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Reviews

The ATP synthase (F_0-F_1) complex in oxidative phosphorylation

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Abstract. The transmembrane electrochemical proton gradient generated by the redox systems of the respiratory chain in mitochondria and aerobic bacteria is utilized by proton translocating ATP synthases to catalyze the synthesis of ATP from ADP and P_i. The bacterial and mitochondrial H^+-ATP synthases both consist of a membranous sector, F_0, which forms a H^+-channel, and an extramembranous sector, F_1, which is responsible for catalysis. When detached from the membrane, the purified F_1 sector functions mainly as an ATPase. In chloroplasts, the synthesis of ATP is also driven by a proton motive force, and the enzyme complex responsible for this synthesis is similar to the mitochondrial and bacterial ATP synthases. The synthesis of ATP by H^+-ATP synthases proceeds without the formation of a phosphorylated enzyme intermediate, and involves co-operative interactions between the catalytic subunits.

Key words. ATP synthase; oxidative phosphorylation; mitochondrial ATPase; bacterial ATPase; F_0-F_1-ATPase.

Background

In eukaryotic cells and aerobic bacteria, most of the ATP utilized in endergonic reactions is synthesized via oxidative phosphorylation. The enzyme which catalyzes the synthesis of ATP, in the final step of oxidative phosphorylation, is located in the inner membrane of mitochondria, and in the plasma membrane of bacteria. Although its main function is the synthesis of ATP from ADP and P_i, it may also catalyze the reverse reaction, i.e. ATP hydrolysis. Therefore, the terms H^+-ATP synthase and H^+-ATPase are used interchangeably.

Our knowledge of the properties of H^+-ATPases has evolved by stages. For many years, oxidative phosphorylation was studied exclusively in mitochondria and submitochondrial particles. The isolation of a soluble ATPase from beef heart submitochondrial particles was an important breakthrough. The demonstration that this enzyme was able to restore ATP synthesis in non-phosphorylating particles led to its being called coupling factor 1, F_1-ATPase, or simply F_1[107]. Later it was recognized that F_1 is one of two sectors of the H^+-ATP synthase complex. The extramembranous F_1 is detachable from the membrane, whereas the other sector, F_0, is embedded in the membrane (fig. 1). The term F_0 was introduced to indicate that the membranous sector binds oligomycin, an antibiotic recognized as an inhibitor of ATP synthesis. By analogy, the term F_0 was applied to the membranous sector of *E. coli* H^+-ATPase, although the latter enzyme is only weakly inhibited by oligomycin[104]. Whereas F_1 is responsible for catalysis, F_0 is organized in such a way that protons can pass through it easily.

The chemiosmotic theory was elaborated in parallel with topographical studies, and it explained how the proton motive force generated by the functioning of the respiratory chain in mitochondria or bacteria is used, via the channeling of protons through the F_0 sector of H^+-ATPase, to drive ATP synthesis at the catalytic site of F_1, without the need for a high energy chemical precursor[85]. However, it is now assumed that protons do not directly interact with substrates to promote the coupling between P_i and ADP as proposed by Mitchell[86], but rather promote substrate binding and/or product release. This latter view is supported by the finding that binding changes are conformationally transmitted between F_0 and F_1[83,100,130].

During the last ten years, further advances in the analysis of the structure and function of the F_0-F_1-ATPases have been made, resulting from the combined use of physical, chemical and genetic techniques. The amino acid sequences of the different subunits of the two sectors have been determined. The stoichiometry of the subunits, and their relative topographical relationships, have been assessed. The catalytic site has been found to be located in one of the five types of subunits composing F_1, namely the β subunit, and the identification of strategic amino acid residues has been undertaken by chemical modification, and, more recently, by site-directed mutagenesis. From kinetic experiments, the idea has emerged that the main energy-requiring step in ATP synthesis is not the chemical condensation of P_i and ADP within the catalytic site, but rather a conformational change of the catalytic site which results in release of the newly synthesized ATP. A cooperative mechanism based on interactions between the three β subunits (catalytic subunits) has been postulated (multisite catalysis) to explain how ATP is continuously synthesized from medium ADP and P_i. At present, much effort is being directed towards the understanding of the mechanism of coupling between the H^+ flux in the F_0 channel and ATP synthesis. In this short review, we shall focus on recent data on the structure and the functioning of the mitochondrial and bacterial ATPases. For the sake of clarity, we shall refer mainly to *Escherichia coli* and beef heart H^+-ATPases as representative of bacterial and mitochondrial H^+-ATPases, respectively. Only some references concerning the chloroplast enzyme will be quoted. For more details on earlier studies, and comparison with different kinds of ATPases, the reader should refer to the excellent review by Penefsky and Cross[103].

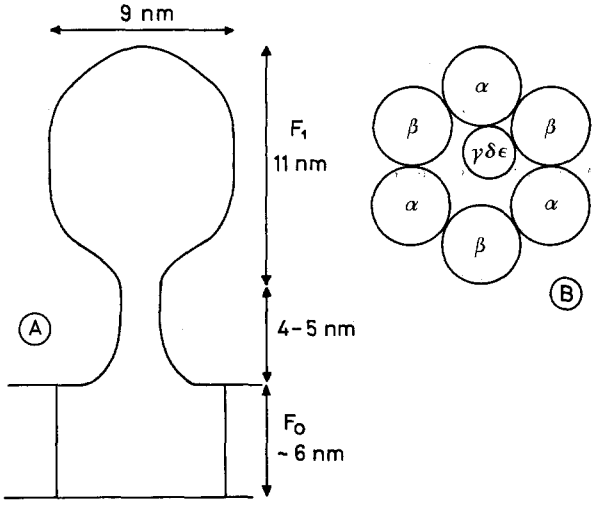

Figure 1. Schematic representation of membrane-bound H^+-ATPase (*A*) and soluble F_1-ATPase (*B*).

