channel ac_{10}.

Fig. 1. Schematic representation of bacterial and mitochondrial F_0F_1 ATP synthase.

minor subunits e, f, g and A6L all span the membrane and, apart for subunit e (Bisetto et al, 2008), their exact stoichiometries are poorly defined (Fig. 1B). Other subunits are species-specific, such as subunit i and k in *Saccharomyces cerevisiae* (Wittig and Schägger, 2008) and coupling factor B in *Bos taurus* (Lee et al,. 2008).
Besides these subunits, in some mammals such as beef, rat and man two hydrophobic proteins namely MLQ/6.8-kDa proteolipid (Chen et al,. 2007; Meyer et al,. 2007), and AGP/DAPIT (Ohsakaya et al,. 2011) are associated to the F_0 part when phospholipids are not extracted. All together, the mitochondrial membrane domain is constituted by approximately 30 trans-membrane α-helices (Carroll et al,. 2009). In addition, the mitochondrial complex can bind the inhibitor protein IF_1, which reversibly binds to F_1 with a 1:1 stoichiometry and fully inhibits the enzyme activity (Bason et al,. 2011; Harris and Das, 1991). In yeast, along with IF_1, the enzyme can be regulated by two additional proteins, namely Stf1 and Stf2 (stabilizing factor 1 and 2) (Andrianaivomananjaona et al,. 2010). The subunit composition of the ATP synthase from prokaryotic and eukaryotic sources along with the subunit homology and the corresponding nomenclature is reported in Table 1. The molecular masses of the different subunits are reported in Table 2, based on ATP synthase from *Bos taurus* and *Bacillus pseudofirmus*.
Despite the differences in the complexity, functionally important subunits are conserved and in all sources the enzyme catalyses the synthesis of ATP by using the energy of the electrochemical gradient of protons (or less commonly of sodium) generated by the respiratory chain. F_0F_1 is an unusually efficient rotary motor that synthesizes ATP at rates exceeding 100 molecules per second (Senior, 2007). Protons traveling down the H^+ gradient generate the rotation of the F_0 c-ring, making the central stalk also rotates, which in turn drives ATP synthesis from ADP and Pi by forcing different conformations sequentially on each of the catalytic sites in the three F_1 subunits β. The enzyme is able to work in the

Homologous subunits	Stoichiometry	Prokaryotes	Eukaryotes		
		E.coli	*S. cerevisiae*	*B. taurus*	*H. sapiens*
F_1	3	α	α	α	α
	3	β	β	β	β
	1	γ	γ	γ	γ
	1	ε	δ	δ	δ
	1	-	ε	ε	ε
F_0	1	δ	5	OSCP	OSCP
	1	a	6	a	a
	1	-	8	A6L	A6L
	10-14	c	9	c	c
	1-2	b	4	b	b
	1	-	d	d	d
	1	-	h	F6	F6
	1	-	f	f	f
	1-2	-	e	e	e
	n.d*	-	g	g	g
Species specific subunits	1		i	-	-
	n.d*	-	k	-	-
	1	-	-	Coupl. fact.B	-
Inhibitor Protein	0-1	-	Inh1	IF_1	IF_1
Stabilizing factor for IF_1	n.d*		Stf1	-	-
	n.d*	-	Stf2	-	
Associated proteins		-	-	AGP or DAPIT	AGP or DAPIT
		-	-	MLQ or 6.8 PL	MLQ or 6.8 PL

*n.d.- not determined

Table 1. Subunit composition and nomenclature of the ATP synthases from prokaryotes (*Escherichia coli*) and Eukaryotes (*Saccharomyces cerevisiae*, *Bos taurus* and *Homo sapiens*).

direction of ATP hydrolysis, sustaining the formation of the proton gradient, when there is loss of membrane potential (Fig. 2).

First direct visualization of ATP-driven rotation of *Bacillus* F_1 immobilized on the glass surface via the N-termini of its β-subunits was obtained more than 10 years ago (Noji et al,. 1997). These experiments showed that a fluorescent actin filament attached on the γ-subunit rotates uni-directionally, counterclockwise when viewed from membrane side, upon addition of ATP. A further technical sophistication used a sub-millisecond resolution camera to detect the rotation of gold beads attached to the γ subunit of the $\alpha_3\beta_3\gamma$ sub-complex along with fluorescence changes of an ATP hydrolysable analog. This technique allowed to display in real time the binding and release of nucleotides at the three catalytic sites simultaneously with the γ rotation (Adachi et al,. 2007). The rotation probe reports a pause, which corresponds to the period during which ATP binds to the empty catalytic site,

Subunits	*Bos taurus*		*Bacillus pseudofirmus*	
	Accession Numbers*	Molecular mass+	Accession Numbers*	Molecular mass+
α	P19483	55263.39	AAG48361.1	54674.48
β	P00829	51562.97	AAG48363.1	51752.14
γ	P05631	30255.71	AAG48362.1	31835.49
δ	P05630	15064.93	AAG48360.1	20534.63
ε	P05632	5651.67	AAG48364.1	14327.68
a	P00847	24787.91	AAG48358.1	26863.77
b	P13619	24668.72	AAG48359.1	18510.16
c	P32876	14223	AAC08039.1	6956.06
c	P07926	15029	-	-
c	Q3ZC75	14693	-	-
d	P13620	18561.28	-	-
e	Q00361	8189.47	-	-
f	Q28851	10165.99	-	-
g	Q28852	11286.26	-	-
8 or A6L	P03929	7936.56	-	-
F6	P02721	8958.09	-	-
OSCP	P13621	20929.75	-	-
Total F_0F_1	583442[a]		540290.53[b]	

*Accession numbers were obtained from UniProt

+Molecular masses are shown in Dalton

[a]Assuming the assigned stoichiometry for *Bos taurus* including the subunits e_1 and g_1 (excluding the amino-terminal modifications). If proteins MLQ and AGP are considered, the total protein mass increases to 596579 Da (Wittig and Schägger, 2008).

[b]According to the stoichiometry ($\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{13}$) for *Bacillus pseudofirmus*

Table 2. Molecular masses of subunits of *Bos taurus* and *Bacillus pseudofirmus* F_0F_1 ATP synthase

and a 120° step rotation, constituted by two sub-steppings, whose duration is still debated, during which ATP hydrolysis and release occur. Single molecule technology studies have been applied also to the whole F_0F_1 complexes from *Propionigenium modestum* and *Escherichia coli*. Rotation was probed with probes attached to the c-ring in the immobilized F_0F_1 and, as expected, occurred in the opposite direction when c-ring rotation was driven by ATP or by proton-flow (Ueno et al,. 2005).

2.1 ATP synthase biogenesis

The mitochondrial F_0F_1 complex is composed of both nuclear and mitochondrial gene products. In yeast the three F_0 core proteins a (Su6), A6L (Su8) and c (Su9) are encoded by mDNA, while in mammals only subunits a and A6L are encoded by mitochondrial genome. This arrangement highlights the complexity of enzyme assembly, which requires accessory factors, whose definition is still under investigation (Wittig and Schägger, 2008). Altogether 9 factors have been identified in yeast, but, until now, the role of only five of them has been defined. Three factors mediate the F_1 formation (Atp11p, Atp12p and possibly Fmc1p) (Ackerman, 2002; Lefebvre-Legendre et al,. 2001) and two the F_0 assembly (Atp10p and