Current status

Structural data on mitochondrial and bacterial H^+-ATPases

Subunit composition and stoichiometry. During the past ten years, a number of comparative studies of H^+-ATPases from bacteria, and yeast or mammalian mitochondria, have been made. The overall subunit composition and stoichiometry of these F_0-F_1 ATPases are similar.

Table 1. Molecular masses of the 8 subunits from *E. coli* F_0-F_1 ATPase

Subunits	F_1 sector					F_0 sector		
	α	β	γ	δ	ε	a	b	c
Molecular mass (kDa)	55.2	50.2	31.4	19.3	14.9	30.3	17.2	8.3
Stoichiometry	F_1: $\alpha_3\,\beta_3\,\gamma\delta\varepsilon$					F_0: a, b_2, c_{10-15}		
Total mass	382 kDa					160 kDa		

The *E. coli* enzyme is a multimeric complex which consists of eight types of subunit[117,131]. The F_1 sector is composed of five types of subunit, referred to as α, β, γ, δ and ε, and the F_0 sector contains three types of subunit known as a, b and c. The stoichiometry of the *E. coli* enzyme is $\alpha_3\,\beta_3\,\gamma_1\,\delta_1\,\varepsilon_1\,a_1\,b_2\,c_{10-15}$[37,129]. The primary structure and the precise molecular masses of all the subunits have been determined by DNA sequencing[131]. The total molecular mass of F_1 is 382 kDa and that of the entire F_0-F_1 complex is ca 540 kDa (table 1).

The structural organization of the mitochondrial enzyme is more complex than that of the *E. coli* enzyme. For example, the mitochondrial beef heart F_0-F_1 complex consists of at least fourteen different subunits[132], i.e. in addition to the eight subunits present in the *E. coli* enzyme, namely $\alpha\,\beta\,\gamma\,\delta\,\varepsilon$, a, b, c, at least six new subunits are present in the beef heart F_0-F_1. These extra subunits are called A6L, d, e, the natural inhibitor (IF_1), the oligomycin-sensitivity conferring protein (OSCP) and F6[132]. Bovine mitochondrial F_0 is composed of at least four genuine transmembranous components: subunits a (also known as subunit 6), b, c (also known as subunit 9) and A6L. On the basis of their hydrophobic characteristics, the mitochondrial subunit b and the *E. coli* subunit b are probably related. The beef heart mitochondrial subunit A6L, identified as chargerin II in rat liver[56] and also related to the subunit 8 in *Saccharomyces cerevisiae*[125], has no equivalent in *E. coli* F_0. Likewise, bovine d and e subunits have no *E. coli* equivalents.

Topographical data. The shape of the H^+-ATPase complex has been determined by electron microscopy. The face of the mitochondrial inner membrane exposed to the matrix space and that of the bacterial membrane exposed to the cytoplasm are lined with knobs of 9–11 nm diameter, which correspond to the F_1 sector. In unstained samples of *E. coli* F_1-F_0 ATP synthase, the protruding F_1 appears to be linked by stalks to the membrane-embedded F_0 sector[47]. The majority of front view projections of electron micrographs of isolated F_1 particles shows a hexagonal arrangement of six large peripheral masses, corresponding to the major subunits α and β. At the center, an aqueous cavity extends nearly through the length of the F_1 complex[48].

The existence of an alternating distribution of the α and β subunits was clearly shown by immunodecoration with specific antibodies directed against α and β subunits[82]. It was suggested that the α and β subunits display a staggered arrangement[9] or overlap in two layers[123,124]. The minor subunits (γ, δ and ε) are located in the central cavity of F_1, and they partly obstruct it. The way they interact with the major α and β subunits is not fully understood[122]; any off-centered position might lead to a structural and possibly functional asymmetry of the molecule.

Early cross-linking studies with bifunctional reagents showed that in mitochondrial F_1, the α subunits are close to each other and to the β subunits. In contrast, the β subunits were found to be apart from each other[12,113]. Consistently with this rough observation, the X-ray diffraction analysis of the quaternary structure of the rat liver mitochondrial F_1 at 3.6 Å revealed an arrangement of interacting α-β subunits with three-fold symmetry. The α and β subunits each exist as a trimeric layer, with the β subunits interacting strongly with the α subunits but little or not at all with each other[8]. Chemical cross-linking experiments with the detection of the following dimers $\beta\gamma$, $\alpha\delta$, $\beta\varepsilon$ and $\gamma\varepsilon$ provided additional information concerning the spatial arrangement of the minor subunits within F_1[12,78]. Mg^{++}-induced dissocation of the F_1 subunits[137], reconstitution experiments[28,32], and direct measurement of binding parameters between isolated subunits[26], have also revealed close contacts between α and γ, β and γ, α and δ, β and δ as well as between γ and ε. In beef heart mitochondrial F_1, interactions between IF_1 and the β subunits as well as interactions between OSCP and both β and α subunits have been demonstrated by cross-linking[29,30,69,70].

In F_0, all the subunits contain hydrophobic portions that span the lipid core of the membrane[91]. In *E. coli* F_0 it is generally assumed that these subunits are arranged in the form of a crown made up of a number of c subunits with the a and b subunits located within this structure. Subunit a contains at least five transmembrane α-helices, and subunit c is an extremely hydrophobic molecule which forms a hairpin, with the two branches spanning the membrane. The latter protein is also referred to as the dicyclohexyl-carbodiimide (DCCD) binding protein[7]. Subunit b is an amphiphilic protein, which crosses the membrane only once, and has a protruding hydrophilic C-terminal portion which is involved in the link with the F_1 sector[58]. In mitochondria the subunit OSCP and factor F_6 are thought to constitute with subunit b, the stalk linking F_1 to F_0 in mitochondria. As a matter of fact, OSCP confers oligomycin sensitivity to the F_1 sector even though oligomycin binds to F_0. The precise localization and function of A6L, F_6, d and e are not known.

Catalytic properties of F_1

Isolated F_1 preferentially hydrolyzes ATP[107], but other nucleoside triphosphates are also hydrolyzed. Magnesium ions are necessary for efficient catalysis, and in the absence of Mg^{++} the enzyme turnover is reduced by more than 100,000 fold[36]. Feldman and Sigman[34] reported that enzyme-bound ATP was synthesized by chloroplast F_1-ATPase from tightly-bound ADP and medium P_i provided the enzyme was incubated in buffer containing a high concentration of P_i. When solubilized in a medium supplemented with the aprotic solvent dimethyl sulfoxide, isolated F_1 or F_0-F_1 complexes from thermophilic bacteria[142,143], and mitochondrial F_1[112], are able to synthesize small amounts of ATP, which remains trapped in the catalytic site. In the latter case, the ratio of bound ATP to F_1 (mol/mol) reaches values close to 0.5. Additional evidence that isolated F_1 has the potential to synthesize ATP stems from the two following observations. 1) During ATP hydrolysis, F_1 catalyzes the incorporation of more than one ^{18}O from $H^{18}OH$ into P_i, a result which reflects an in-site reversible synthesis of ATP bound to F_1[17]. 2) Incubation of isolated F_1 with the metallocomplex Cr(III)ADP and $^{32}P_i$ results in the formation of the ternary complex P_i-Cr(III)ADP bound to F_1[10].

Although isolated F_1 has the potential to synthesize ATP, it is unable to ensure the steady state synthesis of ATP from medium ADP and P_i. In the steady accumulation of ATP which is catalyzed by the F_0-F_1 complex during oxidative phosphorylation, the condensation of bound ADP and P_i to form bound ATP does not require energy, but the endergonic steps are 1) the tight binding of substrates ADP and P_i to F_1, 2) the release of bound ATP from F_1 to the medium. These two steps consume the energy of the electrochemical proton gradient, which is generated across the coupling membrane by the redox systems of the respiratory chain of mitochondria and bacteria. Furthermore, substrate binding and product release occur simultaneously, on separate but interacting catalytic sites.

Number of substrate binding sites in F_1-ATPase. The maximum number of nucleotide binding sites has been shown to be six in F_1 isolated from *E. coli*[61,104], bovine heart mitochondria[21,135] and chloroplasts[46,141]. In the case of *E. coli* and bovine heart mitochondrial F_1, three out of these six binding sites rapidly exchange their bound nucleotides[21,61,104]. This exchange occurs, for example, during ATP hydrolysis in the presence of Mg^{++}, which is consistent with the hypothesis that exchangeable sites are potential catalytic sites. The three non-catalytic sites are highly specific for adenine nucleotides[104] although they also bind some base-modified analogs of ADP, like the photoactivable derivative $2N_3$ ADP[68] and the fluorescent derivative lin benzo ADP[136]; they do not exchange their nucleotides during ATP hydrolysis. Similar behavior of the nucleotide-binding sites has been reported for chloroplast F_1 and rat liver mitochondrial F_1[141].

A typical feature of many isolated F_1-ATPases is the presence of tightly bound nucleotides. Although non-covalently bound to the enzyme, these nucleotides remain associated with the enzyme during gel filtration, charcoal treatment and repeated ammonium sulfate precipitations[52,54]. When purified beef heart mitochondrial F_1, stored as an ammonium sulfate suspension in the presence of EDTA and ATP, is carefully desalted, it retains three tightly bound nucleotides[45,62,68,80,121]. Binding experiments have revealed that two out of these three nucleotides are bound at non-exchangeable sites and one at an exchangeable site[68,84]. Sieve chromatography in the presence of a high concentration of glycerol promotes the release of the tightly bound nucleotides[45].

Early titration studies revealed the presence of one major P_i binding site in native mitochondrial F_1, which was characterized by a K_d value close to 80 μM[99]. However, it is noteworthy that F_1-ATPase depleted of tightly bound nucleotides loses the ability to bind inorganic phosphate[71]. Binding of P_i to the native mitochondrial F_1 is prevented by nucleotides[66], which suggests that the P_i and nucleotide binding sites are close to each other. This is in agreement with the fact that covalent binding of the photoactivable derivative of P_i, azido nitrophenyl phosphate (ANPP) upon photoirradiation[73] results in the labeling of amino acids located at the level of the nucleotide binding sites[44].