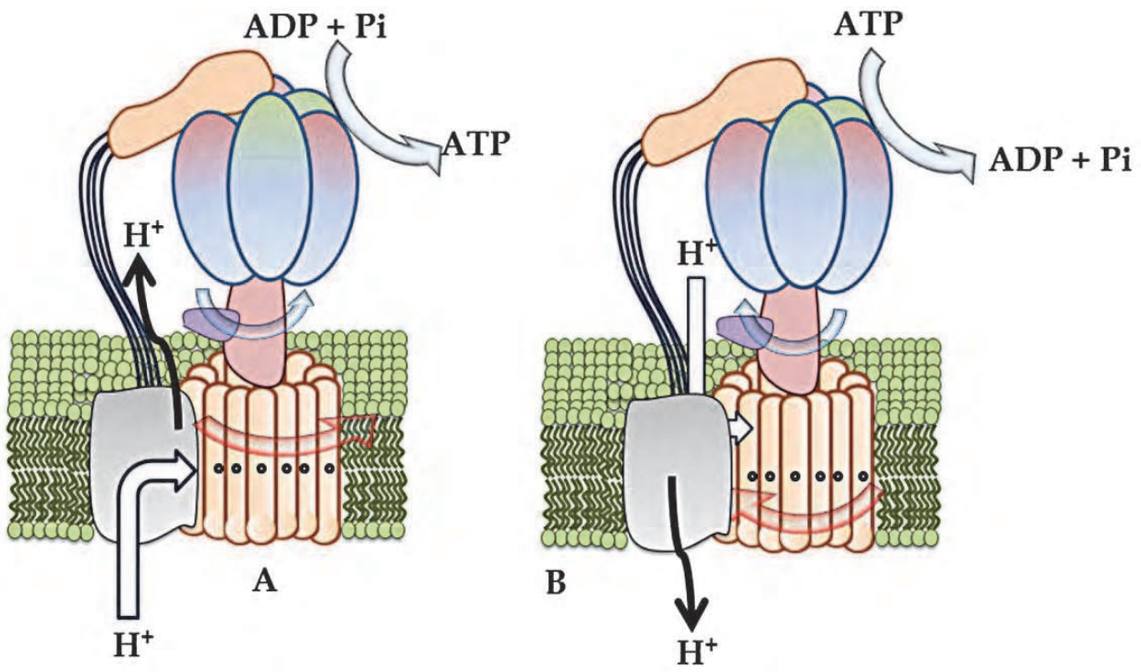


Fig. 2. Schematic representation of ATP synthesis and hydrolysis by F_0F_1 ATP synthase (A) Proton powered rotation of c ring makes the central stalk turn with it, generating torque and conformational changes in the catalytic αβ domain to synthesize ATP from ADP and Pi. (B) The hydrolysis of ATP sustains proton flow in opposite direction.

Eukaryotes	Factors	Mass (Da)	Swiss-Prot Accession
Yeast	Fmc1p	≤18364	P40491
Yeast	Atp10p	32093.91	P18496
Yeast	ATP11p	≤36581	P32453
Yeast	ATP12p	≤36554	P22135
Yeast	ATP22p	≤79756	A6ZYV0
Yeast	ATP23p	26890.37	P53722
Yeast	Mdm38p	58610.83	Q08179
Yeast	Aep3p	70310.03	Q12089
Yeast	Oxa 1p	40000.09	P39952
Human	ATPF2	32772	**Q8N5M1***
Human	ATPF1	36437	Q5TC12*

* UNIPROT accession number

Table 3. Assembly factors of yeast and human F_0F_1 ATP Synthase. The assembly factors for F_1 are in blue and F_0 are in red (Wittig and Schägger, 2008).

Atp22p) (Helfenbein et al,. 2003; Rak et al,. 2011). In mammalian cells only two factors are known, which are orthologus to yeast F_1 assembly factors (Table 3).

The assembly process is best characterized in yeast, where recent *in organello* pulse-labeling and pulse-chase experiments have enabled to identify three different assembly intermediates and to demonstrate that the whole enzyme is formed by two separate

pathways that converge to form the ATP synthase from their respective end-products. One pathway leads to the formation of F_1, which was already known to assemble as an independent unit (Tzagoloff, 1969), and of the Su9-ring. These two sub-complexes subsequently combine to constitute the F_1/Su9-ring end-product. The other pathway leads to the formation of the Su6/Su8/stator sub-complex, which, in addition to Su6 and Su8, contains the chaperone Atp10p and additional still undefined proteins of the lateral stalk (Rak et al., 2011) (Fig. 3).

Because in yeast the interaction between Su6 and Su8 is kinetically much more rapid, the entire process is regulated by the control of the Su6 and Su8 translation by F_1 in order to obtain a balanced production of the different intermediates (Rak et al., 2011). Conversely, in mammals the amount of ATP synthase seems to be controlled by the availability of subunit c, as demonstrated in brown fat (Houstek et al,. 1995) and other tissues (Andersson et al,. 1997).

Independent of the mechanism, it has been proposed that the ATP synthase assembly may recapitulate some of the evolutionary events that gave rise to this enzyme. In fact, there is evidence that F_1 evolved from an ATP-dependent helicase (Gomis-Rüth et al,. 2001), while the Su9 derived from an ion channel (Rak et al., 2011), so that their combination converted a passive channel into an active pump.

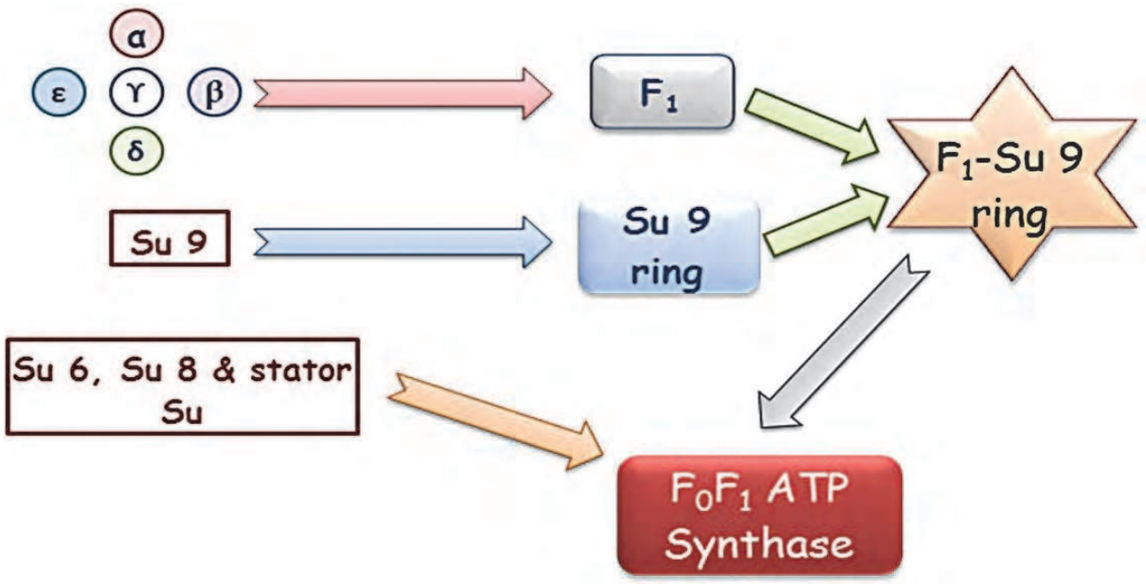


Fig. 3. Assembly of F_0F_1 ATP synthase in yeast. The scheme shows the two separate pathways, leading to two separate end-products, i.e. the F_1/Su9-ring and Su6/Su8/stator sub-complexes that converge at the end to form the whole F_0F_1 complex (Rak et al., 2011).

So far there is little evidence for tissue-specific or developmentally regulated isoforms of ATP synthase subunits. In tobacco plants three isoforms of the F_1 subunit β have been identified, of which only one is exclusively expressed in pollen (Lalanne et al,. 1998). In mammals, two tissue-specific isoforms of the F_1 γ subunit, heart and liver type, were identified in bovine F_0F_1-ATP synthase (Matsuda et al,. 1993). These two isoforms are generated by alternative splicing and their V_{max} and K_m are identical (Matsuda et al,. 1994). In addition, three isoforms (P1, P2, and P3) of the F_0 subunit c have been identified (Vives-

Bauza et al,. 2011). These isoforms differ in their cleavable mitochondrial targeting peptides, whereas the mature peptides are identical. Considering that in mammals c genes determine the ATP synthase content, the existence of iso-genes would be advantageous for regulation of subunit c synthesis, and thus ATP synthase biogenesis, by multiple factors. It appears that much remains to be learned about this argument.