Location and role of the different substrate binding sites in F_1. The F_1 subunits can be dissociated from each other by mild treatments. One of them is the incubation of F_1 with concentrated saline solutions in the cold. From equilibrium binding studies with various ligands it has emerged that α and β are the major functional subunits. In *E. coli*, each isolated α or β subunit contains a single nucleotide binding site[27,60,109]. This is also true for the α and β subunits of F_1 from the thermophilic bacterium PS3[94,110]. In brief, in the case of bacterial F_1, the six nucleotide binding sites are distributed on the 3 α and 3 β subunits of the enzyme.

The purified *E. coli* α subunit binds ATP and ADP in a magnesium-independent manner, with K_d values of 0.1 and 0.9 μM respectively, but does not bind GTP or ITP[27,104]. On the basis of chase experiments performed with nucleotide-depleted or native F_1, it has been inferred that non-exchangeable nucleotide binding sites in *E. coli* F_1 are located in the α subunits[61,104]. Moreover, the binding of ATP promotes a large change in the conformation of the isolated α subunit, as is revealed by the modification of the physicochemical parameters of this subunit or its dramatic decrease in sensitivity to trypsin digestion[25,96,119]. Nucleotide-depleted *E. coli* F_1 is able to rebind to F_1-depleted membranes, and then to promote a GTP driven proton pumping or GTP synthesis. Under these conditions, F_1 remains free of tightly bound nucleotides in the non-exchangeable sites[104,139]. Furthermore, if *E. coli* F_1-ATPase is rebound to F_1-depleted membranes with their non-catalytic sites either empty or

filled with ADP, ATP or AMPPNP, the F_1 catalyzes GTP synthesis in a similar manner.

These observations would suggest that tightly bound non-exchangeable nucleotides do not play any regulatory role in catalysis. This is, however, in contrast with the finding that the occupancy of one or two non-catalytic sites in chloroplast F_1 by ATP markedly increases the rate of GTP hydrolysis [141]. Moreover, in the case of pig heart F_1 hydrolyzing ATP, the addition of ADP in combination with P_i and Mg^{++} promotes a progressive shift towards an inhibited state of the enzyme. This event has been termed hysteretic inhibition [24]. Hydrolytic activity of such an inhibited F_1 is no longer subject to activation by anions which are positive effectors of the unmodified enzyme [6]. The fact that guanosine nucleotides are not able to promote such inhibition [5] suggests that non-catalytic site(s) are responsible for the hysteretic inhibition. These results, taken together, make it clear that the role of the non-catalytic sites is far from being understood.

Significant advances in the understanding of the function of the different subunits of the ATPase complex have been made possible through binding studies with different types of radiolabeled ligands, including affinity and photoaffinity labeling reagents and chemical modifiers [127]. Photoaffinity and affinity probes are substrate analogs containing a chemically reactive group. This reactive group does not alter the specific binding of the probe to the enzyme, but it provides the means to make a covalent bond with amino acid residues in the vicinity of the substrate binding sites and to identify them. In the case of nucleotide analogs, the modifying group can be introduced into the phosphate chain, the ribose moiety, or the purine ring.

Representative nucleotides modified in the phosphate chain are p-fluorosulfonylbenzoyl-5'-adenosine (FSBA) and p-fluorosulfonyl-benzoyl-5'-inosine (FSBI). In the case of the beef heart mitochondrial F_1, full inactivation of ATPase activity was attained for one mole of FSBI bound to a catalytic site, compared to three moles of FSBA bound to the non-catalytic sites per mole of F_1 [14, 15]. Irrespective of their binding stoichiometry, both probes bind to the β subunit of F_1 (see table 2). In contrast, the inactivation of F_1 by p-fluorosulfonylbenzoyl ethenoadenosine ($FSB_\varepsilon A$) was correlated with the modification of residue Tyr 244 in subunit α. This residue probably belongs to the non-catalytic site [126]. Other nucleotides modified at the level of the phosphate chain are adenosine diphosphopyridoxal and adenosine triphosphopyridoxal [59, 109]. Studies conducted on the *E. coli* F_1 with the adenosine triphosphopyridoxal pointed to the presence of α Lys 201, β Lys 155 and β Lys 201 close to the γ phosphate group of ATP bound to the catalytic site [59].

Ribose-modified and base-modified nucleotides include photoactivable derivatives of ADP or ATP, with the photoreactive adduct attached either to the 3' hydroxyl group of ribose, or directly to the purine ring. Enzyme inactivation by these reagents is usually correlated with the binding of one or two moles of photoactivable probe per mole of enzyme. Ribose-modified derivatives photolabel both the α and β subunits [79, 80]. This is also the case for the 8-azido nucleotides [57], whereas 2-azido nucleotides loaded at either catalytic or non-catalytic sites appear to recognize exclusively the β subunit [23, 43].

Covalent binding of azidonitrophenyl phosphate (ANPP), a photoactivable derivative of P_i, to one β subunit of F_1 results in complete inactivation of the ATPase [73] (cf. table 2).

Fluoroberyllate and fluoroaluminate were other chemical probes used as analogs of P_i [81]. Mitochondrial F_1 and *E. coli* F_1 were strongly and quasi irreversibly inhibited by these fluorometals, provided incubation was performed in the presence of a diphosphonucleoside. ADP but also GDP or IDP, which are recognized only by the catalytic sites of F_1, were able to promote inhibition. The stoichiometries of the bound species in the ADP-fluorometal complexes, ($ADP_1\ Mg_1\ Al_1\ F_4$ and $ADP_1\ Mg_1\ Be_1F_x\ (H_2O)4\text{-}x$ with $1 < x < 3$), are consistent with the fact that tetracoordinated aluminium or beryllium mimick P_i and may form abortive complexes with ADP at the catalytic sites of F_1. Complete inhibition required the binding of 2 mol beryllium or aluminium and 2 mol ADP per mol F_1 [31, 63].

The catalytic and non-catalytic nucleotide binding sites are probably located closely to the α-β subunit interfaces.

Table 2. Amino acid residues of the β subunit of beef heart mitochondrial F_1 labeled by chemically modified substrates and chemical reagents

Chemical reagents and substrate analogs	Labeled amino acid residue in β subunit of beef heart F_1	Equivalent residue in β subunit of *E. coli* F_1
NBF pH > 9	Lys 162	Lys 155
FDNP ADP	Lys 162	
DCCD	Glu 199	Glu 192
EEDQ	Glu 199	
8 azido ATP	Tyr 311 [a]	Tyr 297
Nbf (pH 7)	Tyr 311	
ANPP	Tyr 311 [b]	
2 azido ADP	Tyr 345 [c]	Tyr 331
FSBI	Tyr 345	
FSBA	Tyr 368 [d]	Tyr 354

[a] Additional labeled residues: Lys 301, Ile 304
[b] Additional labeled residues: Ile 304, Gly 308
[c] Additional labeled residues: Leu 342, Ile 344, Pro 346 (catalytic site only)
[d] Additional labeled residue : His 427.
Full inactivation required one or at most two moles of chemical reagent bound per mole of F_1, except for FSBA. In this case, 3 moles of modifier per mole of F_1 were needed.
Abbreviations and references:
FSBA: p-fluorosulfonylbenzoyl-5'-adenosine [14]
FSBI: p-fluorosulfonylbenzoyl-5'-inosine [15]
DCCD: dicyclohexyl-carbodiimide [33]
EEDQ: N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline [72]
ANPP: 4-azido-2-nitrophenyl phosphate [44]
Nbf : 4-chloro-7-nitrobenzofurazan [2, 3]
FDNP ADP: 3'-O-(5-fluoro-2,4-dinitrophenyl)ADP ether [18]
2 azido ADP [43]
8 azido ADP [57]

This idea was inferred from the results of labeling experiments involving bifunctional diazido nucleotides[116] or, as reported above, 2-azido adenine nucleotides[68], ribose-modified derivatives[79,80], or adenosine triphosphopyridoxal[59]. Finally, recent cross-bridging experiments carried out with 5'-5' diadenosine pentaphosphate (AP_5A), a traditional inhibitor of adenylate kinase, favored the hypothesis of an adenylate kinase-like orientation of the catalytic and non-catalytic sites on beef heart mitochondrial F_1[128].

Chemical modifiers such as DCCD, N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and nitrobenzofurazan (Nbf) have been shown to inactivate F_1. Interestingly, inactivation by any one of these modifiers was correlated with the specific covalent modification of one amino acid residue located in one or at most two β subunits (table 2).

The presence in the β subunits of binding sites for many inhibitors or inactivators of F_1-ATPase is strongly indicative of the fact that the β subunit is the catalytic subunit. This conclusion is also supported by the following observations. 1) The β subunit from *Rhodospirillum rubrum* F_1 binds P_i, natural substrate of the enzyme[67]. 2) Aurovertin, an inhibitor of the enzyme, binds to the β subunit of *E. coli* F_1[115] and specifically enhances the affinity of the liganded β subunit for ADP and ATP[60]. 3) IF_1, the natural protein inhibitor of mitochondrial F_1-ATPase, originally discovered by Pullman and Monroy[106], is known to interact with the β subunit[65,69,70]. In addition, data from mapping studies provided a firm basis for the elaboration of a rough model of the topography of the active sites of F_1 with the identification of essential residues, and also an approach to the mechanism of catalysis with the characterization of the relationships between the binding stoichiometry of the probes and the inhibitory effects. Both aspects will be documented in the following chapters.

Mechanism of catalysis. From the study of the stereochemical course of ATP hydrolysis by beef heart F_1-ATPase in the presence of (^{17}O)water and ATPγS, it has been concluded that hydrolysis proceeds by transfer of the γ phosphoryl group of ATP directly to water, without the formation of a phosphoenzyme intermediate[134]. Two and probably three catalytic sites of F_1 exhibit negative cooperativity with respect to substrate binding, but show positive cooperativity with respect to catalysis. The first of these catalytic sites exhibits a very high affinity for substrate. The K_d value for ATP is close to 10^{-12} M in the case of the beef heart mitochondrial F_1-ATPase[51], and to 10^{-10} M in that of *E. coli* F_1[1]. When the molar ratio of ATP to F_1 is lower than 1, resulting in unisite catalysis, the F_1 turnover is lower than 10^{-4} s^{-1} [1,51]. Under steady state conditions of catalysis with ATP concentrations high enough to saturate this high affinity site, ATP hydrolysis is characterized by the following parameters: K_M close to 10^{-6} M, and V_{max} in the range of 1 to 5 μmol/min/mg (turnover ~5 to 30 s^{-1})[49,53,111,140]. Increasing the ATP concentration results in multisite catalysis, with at least one and probably two additional catalytic sites being occupied, the K_M ATP increasing to 10^{-4}–10^{-5} M, and the turnover reaching values of 600 s^{-1} [22,49,111,140]. The increase in turnover rate which is linked to the shift from unisite catalysis to multisite catalysis clearly reflects the presence of interactions between the catalytic sites[22,49,50,102]. However, Bullough et al.[16] have argued that the high affinity site is not a normal catalytic site, and that it contributes only in part to the high rate of hydrolysis of ATP under steady state conditions in the presence of high concentrations of ATP.