2.2 ATP synthase self-association

Biochemical evidence and electron microscopy studies recently demonstrated that within the inner mitochondrial membrane the enzyme is organized in dimers and oligomers, which possibly associate with other inner mitochondrial membrane proteins, e.g. with phosphate and adenine nucleotide carriers in the "phosphorylating assemblies"– the so called ATP synthasome (Chen et al,. 2004; Wittig and Schägger, 2008). Elucidation of dimer/oligomers structural properties and of their formation process is quite important. In fact, proposed roles of the ATP synthase oligomers are higher efficiency and higher stability. In this regard, we demonstrated that dimers have a greater specific activity than monomers (Bisetto et al,. 2007). In accordance, a recent numerical simulation indicated a significant increase in charge density in regions of high membrane curvature induced by ATP synthase dimerization, thus favoring effective ATP synthesis under proton-limiting conditions (Fig. 4) (Strauss et al,. 2008). Moreover, these oligomers appear to play a special role for mitochondrial morphology, being involved in cristae formation (Couoh-Cardel et al,. 2010; Paumard et al,. 2002).

The structural properties of dimers/oligomers were initially characterized in yeast where genetic approaches, cross-linking analyses and electron microscopy images established preferential interactions within the inner membrane (Thomas et al,. 2008) mainly through the subunits Su6 (Wittig et al,. 2008), Su4 (Spannagel et al,. 1998), e (Everard-Gigot et al,. 2005) and g (Bustos and Velours, 2005), which are conserved in mammals, and also through the F_0 subunits h and i in yeast (Fronzes et al,. 2006). High-resolution images showed that both in yeast and mammals the dimers display angles between two F_1-F_1 ranging from 35° to 180°. Recent images of yeast dimers at 27 Å resolution showed that the dominant angle is 42°, suggesting that this is the most stable conformation (Couoh-Cardel et al., 2010).

We recently demonstrated by a structural proteomic approach that also in mammals the e subunit is essential for ATP synthase self-association in dimers and oligomers. Selective degradation by *in situ* limited proteolysis caused an alteration of the oligomeric distribution of ATP synthase by eliminating oligomers and reducing dimers in favor of monomers (Bisetto et al., 2008).

A critical aspect of F_0F_1 dimerization is related to the role of IF_1, which is still controversial (Wittig and Schägger, 2008). IF_1 is well known to bind ATP synthase under energy deficiency, i.e. at low pH and membrane potential, when the enzyme hydrolyzes rather than synthesizes ATP. Therefore, IF_1 is considered responsible for the beneficial down-regulation of F_0F_1 during ischemia in *in vitro* experimental models, but also *in vivo*, as demonstrated by our group in anaesthetized open-chest goat heart (Di Pancrazio et al,. 2004). Nevertheless, because isolated IF_1 from bovine heart is present in dimeric form in solution where it has been shown to link two F_1-subcomplexes (Cabezón et al,. 2003), it seemed conceivable that dimeric IF_1 might also be able to link two F_0F_1 complexes in the inner mitochondrial membrane. However, in yeast deletion of IF_1 and of the associated proteins Stf1 and Stf2 did not eliminate dimers and oligomers (Dienhart et al,. 2002), thus excluding an essential role of IF_1 in ATP synthase self-association. On the other hand, a very recent study revealed that

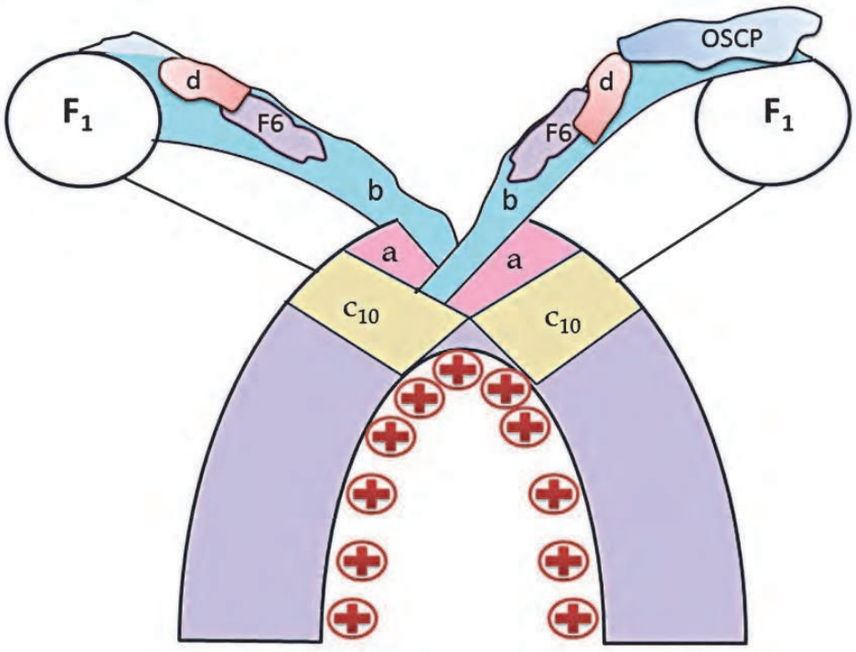


Fig. 4. Model of ATP synthase dimers. Dimers mainly interact through F_0 sector and enforce a strong local curvature on the inner mitochondrial membrane, where an increased charge density favors effective ATP synthesis (Strauss et al., 2008).

also in yeast oligomers contain considerable amounts of IF_1, raising the question of whether bound IF_1 inhibits the oligomer activity (Couoh-Cardel et al., 2010).
In bovine heart mitochondria, we demonstrated that physical release of IF_1 from the inner membrane did not markedly alter the amount of dimers separated by Blue Native electrophoresis (Tomasetig et al,. 2002), suggesting that F_0F_1 dimers could form independently from IF_1. Nevertheless, in human HeLa cells IF_1 overexpression increased ATP synthase dimers, as revealed by native electrophoresis, and this was paralleled by a higher ATP synthesis efficiency (Campanella et al,. 2009). However, it should be noted that in this paper the identification and quantification of ATP synthase dimers, as well as of their IF_1 content, seems questionable due to the use of dodecylmaltoside as detergent, which is well known to alter the dimer/monomer ratio (Tomasetig et al., 2002).

2.3 Disorders related to ATP synthase

In spite of the fact that the assembly process of the mitochondrial ATP synthase is still poorly characterized in humans, its defects have been recognized as a cause of human diseases (Houstek et al,. 2006; Kucharczyk et al,. 2009). Alteration of ATP synthase biogenesis leading to mitochondrial ATP synthase deficiency may cause two types of defects: qualitative when the enzyme is structurally modified and does not function properly, and quantitative when it is present in insufficient amounts. Examples of qualitative defects are those caused by missense mutations in the mitochondrially-encoded subunit a gene. Eight point mutations and a two-nucleotide micro-deletion in the ATP6 gene have been identified, of which the most common and best studied is the T8993G mutation that leads to replacement of a highly conserved leucine by arginine (Kucharczyk et al,. 2009). These mutations prevent ATP synthesis but not ATP hydrolysis

because mutated F_0 can translocate protons from the cytosol to the mitochondrial matrix, thus sustaining membrane potential (Sgarbi et al,. 2006). The impaired ATP synthesis mainly affects brain tissue and at high mutation load, up to approximately 95%, the heteroplasmic ATP6 gene mutations manifest as neuropathy, ataxia, retinitis pigmentosa (NARP) or as fatal encephalopathy known as Leigh syndrome (Houstek et al., 2006). Examples of quantitative defects are those in which the cellular content of the enzyme is reduced to less than 30%. Apparently, these disorders are caused by different nuclear genetic defects that remain to be identified, but most of them display a uniform fatal phenotype with onset in newborns characterized by lactic acidosis and hypertrophic cardiomyopathy (Houstek et al., 2006). In both types of ATP synthase disorders, hyperpolarization due to decreased ATP synthesis promotes ROS production by the respiratory chain, an event that can contribute to the clinical phenotypes as suggested by the beneficial effect of antioxidants observed in NARP cells (Mattiazzi et al,. 2004). This finding is quite important, considering that, in spite of the considerable progress in understanding of the molecular mechanisms of ATP synthase disorders, the available therapeutic approaches are still extremely limited (Kucharczyk et al., 2009). It has been proposed that other secondary effects possibly involved in the pathogenic pathways of ATP synthase deficiency could be changes in mitochondrial cristae morphology, which is mediated by ATP synthase oligomerization (Couoh-Cardel et al., 2010; Paumard et al., 2002), and/or a concomitant impairment of an ectopic function of ATP synthase localized on cell surface (see paragraph 4) (Kucharczyk et al., 2009).