The question of the number of functional catalytic sites of F_1 is still debated. In an attempt to get an answer, a number of approaches have been used; namely, binding of substrate analogs, chemical modifications of F_1 and use of mutated enzymes from procaryotes. The results and models reported in the literature have recently been scrutinized[20]. In brief, three catalytic models have been proposed in which either zero, one or two β subunits of F_1 are assumed to play a regulatory function, with catalysis being ensured by three, two or one β subunits, respectively[20,35,133]. Although not firmly established, the model which postulates that all three interacting β subunits participate equally in catalysis is favored[20]. There are different possible ways for three equipotent catalytic sites to operate in a cooperative manner. For example, all three sites may perform catalysis in a strictly coordinated and synchronized manner: this model, proposed by Tiedge and Schäfer[122] as a working hypothesis, needs further investigation. On the other hand, catalytic units may pass through identical states in a strictly ordered sequence[11], or alternatively the catalytic subunits may interact in a random fashion[63,79].

In order to take into account the possibility of a strictly ordered sequence in the turnover of catalytic subunits, as well as the asymmetrical interaction of β subunits with the minor subunits γ, δ and ε, the idea of rotational catalysis has been put forward[19,49,55,87]. In favor of such an ordered catalytic pathway are results concerning a number of reagents which are able to fully inactivate F_1 when one mole of reagent has bound to one mole of F_1[20]. In these experiments there is, however, no indication of whether the single modified catalytic subunit induces conformational changes in the other subunits of F_1, leading to ATPase inhibition. On the other hand, it has been reported that in *E. coli*, the three β subunits are structurally asymmetrical and that this asymmetry is not due to the association of the δ and ε subunits with specific β subunits[13]. Moreover, the α_3 β_3 catalytic complex reconstituted from purified F_1 subunits isolated from the thermophilic bacterium PS3 exhibits cooperative kinetics as does native F_1, indicating that the minor subunits (γ, δ and ε) are probably not responsible for the control of the catalytic properties of the enzyme and are therefore not involved in putative asymmetrical interactions with the β subunits[88]. These experiments suggest that the co-

operative control of catalysis might be achieved by the binding of the substrates to the β subunits.

Other reconstitution experiments were performed with mixtures of wild type and mutated subunits from either *E. coli* or thermophilic bacterium F_1. In the case of mutated *E. coli* β subunits, hybrids were unable to perform multisite catalysis[92]. Hybrid *E. coli* F_1 reconstituted with one third mutant α subunit and two thirds normal α subunits, however, had substantial though not maximal activity[108]. All three intact catalytic units (containing 1α and 1β) therefore appeared to be required for maximal activity. These results can be interpreted to mean that cooperative interaction between pairs of catalytic sites is required for effective catalysis. The same conclusion could be drawn from an experiment in which hybrid thermophilic bacterium F_1, containing one mutated β subunit per $\alpha_3\,\beta_3\,\gamma$ complex, exhibited nearly 50% of the activity of the wild-type enzyme[89]. A stochastic model was proposed by Lübben et al.[79] in which each catalytic subunit interacted cooperatively with one of the other two vicinal catalytic subunits. This model might easily explain why the multisite catalysis of F_1 is inhibited upon the binding of two moles of photoactivable analog of ADP per mole of F_1. This stochastic model with the three catalytic subunits functioning by pairs in a random sequence is also favored by the results of recent experiments in which mitochondrial F_1 was non-competitively inhibited by fluoroberyllate or fluoroaluminate in the presence of ADP[63]. As previously mentioned, full inhibition was obtained for two moles of fluorometal bound per mole F_1.

Strategic amino acids in catalysis. Numerous reports have now been made concerning the identification of the amino acid residues modified by chemical modifiers or affinity-labeling reagents (table 2). The 2- and 8-azido nucleotides, which are hydrolyzed by F_1 in their triphosphate form, have proved to be valuable tools in the mapping of catalytic sites. In beef heart F_1, 8-azido ATP labels a few amino acid residues around Tyr 311 of β (β-Tyr 311)[57], and 2-azido ADP(ATP) labels other amino acids around β-Tyr 345[43]. These results have led to the interpretation that, in the catalytic site, the adenine moiety of ADP or ATP is embedded in a crevice of the β subunit. The edges of this crevice, containing Tyr 311 and Tyr 345 respectively, would be in the close vicinity of the 2- and 8-carbons of the adenine ring. β-Tyr 345 was also shown to react with FSBI[15]. Several amino acid residues close to β-Tyr 311 are photolabeled by ANPP, the photoactivable derivative of P_i[44]. The β-Tyr 311 residue can also be chemically modified by Nbf at neutral pH. Bound Nbf can be transferred from β-Tyr 311 to β-Lys 162 by shifting the pH to alkaline values, which suggests that β-Tyr 311 and β-Lys 162 are in close proximity in the protein[2,3]. β-Lys 162 belongs to a glycine-rich sequence called the Gly-loop[39], which has been found to interact with the phosphate chain of nucleotides in a number of nucleotide-binding proteins. These topographical data are summarized in the scheme presented in figure 2.

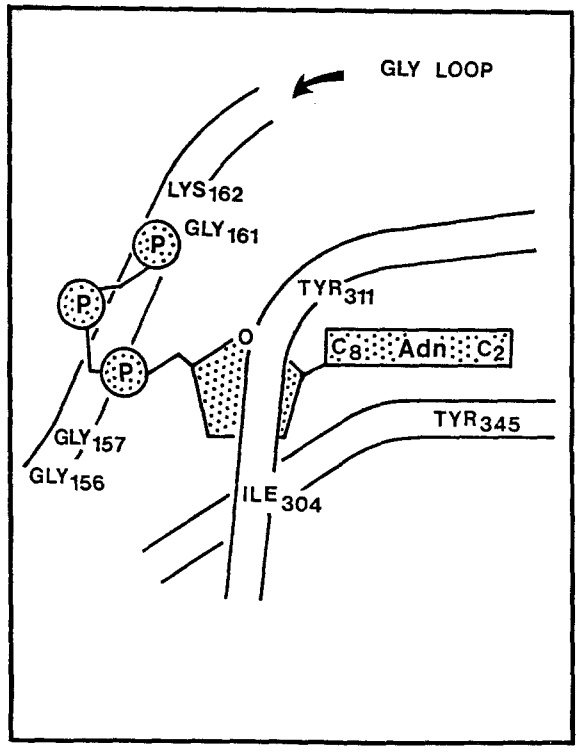

Figure 2. Model for the catalytic site of mitochondrial F_1-ATPase (for detail, see 'Strategic amino acids in catalysis' in text).

The role played by the Gly-loop in the catalytic mechanism has been emphasized by mutagenesis studies in *E. coli*. Directed mutagenesis of amino acid residues present in the Gly-loop sequence of the β subunit of *E. coli* F_1 results in a very strong impairment of either multisite or unisite catalysis[75,120]. In other experiments, wild type and mutant β subunits of rat liver mitochondrial F_1-ATPase were purified from overexpressing *E. coli* strains, and their ability to bind the fluorescent 2′(3′)-O-2,4,6-trinitrophenyl) adenosine 5′ triphosphate (TNP-ATP) was analyzed. A deletion mutant lacking the entire Gly-loop sequence displayed a marked reduction in affinity for TNP-ATP, but interaction still occurred, indicating that one or more additional regions of the β subunit contribute to the nucleotide binding site[42]. In the yeast *Saccharomyces cerevisiae*, the replacement of the threonine residue at the end of the Gly-loop by a serine residue resulted in an increase in the ATPase activity and also in a modulation of other kinetic parameters[90]. The mutagenesis of the equivalent threonine residue in *E. coli* leading to ATPase inhibition suggests that the hydroxyl moiety is essential for the catalytic activity[64].

The replacement of β-Tyr 297 (homologous to bovine β-Tyr 311) with Phe has little effect on catalysis[97]. Thus, the inhibition of ATPase observed after chemical derivatization of this Tyr residue with Nbf, ANPP, or 8-azido ATP is probably due to steric effects inherent in the

bound probe. On the other hand, the alteration of ATPase activity due to replacement of β-Tyr 331 (homologous to bovine β-Tyr 345) suggests that an aromatic residue is required at this position[138]. In the case of the thermophilic F_1-ATPase, replacement of Tyr residues corresponding to Tyr 311, Tyr 345, and Tyr 368 (which in the bovine enzyme are target residues for $8N_3ADP$, $2N_3ADP$ and FSBA, respectively) has no effect on the ATPase activity. This means that neither the aromatic rings nor the hydroxyl groups are required for ATPase activity[93]. Directed mutagenesis in *E. coli* has shown that β-Glu 192, the target of EEDQ and DCCD (corresponding to bovine β-Glu 199) is not absolutely essential for catalysis[98]. This highlights an important difference between chemical modification and the mutagenesis approach. In the latter case, a lack of inhibition resulting from an amino acid substitution does not rule out the possibility of steric interaction with the substrate, but only shows that the amino acid is not directly involved in catalysis. In fact, if one assumes that the main feature of the catalytic site of the F_0-F_1-ATPase is to bring together MgADP and P_i in an anhydrous environment to promote their chemical condensation into ATP, one may expect that the catalytic properties of the enzyme are more related to the general architecture of the site than to the properties of any individual amino acids.

Genetic studies of *E. coli* mutant strains have pinpointed several amino acids in the α subunit of F_1 (namely Gly 351, Ser 373, Ser 375 and Lys 201) whose mutation causes impairment of enzyme cooperation[40, 59, 120]. It is noteworthy that mutagenesis of Arg 171 and Glu 172, belonging to the Gly-loop of the α subunit of *E. coli* F_1 subunit (which is probably involved in the binding of ADP or ATP at non-catalytic sites), did not result in any significant effect on the tight binding of ADP or ATP to the α subunit[95].