Microarray analyses have been performed in an attempt to gain a more global view of the cellular consequences of ATP synthase deficiency. In fibroblast cell lines from 13 genetically heterogeneous patients, 1632 human genes involved in mitochondrial biology, cell cycle regulation, signal transduction and apoptosis have been analysed. Surprisingly, only minor changes in expression of ATP synthase related genes were shown. Moreover, the cellular gene expression phenotypes were different depending on the site (mtDNA vs nuclear DNA) and the severity (ATP synthase content) of the underlying defect, indicating the need for further investigation of these pathways in other ATP synthase disorders (Cížková et al,. 2008). As far as our knowledge is concerned, the proteomic profiles of ATP synthase-related diseases have not yet been reported.

Other intriguing examples of ATP synthase-related diseases are Batten disease in man or ceroid lipofuscinosis in sheep. Both are storage diseases with abnormal accumulation of subunit c in lysosomes occurring in the brain and liver, respectively. MS and protein sequencing have shown that the stored protein is structurally identical to the normal mitochondrial subunit c (Chen et al,. 2004).

Up- and down-regulation of ATP synthase biogenesis has been observed under different pathophysiological conditions (Houstek et al., 2006). We developed polyspecific antibodies directed against the whole human mitochondrial subproteome by hyperimmunization of rabbits with purified skeletal muscle mitochondria, which allowed detection of up-regulation of ATP synthase, in muscle biopsies from patients affected by MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes) which are characterized by a drastic reduction of OXPHOS complex 1 (Loro et al,. 2009). MS approaches have also been applied to compare the ATP synthase expression levels *in vivo* and *in vitro*. Recent examples of ATP synthase down-regulation have been obtained by the mitochondrial proteome analyses in heart from type 2 diabetic patients (Heather and Clarke, 2010) and in pancreatic β-cells exposed to high glucose (Ahmed et al,. 2010).

3. Mass spectrometry data of F_0F_1 ATP synthase

The whole F_0F_1 complex, as well as individual subunits or sub-complexes, have been purified by classical approaches and their amino acid sequences determined almost entirely by direct protein sequence analysis (Walker et al,. 1991). The detailed molecular structures of F_0F_1 from several species have been studied intensively by different groups (Chen et al,. 2006; Cingolani and Duncan, 2011; Dautant et al,. 2010), and the importance of these studies is highlighted by the award of the Nobel Prize to John Walker in 1997. The structural analyses by X-ray diffraction required the identification of the subunit composition by measurement of accurate molecular masses by mass spectrometry, allowing the detection of posttranslational modifications. The exploration of the exposed regions by limited proteolysis had similar analytical requirements.

Mass spectrometry of F_0F_1 has been a methodological challenge due to the presence of both hydrophilic parts and hydrophobic subunits, which are difficult to detect by standard ionization techniques, and the fact that all subunits are bound non-covalently. More than 15 years ago, reverse phase liquid chromatography methods were applied to purify the hydrophilic subunits and also some membrane-bound subunits (b, d, F6, e, f, g and A6L) of the bovine heart enzyme, so as to allow their molecular masses to be measured by a mass spectrometer with electrospray ionization (Collinson et al,. 1994). Over the years, direct identification of all the hydrophilic and most of the hydrophobic subunits of ATP synthase from many sources has been obtained by MALDI- and ESI-MS/MS analyses of the tryptic peptides of individual bands or spots on native polyacrylamide or SDS gels. However, the most hydrophobic membrane proteins, such as subunit c and A6L, could not be detected by these approaches (Wittig et al,. 2010). All of the hydrophobic subunits have been identified by tandem mass spectrometry after optimization of their purification in organic solvents and by fragmenting the intact protein ions by collision induced dissociation (CID) with argon (Carroll et al,. 2007). Moreover, a procedure that allows to measure the molecular masses of all of the 17 subunits of F_0F_1 from bovine heart in a single experiment has been published, this approach is based on the use of a mobile phase during liquid chromatography separation, in which the hydrophilic and hydrophobic components remained soluble, linked directly via an electrospray interface to a triple quadrupole mass spectrometer operated in positive ion mass spectrometry. The method has been used to characterize the ATP synthase subunits from a variety of species and to follow the progress of mild trypsinolysis of the enzyme (Carroll et al., 2009).

3.1 Phosphoproteome of ATP synthase

The measurement of the mass of a protein allows the presence but not the location of any posttranslational modifications (PTMs) to be detected. Phosphorylation of serine, threonine and tyrosine residues is one of the most prominent PTMs and a key regulator of nearly all biological processes including mitochondrial oxidative phosphorylation (Hüttemann et al,. 2007). However, phosphorylation is often a sub-stoichiometric process and usually only a low percentage of a given protein is present in phosphorylated state at a given time, making its observation challenging. In the past, phosphorylation analysis was mostly done by radiolabeling with $^{32/33}P$ (Bendt et al,. 2003; MacDonald et al,. 2002) combined with amino acid analysis. In the last decade, considerable effort has been devoted to improving the analysis of phospho-proteome by MS (Eyrich et al,. 2011; Gerber et al,. 2003). The main criticisms are ion suppression of phosphorylated species in a high background of non-phosphorylated ones,

specificity lack of the proteolytic cleavage and lability of phosphoester bonds during MS analysis. Different enrichment strategies for phosphorylated peptides or proteins, such as immunoaffinity chromatography (IMAC) or metal oxide affinity chromatography (MOAC), have been established to reduce sample complexity. Concurrently, attention has been paid to the LC-MS instrumentation to avoid loss of phosphorylated peptides within the analytical system. In addition, specific MS techniques have been developed for the identification and relative quantification of phosphorylation sites down to the femtomole range. Nevertheless, phospho-proteomics still remains far from being routine.

Regarding ATP synthase, in a recent phospho-proteomic study an improved protocol called BEMAD enabled to identify in a cytosolic lysate from mouse brain Ser76 of the F_1 subunit α as being phosphorylated (Vosseller et al,. 2005). This method involved differential isotopic labeling of O-phosphate-modified serine/ threonine residues through Michael addition with normal or deuterated dithiothreitol and enrichment of these peptides by thiol chromatography. Specificity of O-phosphate mapping was achieved by blocking of cysteine labeling by prior oxidation and by subsequent enzymatic dephosphorylation of O-phosphate-modified peptides. In a single mass spectrometry analysis along with αSer76 other 20 phosphorylation sites (5 previously reported) were identified and quantified.

MS allowed to identify phosphorylated tyrosine and serine residues in the F_1 subunits α and β from yeast, which was long considered a "zero background" organism for tyrosine phosphorylation (Krause-Buchholz et al,. 2006). A novel screening technique was applied in combination with Blue Native electrophoresis to separate the ATP synthase complex in native state and second dimension SDS-PAGE to resolve its subunit composition (see below). LA–ICP–MS (Laser ablation inductively coupled plasma mass spectrometry) was used to rapidly screen for the presence of phosphorus in the subunits using sulfur as the internal standard element for quantification. The subunits containing phosphorus were then identified by MALDI–FTICR–MS (matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry) as Tyr434, Ser 413 and Ser426 of the α subunit and Tyr7 of the β subunit (Krause-Buchholz et al., 2006).

At variance from yeast, by combining Blue Native electrophoresis and second/third dimension SDS-PAGEs with LC-ESI/MS analysis we found that in bovine heart mitochondria only the F_1 γ subunit contained one phosphorylated tyrosine (Di Pancrazio et al,. 2006). Moreover, the tyrosine residue was phosphorylated only in the monomeric form of ATP synthase and was present in low amount (about 6% of the total protein monomer), while the ATP synthase dimers were lacking. Interestingly, this finding suggested that the oligomerization process might be regulated through cell signaling (Di Pancrazio et al., 2006), but the pathway is still to be clarified. To obtain these results a novel procedure was developed because, due to the low percentage of the phosphorylated species, standard MS/MS analysis failed to detect phosphorylated peptides. The screening of the phosphorylated subunits was done by immunoblotting using anti-phosphotyrosine antibody after the third SDS-PAGE and after trypsin digestion the phosphorylated fragment of γ subunit was identified and quantified by a novel LC-ESI/MS method. This latter was based on the use of two different de-clustering potential values that allowed to obtain, with a single LC-ESI/MS run, the pattern of the phosphorylated and unphosphorylated species. These species were further analyzed by tracing back the origin of the HPO_3-deprived forms using tandem MS (Alverdi et al,. 2005).

Several other studies have shown that phoshorylation can occur on different sites of ATP synthase in mammals (HÃ¸jlund et al,. 2003; Ko et al,. 2002), yeast and plants (Struglics et

al,. 1998). Nevertheless, a comprehensive mapping is still lacking. The constant technological progress might soon enable to generate the quantitative and temporal phosphorylation pattern of the enzyme in all organisms and under different pathophysiological conditions, thus allowing understanding of the regulation of ATP synthase in light of cell signaling.

3.2 Native mass spectrometry

ESI-MS and the novel LILBID-MS (laser induced liquid bead ion desorption mass spectrometry) are two techniques that can be used under native conditions to determine the molecular mass of non-covalently assembled complexes up to the MDa-range with high accuracy. The techniques are complementary, LILBID being more tolerant than nESI to addition of detergents, which are necessary to solubilise membrane proteins such as ATP synthase. Very recently, LILBID-MS combined with Blue Native electrophoresis was successfully applied to compare the subunit composition of the whole F_0F_1 from *Bacillus pseudofirmus* and from bovine and human heart (Hoffmann et al,. 2010), but also to determine the subunit composition of other even larger membrane complexes such as the NADH-dehydrogenase (complex I of the OXPHOS system) from *Yarrowia lipolytica* (Sokolova et al,. 2010). LILBID-MS can be applied in several modes, from soft laser desorption (yielding the intact macromolecular complexes) to medium to high laser intensity (which disassembles the protein complexes partially into sub-complexes and these latter into the single subunits). The analysis of the bacterial enzyme revealed at low laser intensity the masses of the F_1 sub-complex, the F_1 sub-complex lacking the δ-subunit and the F_0 sub-complex, while at high laser energy the signal of the 8 subunits appeared. In the case of the mammalian enzymes, the spectra evidenced all of the 15 subunits, of which the masses agreed within ± 150 Da with theoretical masses (see Table 2). While functionally important subunits are conserved, others, such as the so-called minor F_0 subunits, show differences in their masses among the species. Determination of their masses from many sources might help to clarify their structural and functional roles, which are still only partially known, and LILBID-MS certainly offers a novel and rapid way to obtain such results using very low material and in detergent solution.

LILBID-MS has also provided an accurate determination of stoichiometry (c_n) of the sub-complex formed by the c-ring from the thermoalkaliphilic bacterium *Bacillus sp*. (Meier et al,. 2007). This result is particularly interesting, considering the difficulty to detect c subunit by classical MALDI-MS and LC-MS/MS (Bisetto et al., 2008; Wittig et al., 2010). Moreover, it represents an important MS application in cell bioenergetics, the number of c-subunits being in principle equals to the number of H^+ transported across the membrane for every 360° rotation of the rotor in which three ATP molecules are synthesized in the three β subunits. Hence the H^+/ATP ratio can be expressed by $c_n/3$.

At variance from LILBID, nESI is ideal for soluble complexes and we recently applied this technique to determine the exact molecular mass of the non-covalent complex formed in solution by the ATP synthase inhibitor IF_1 and Calmodulin, the archetypal EF-hand calcium sensor. Interestingly, nESI established a 1:1 stoichiometry between IF_1 and Calmodulin, suggesting that binding to Calmodulin promotes the dissociation of the pre-existing dimeric form of IF_1 (Cabezon et al,. 2001). Furthermore, native mass analysis was paralleled to the definition of the IF_1-CaM complex topology by combining limited proteolysis and cross-linking data with MALDI-MS and LC-MS/MS analyses (Pagnozzi et al,. 2010).

3.3 AQUA workflow

Native mass spectrometry is a powerful technique to define the stoichiometry of protein complexes, but this can be defined also by quantifying the absolute amounts of the different subunits and therefore by calculating their molar ratio. An interesting approach is the AQUA workflow, which is a variation of isotope dilution MS techniques used for decades for quantification of small molecules and more recently successfully applied in the proteomics context to the absolute quantification of proteins and their modification states in whole cell lysates (Gerber et al., 2003). This approach is based on the addition of synthetic isotopically labeled reference peptides in known amounts to a protein sample in solution prior to tryptic digestion and LC-MS analysis. Being gel-free, this approach avoids errors due to incomplete peptide extraction from the gel or impaired protein digestion within the gel matrix.

Regarding ATP synthase, we recently applied the AQUA workflow to determine the stoichiometry of the F_0 subunit e in bovine heart mitochondria (Bisetto et al., 2008). This subunit is involved in dimer/oligomer formation both in yeast (Fronzes et al., 2006) and mammals (Bisetto et al., 2008), but its stoichiometry was still unknown (Arakaki et al,. 2001; Hong and Pedersen, 2003). A critical point of the AQUA approach is the design of the heavy-labeled peptides, which must be unique to the proteins of interest and show high ionization efficiency (proteotypic peptides). In addition, such peptides should have a good fragmentation pattern with reliable matching of b- and γ- ion series, a preferable length between 7 and 15 amino acid residues and contain no chemically unstable residues (M,W, C or N-terminal Q or N) or unstable peptide bonds (e.g. D-P). Moreover, it is necessary to choose reference subunits, whose stoichiometry is already known, to validate the method. We have chosen the F_0 subunit b and the F_1 subunit γ as reference subunits which are present in single copies in the whole F_0F_1 complex, as defined by crystal structures. After having chosen among the proteotypic peptides characterized by LC-MS/MS three peptides - one from subunits e, b and γ-, the corresponding isotopically labeled analogues were added in known amounts to the detergent extracts of bovine heart mitochondria prior to tryptic digestion. The absolute quantification of the three subunits was then achieved by comparison of the areas under the curve (AUC) of the extracted ion chromatograms of the endogenous and labeled peptides in LC-MS mode. Accuracy of the method was demonstrated by confirming the 1:1 stoichiometry of subunit γ and b and by the low coefficients of variation which were <12% for technical and biological replicates. In the samples analyzed, which contained extracts of mitochondria in resting state, subunit e was present in 1:1 molar ratio with respect to subunit b or γ, demonstrating that in F_0F_1 it is contained as unique copy.

4. Native electrophoresis of F_0F_1 ATP synthase

Classical 2D IEF-SDS-PAGE do not resolve all the subunits of ATP synthase, because they are quite often small, hydrophobic and basic (pI>9). For this reason, in some studies MS analysis has been run on peptide mixtures obtained after in-solution trypsinization of sample extracts (Bisetto et al., 2008). Alternatively, a powerful approach for efficient separation from tissue homogenates, tissue biopsies and cell cultures of the whole complex of ATP synthase, as well as of assembly intermediates and supra-molecular structures is the native polyacrylamide gel electrophoresis after mild detergent extraction (Wittig et al,. 2006). Following native PAGE, proteins of interest can be extracted in native state and

analyzed by MS or electroblotted for immunodetection or analyzed by in-gel catalytic activity assays. In addition, subunit composition of the complexes can be identified by various denaturating techniques: SDS-PAGE, doubled SDS-PAGEs, as we used to detect phosphotyrosine in monomer/dimers of ATP synthase (Di Pancrazio et al., 2006) and IEF/SDS PAGEs (Wittig et al., 2006), as shown in Fig. 5. These advantages make this approach superior for functional proteomic analyses. For this reason, a brief introduction to native electrophoresis will be presented.