An interesting approach to the study of the structure of the catalytic site is the synthesis of peptides corresponding to sequences thought to belong to this site, followed by assay of their reactivity with respect to different F_1 substrates. Thus, a peptide has been synthesized, corresponding to the sequence Asp 141-Thr 190 of the β-subunit of rat liver F_1, which encompasses the Gly-loop and is therefore presumed to interact with the phosphate moiety of ATP. This synthetic peptide was indeed found to interact with ATP and with a fluorescent derivative of ATP, confirming the predictions that the peptide forms part of the catalytic site of F_1[41].

Mutations in the aurovertin binding site of *E. coli* have also been examined. An aurovertin-resistant mutant[115, 116] has recently been identified as being the result of the replacement of Arg 398 by His[74]. This mutation does not affect the ATPase activity of F_1, indicating that this region of the β subunit does not participate in nucleotide binding.

Translocation of protons through F_0 and coupling of ATP synthesis

In *E. coli*, genetic and biochemical studies have shown that all three subunits, a, b and c, are necessary for an optimal proton conduction through the F_0 sector of the H^+-synthase complex[40, 120]. Subunit b may play a structural role, whereas the a and c subunits are probably involved in proton conduction per se. Mutation and chemical derivatization analysis have identified a number of amino acids involved in proton conduction in the subunit a (Ser 206, Arg 210, Glu 219, His 245)[76], and in the subunit c (the DCCD reactive residue, namely Asp 61)[118]. Examination of a large number of mutations in the polar loop region of the hairpin-like subunit c (from Glu 37 to Leu 45) led to the conclusion that Arg 41 is the only essential residue in this region[38]. Many of the above residues may line a pore in which protons could be translocated across a network of H bonds localized at the interface of the a and c subunits[76]. This model implies that the single copy of subunit a must interact with one of the multiple copies of subunit c to form the conducting pore. Interestingly, the derivatization of only one copy of the subunit c by DCCD is sufficient to block the flux of protons. This partial site reactivity may be related to the functioning of F_1. On the other hand, protonation of specific amino acid residues might not occur, in which case, F_0-F_1 activity could be linked to a movement of H_3O^+ molecules[11].

It is generally agreed that the synthesis of one molecule of ATP requires the translocation of three protons across the membrane. How the energy of the proton flux through F_0 is converted, at the level of F_1, into chemical work in the form of ATP, has not yet been satisfactorily explained. Two possible mechanisms have been proposed: 1) a direct coupling mechanism in which the translocated protons are directly involved in the chemical reaction of condensation of ADP and P_i; 2) an indirect coupling mechanism in which the energy of the electrochemical proton gradient is used to induce conformational changes in F_1, so that bound ATP at the catalytic site is released from F_1 to the medium. The second alternative is favored by the finding that the selective modification of F_0 by DCCD results in a large decrease in the affinity of F_1 for ATP[100, 101]. As the DCCD target site in F_0 is located 2 nm from the nucleotide binding site in F_1, a direct interaction between the two sites is unlikely[4]. Additional evidence of the existence of a conformational coupling between F_0 and F_1 comes from experiments involving the use of oligomycin and aurovertin[83], as well as experiments based on the measurement of the inhibitory effects of fluorometals on energized submitochondrial particles[81], and the release of IF_1 from submitochondrial particles after generation of a proton motive force[77].

Perspectives

Over the last ten years, both chemical probes and mutagenesis have been used to explore the structure and the

catalytic mechanism of mitochondrial and bacterial H^+-ATPases, and the value of these approaches has been clearly demonstrated. Although much remains to be done along these lines, the establishment of the 3D structure of the F_1 sector and the F_0-F_1 complex at high resolution is the next important goal. Mutational studies extended to an eucaryotic system, such as the yeast ATPase complex, should also reveal additional details of the catalytic site of the enzyme and about possible regulatory mechanisms. A number of questions pertaining to catalysis also remain unanswered. Thus, the role of the tightly bound nucleotides is still not understood, and the nature of regulatory mechanisms in the F_1 complex require further analysis. Detailed information is still lacking on the molecular mechanism of proton channeling through F_0, and the nature of the conformational changes in F_1 resulting from proton translocation through F_0. The mitochondrial and bacterial H^+-ATPases, which have been surveyed in this short review, have many structural and kinetic features in common with the chloroplast H^+-ATPase, but also a number of differences from it. Since the basic mechanism of action of the three H^+-ATPases is likely to be similar, valuable insights should be obtained from a comparative study of these enzymes.

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Mini-Review

The importance of microbiology in waste management

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Abstract. Until a hundred years ago, the waste products from human activities were returned into the environment and underwent the biosphere's natural elimination processes without there being any long-term charge on the environment. During the last century, the increase in the amount of refuse has been accompanied by a decrease in its quality, mainly due to the production and dispersal of heavy metals and xenobiotic compounds. Both useful and noxious microbial processes have been underestimated in applied research in the field of waste management which, until now, has dealt mainly with artificial technologies. This paper presents some examples of microbiological processes occurring in waste treatment, particularly dumping, waste incineration, composting and biomethanization.
Key words. Microbiology; aerobic processes; anaerobic processes; waste disposal; landfills; landfill topsoil; biogas; biomethanization; percolating waters; tetrachloroethylene, anaerobic biodegradation; vinyl chloride, anaerobic production; incineration; biofilter; composting; *Aspergillus fumigatus*.