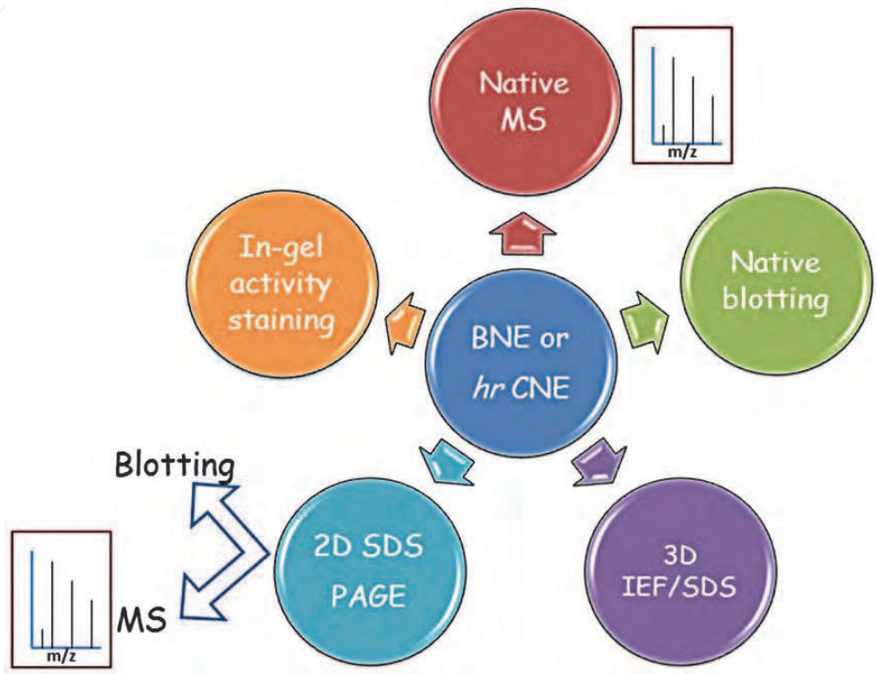


Fig. 5. Applications of native electrophoresis for functional proteomic analyses.

Blue native electrophoresis, abbreviated as BNE, was developed to isolate native membrane proteins and complexes on micro-scale. It separates proteins in the mass range of 10 kDa to 10 MDa. It is a one step technique to isolate proteins from mitochondria, whole cell lysates and tissue homogenates (Wittig et al., 2006). BNE has also been used for the identification of protein-protein interactions, as we recently did to define the new interaction between ATP synthase and Cyclophilin D in mammalian mitochondria (Giorgio et al,. 2009). Besides BNE a similar native electrophoresis method was developed that is called Clear native electrophoresis (CNE) with its variant high resolution CNE (*hr* CNE). Fig. 6 depicts the resolution of the five OXPHOS complexes including ATP synthase with its dimeric form obtained by BNE and *hr* CNE. All these methods are quite similar and have been used for MS analyses, but differ in few aspects, which are discussed below, with their drawbacks and applications.

From a practical stand point BNE, CNE and hr CNE differ in the composition of cathode buffers as well as in the mechanisms by which proteins migrate in the gel. What makes BNE different from CNE and *hr* CNE is the incorporation of Coomassie Brilliant Blue G-250 both in the cathode buffer as well as in loading dye. Coomassie is an anionic dye, it binds to the proteins and imparts negative charge over their surface. In this way proteins migrate with

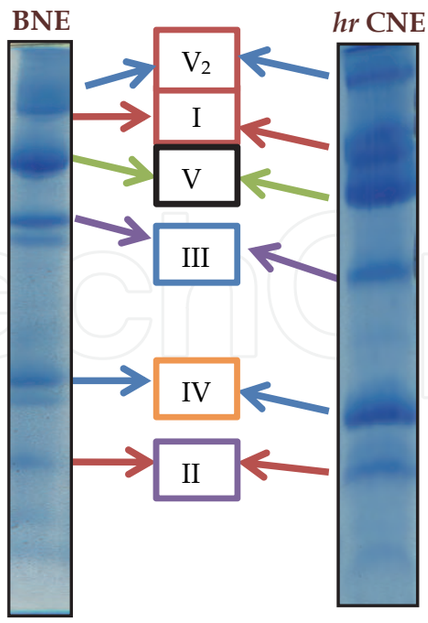


Fig. 6. BNE and *hr* CNE of DDM extracts of bovine heart mitochondria showing OXPHOS complexes.

respect to their native masses independently of their p*I*. Negative charge also helps in preventing protein aggregation as negative charges repel each other and this is the reason for its very good resolution. Furthermore, upon binding the previously detergent-solubilised membrane, proteins lose their hydrophobic character and become water soluble hence no further detergent is required in these gels minimising the risk of detergent-dependent protein denaturation (Wittig et al., 2006). Besides Coomassie, the presence of Imidazole in anode and cathode buffer (BNE, CNE, *hr* CNE) helps in maintaining pH in the range 7.0-7.5, and incorporation of high concentration of 6-aminohexanoic acid (a zwitterionic substance) improves the solubilisation of membranes (Wittig and Schägger, 2008). Separation of proteins as blue bands helps in gel excision and recovery of blue stained native proteins by electroelution (Wittig et al., 2006) for further MS analysis, as recently applied for LILBID MS (Hoffmann et al., 2010).

A rapid way to identify ATP synthase in BNE is to monitor ATP hydrolysis by in-gel activity staining, which was developed in our lab and is based on the formation of a white lead phosphate precipitate from phosphate (Pi) released during the reaction (Fig. 7) (Zerbetto et al,. 1997). The catalytic activity can be obtained by incubating the gels in glycine buffer supplemented with Mg-ATP in the presence of 0.2% $Pb(NO_3)_2$. The native staining of ATP synthase is reproducible and the white bands on gel can be easily quantified by densitometry (Bisetto et al., 2007). Moreover, the bands can be excised and easily destained in acetic acid solution giving a colourless protein complex ready for MS analysis. This method had been successfully applied by our group to analyse skeletal muscle and heart biopsies from patients with oxidative phosphorylation enzyme deficiencies (Zerbetto et al., 1997)

Detection limits of in-gel activity staining are in the microgram range of protein or micromolar phosphate and the resulting white bands are challenging for detection and documentation of low activity due to interference of Coomassie dye. Different strategies of optimization for activity staining of BNE have been applied by us (Bisetto et al., 2007) and

others (Suhai et al,. 2009; Wittig and Schägger, 2005). Alternatively, CNE can be used that was introduced to circumvent this disadvantage of BNE. In fact, it uses the same buffers and conditions for electrophoresis but the difference lies on the absence of Coomassie dye both in cathode buffer as well as in sample buffer. In this way, much higher activity staining has been obtained (Wittig et al,. 2007). However, due to the absence of Coomassie there is no negative charge shift and the movement of proteins totally depends on their intrinsic mass and p*I*. Therefore CNE suffers from a poor resolution and is limited to proteins having a p*I*<7 unlike BNE where even proteins having pI>10.5 migrate towards anode (Wittig and Schägger, 2008). Anyway, CNE offers advantage over BNE for isolation of supra-molecular structures, including ATP synthase dimers and oligomers, being the mildest technique to separate mitochondrial membrane proteins.

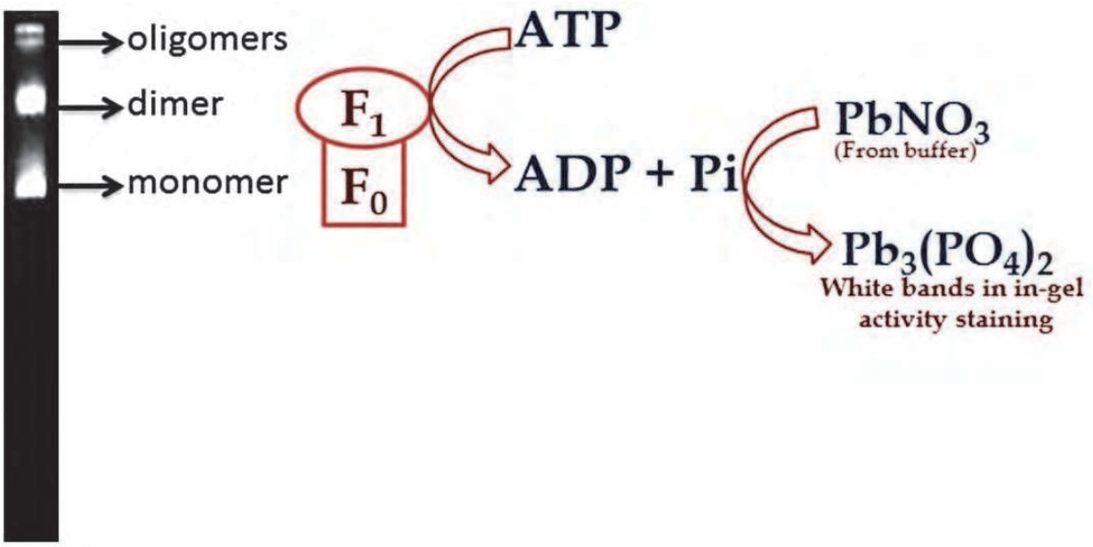


Fig. 7. In-gel ATPase activity staining of the different oligomeric forms of ATP synthase extracted by Digitonin (0.5 g/g protein) from mouse heart mitochondria and analyzed by BNE.

To preserve the advantages of both the techniques Wittig et al introduced *hr* CNE. In this technique cathode buffer is supplemented with a combination of colourless anionic and neutral detergents such as Triton X-100, Deoxycholate, or Dodecyl-β-D-maltoside (DDM). This leads to a charge shift over the surface of the proteins and helps them in migration with a resolution comparable with BNE. Also there is no interference in in-gel assays due to absence of Coomassie.

An interesting recent application of *hr* CNE is related to the molecular characterization of assembly intermediates of ATP synthase in mammals. In fact, using particularly mild detergent conditions and *hr* CNE, Wittig et al. were able to separate the assembly intermediate in human ρ_0 cells, which lack the mitochondrial DNA encoding subunits a and A6L (Wittig et al., 2010). By analyzing its subunit composition by ESI-MS/MS after excising the native band from 1D *hr* CN-PAGE or by MALDI-MS (MS/MS) after 2D *hr* CN-/SDS-PAGE they established that this intermediate contains all the nuclear-encoded subunits. These results allowed to propose that in mammals, differently from yeast (Hoffmann et al., 2010), the assembly of the whole enzyme is a linear process and the subunits of the lateral

stalk combine with the F_1 sector and the c-ring independently from the mitochondrial encoded subunits a and A6L which bind at the late stage. Conversely it has been proposed that in yeast the subunits Su6/Su8, homologous to subunits a and A6L, first combine to the subunits of the lateral stalk, forming the sub-complex Su6/Su8/stator, which finally binds to the sub-complex F_1/c-ring, as already shown in Fig. 3. Moreover, the finding that ρ_0 mitochondria still contain dimers/oligomers of ATP synthase although in lower amounts than control mitochondria, supported the idea that subunits a/A6L contribute, together with subunits e/g (Bisetto et al., 2008), to stabilize the supra-molecular structures, but they are not the most important interface as previously proposed in yeast (Wittig et al., 2008).
Recently Yan et al., using rat brain mitochondria, demonstrated that a non-gradient highly porous BNE of 8% polyacrilamide is an efficient technique to resolve all OXPHOS complex along with other mitochondrial proteins, such as DLDH and Hsp60 polymer. Further, the gel strips can be even used to perform 2D BN-/SDS PAGE or the bands can be excised for MS peptide sequencing (Yan and Forster, 2009).

4.1 Types of detergents and their use in native electrophoresis

The choice of detergent for protein extraction is an integral part of a successful native electrophoresis. The principal effect of detergents during solubilisation is the breaking of lipid-lipid and lipid-protein interactions present on biomembranes. Competing with lipids for the occupation of the surface of integral hydrophobic proteins, they form mixed micelles containing detergent, lipids and proteins. The solubilisation effect is maximum when the detergent is used at a concentration equal or higher to the Critical Micelle Concentration (CMC), the concentration at which the detergent molecules form micelles. These are detergent self associating structures with hydrophobic ends facing inside and hydrophilic groups facing outside the aqueous phase. CMC is a characteristic of each detergent and depends on pH, temperature and ionic strength (Reisinger and Eichacker, 2008).
The anionic SDS is, in principle, not suitable for native electrophoresis as besides solubilising membranes, it dissociates and denatures the enzyme complex leading to loss of activity. Non-ionic detergents are uncharged and milder and hence can be used in membrane solubilisation to isolate mitochondrial complexes with varying degrees of association dependent on the kind of detergent used and its concentration. The most commonly used non-ionic detergents are Triton X-100, Digitonin and Dodecyl-β-D-maltoside (DDM) which form micelles at very low concentration, thus avoiding protein denaturation (Wittig and Schägger, 2008). Regarding isolation of OXPHOS complexes, these non-ionic detergents behave very differently from each other. For example, Digitonin can be used in a very broad concentration range (from 0.5 to 8 g/g proteins), as compared to Triton and DDM which work in the range of 1-2 g/g proteins (Schägger and Pfeiffer, 2000). Besides this, Digitonin is the best candidate for the isolation of supercomplexes due to its milder nature (Reisinger and Eichacker, 2008). They can be used to extract OXPHOS complexes from bacteria, yeast and mammals, as well as from subcellular fractions or total membranes (Wittig et al., 2006). The quantity of detergent required to solubilise membrane proteins vary in the different cells/tissues and optimal solubilisation conditions for each membrane and each membrane complex should be experimentally investigated.
Recently, an interesting modification of classical native PAGE has been proposed. Klodmann et al. reported the treatment of the samples with low amounts of SDS before BNE. This allowed to destabilize the OXPHOS complexes in sub-complexes in a very defined and reproducible manner and to study their internal architectures. SDS was added

to the mitochondrial Digitonin extracts from *Arabidopsis* in the range of 0.05-1.0% just before BNE loading. By combining with 2D BN-/ SDS or 2D BN/BN PAGE, the authors clearly demonstrated the variable effect of SDS on the OXPHOS complexes. As an example, at 0.2% SDS concentration, the ATP synthase complex dissociated in to F_0 and F_1. At 0.3% F_0 even dissociated in to a sub-complex composed of c-ring (Klodmann et al,. 2011).

5. Ectopic F_0F_1ATP synthase of mammalian cells

The application of proteomic analyses to sub-cellular mammalian fractions other than mitochondria revealed the presence of mitochondrial membrane components in unexpected cellular locations, such as plasma membranes or nuclei. Some of these studies ascribed it to cross-contamination, due to the contiguity of the different membranes within the cell and to the high sensitivity of MS which identifies the proteins up to subfentomolar levels, but in others cases the parallel demonstration of such unusual locations obtained in cells and tissues by immunofluorescence and functional studies led the scientists to consider the proteomics results a mainstay to discover new scenarios in the intracellular traffic connections.

Regarding ATP synthase, many proteomics studies have recently reported that subunits of this complex, along with other OXPHOS complexes, are expressed in extra-mitochondrial membranes of different mammalian cell types – especially on the cell surface, but also in the endoplasmic reticulum and nuclear envelope (Panfoli et al,. 2011). In human apoptotic T-leukemia cells the presence of eight ATP synthase subunits was revealed in the nucleus fraction obtained by differential extraction and stable isotope labeling of cell culture followed by LC-MS/MS analysis and it has been ascribed to a dynamic recruitment of mitochondria into nuclear invaginations during apoptosis (Hwang et al,. 2006). In mouse brain a proteomics analysis of microsomal fraction obtained both by 2D-LC-MS/MS and shotgun LC-MS/MS found many subunits of the OXPHOS complexes, including ATP synthase, which were proposed to represent mitochondrial proteins with high turnover rates in the cell (Stevens Jr et al,. 2008). Conversely, ATP synthase is now considered a true resident on the plasma membranes. In fact, the identification by MS of ATP synthase subunits in plasma membrane preparations from different sources has been paralleled by the demonstration of the enzyme expression with the F_1 sector facing outside (and for this reason the enzyme is named ecto-F_0F_1) obtained by cytometry, confocal microscopy and functional studies (Vantourout et al,. 2010). ATP synthase subunits were identified in plasma membranes isolated from cell culture, i.e. in hypoxia-adapted tumor cells where differential $^{16}O/^{18}O$ stable isotopic labeling and multidimensional LC-MS/MS revealed an increased expression of ATP synthase α subunit with respect to normoxia (Stockwin et al,. 2006), and in tissues that were characterized by high purity, i.e. obtained by combining subcellular fractionation with immunoisolation strategies so that no proteins from endoplasmic reticulum and nuclear envelope were detected (Zhang et al,. 2007). In other studies, the presence of ATP synthase subunits was found in the detergent-resistant fragments of plasma membranes, i.e. in the lipid rafts which are cholesterol and sphingolipid-rich microdomains involved in signal trasduction. In particular, in lipid rafts isolated from rat liver and subjected to an efficient *in solution* digestion followed by cRPLC/MS/MS four subunits both of F_1 and F_0 sector were identified (Bae et al,. 2004).

The major limit of the proteomic studies related to plasma membranes is that in no one the complete subunit composition of ecto-F_0F_1 has been recognized, leaving open the possibility of a different assembly of the ectopic enzyme with respect to the mitochondrial ATP

synthase. We recently separated by detergent extraction and BNE the whole ecto- F_0F_1 from plasma membranes of rat liver (Giorgio et al,. 2010) and we found that the low, but constant amounts of F_0F_1 complexes display a similar molecular weight to the monomeric form of the mitochondrial F_0F_1 ATP synthase, as evidenced by in-gel ATPase activity staining and immunoblotting. This suggests that the plasma membranes of normal liver do contain complete, functional F_0F_1 ATP synthase complexes, which display very similar subunit composition and assembly of the mitochondrial enzymes for which MS analysis is in progress in our laboratory.

All together these studies support the view that ATP synthase is mainly located in lipid rafts of plasma membranes, is enzymatically active and functions as a cell-surface receptor involved in different biological effects depending on the cell types (Fig. 8). In hepatocytes ecto-F_0F_1 functions as high affinity ApoA1 receptor and regulates HDL metabolism, in endothelial cells it functions as angiostatin receptor thus mediating angiogenesis, in tumour cells it functions as a pH regulator and participates in tumor recognition by cytotoxic Vγ9/Vδ2 T lymphocytes (Vantourout et al., 2010). Various independent studies have reported that ecto-F_0F_1 can synthesize ATP from ADP and Pi extruding protons from cytoplasm in different cell types, such as endothelial cells or hepatocytes. The resulting ATP can triggers cation influx into the cells through ATP-gated ion channels (P2X purinoreceptors) or can bind to G-protein coupled receptors (P2Y purinoreceptors) activating a downstream signaling pathways (Mowery and Pizzo, 2010). However, whether or not ecto-F_0F_1 can synthesize ATP is still debated, as there are several conflicting reports. Conversely, it is widely accepted that ecto-F_0F_1 catalyses the hydrolysis of ATP, potentially affecting purinergic signaling (Fig. 8). As an example, in hepatocytes, ADP generated by

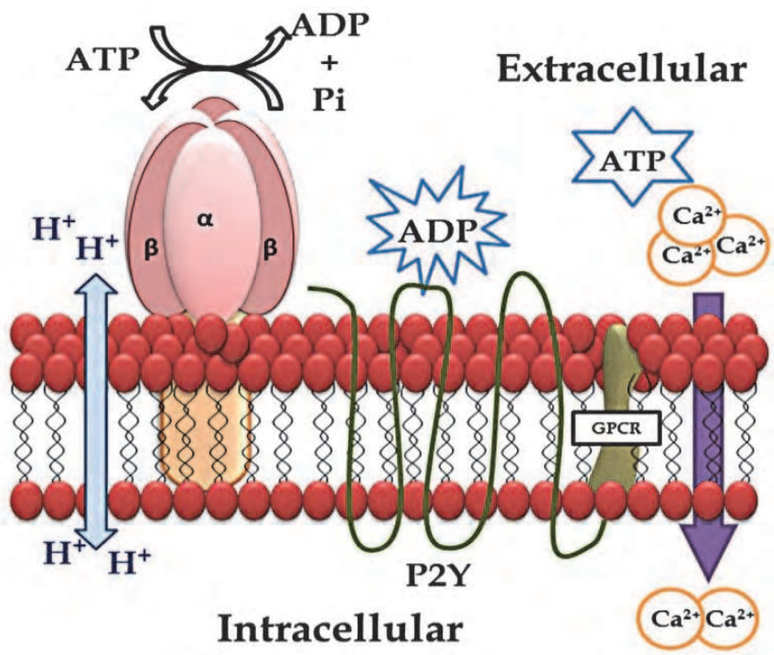


Fig. 8. The model depicts the orientation of ectopic F_0F_1 ATP synthase in eukaryotes with the F_1 sector facing outside. In some cells the ATP synthesized by the enzyme leads to influx of cations such as Ca^{2+} through P2X receptors and in others the ADP generated by ATP hydrolysis triggers signaling pathways through activation of G-protein coupled P2Y receptors.

ecto-F_0F_1 upon binding of ApoA1 activates the P2Y receptors resulting in HDL endocytosis and downstream the small GTPase RhoA and its effector ROCK I (Malaval et al,. 2009).
The mechanism used by ATP synthase to reach the plasma membrane is still unknown (Vantourout et al., 2010). The hypothesis that the single ATP synthase subunits are routed to plasma membrane instead of the mitochondria seems unlikely, because different mRNA isoforms of ATP synthase subunits have not been found in mammals apart for the bovine subunit c (Vives-Bauza et al., 2011). The simpler explanation seems that once assembled into mitochondria, the whole complex reaches the cell surface *via* vescicular transport or fusion of mitochondrial membranes with plasma membranes (Vantourout et al., 2010). It is tempting to hypothesize that the new technology of imaging MS (MALDI MS profiling/imaging), which can acquire individual spectra from the surface of frozen tissue sections (Chaurand et al,. 2006), could give important answers regarding the trafficking of the enzyme to cell surface.

6. Conclusions

F_0F_1 ATP synthase is an intensely studied enzyme complex, for which single molecule studies have allowed to define the fascinating catalysis in great detail. In addition, high-resolution molecular structures have been obtained mainly by X-ray crystal analyses. In spite of this tremendous progress, many aspects of ATP synthase physiology, such as biogenesis or super-complex formation, and its role in pathology are still unknown. The omni-comprehensive nature of proteomics, unlike the more reductionistic approaches of classical biochemistry and genetics, makes it the best candidate for revealing changes in the expression level of the whole complex and/or of its single subunits, but also to define the quantitative and temporal phosphorylation pattern of the enzyme in all organisms and under different physiopathological conditions, thus allowing the understanding of the ATP synthase regulation in a better way. In addition, the constant technological progress will enable to define the intriguing enzyme intracellular trafficking and its translocation to cell surface. In this context, native electrophoresis combined with MS techniques offers a powerful top-down approach for functional and structural analyses of such complicated enzyme using minimal amount of cell lysates or tissue homogenates and making this approach useful also for clinical investigation.

7. Acknowledgements

Authors acknowledge Prof. Paolo Bernardi, Dept. of Biomedical Sciences, University of Padova and Dr. Paola Picotti, Institute of Biochemistry, ETH, Zürich for critical reading of the manuscript.

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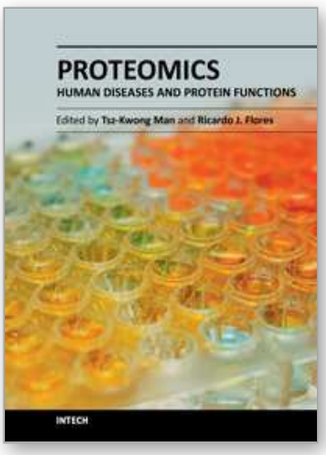

Proteomics - Human Diseases and Protein Functions

Edited by Prof. Tsz Kwong Man

ISBN 978-953-307-832-8
Hard cover, 438 pages
Publisher InTech
Published online 10, February, 2012
Published in print edition February, 2012

Biomedical research has entered a new era of characterizing a disease or a protein on a global scale. In the post-genomic era, Proteomics now plays an increasingly important role in dissecting molecular functions of proteins and discovering biomarkers in human diseases. Mass spectrometry, two-dimensional gel electrophoresis, and high-density antibody and protein arrays are some of the most commonly used methods in the Proteomics field. This book covers four important and diverse areas of current proteomic research: Proteomic Discovery of Disease Biomarkers, Proteomic Analysis of Protein Functions, Proteomic Approaches to Dissecting Disease Processes, and Organelles and Secretome Proteomics. We believe that clinicians, students and laboratory researchers who are interested in Proteomics and its applications in the biomedical field will find this book useful and enlightening. The use of proteomic methods in studying proteins in various human diseases has become an essential part of biomedical research.

How to reference

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Federica Dabbeni-Sala, Amit Kumar Rai and Giovanna Lippe (2012). F0F1 ATP Synthase: A Fascinating Challenge for Proteomics, Proteomics - Human Diseases and Protein Functions, Prof. Tsz Kwong Man (Ed.), ISBN: 978-953-307-832-8, InTech, Available from: http://www.intechopen.com/books/proteomics-human-diseases-and-protein-functions/f0f1-atp-synthase-a-fascinating-challenge-for-proteomics

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