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Review

ATP hydrolysis in ATP synthases can be differently coupled to proton transport and modulated by ADP and phosphate: A structure based model of the mechanism

Manuela D'Alessandro, B. Andrea Melandri *

Department of Biology, Laboratory of Biochemistry and Biophysics, University of Bologna, Via Irnerio 42, 40126 Bologna, Italy

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ABSTRACT

In the ATP synthases of *Escherichia coli* ADP and phosphate exert an apparent regulatory role on the efficiency of proton transport coupled to the hydrolysis of ATP. Both molecules induce clearly biphasic effects on hydrolysis and proton transfer. At intermediate concentrations (~0.5–1 μM and higher) ADP inhibits hydrolysis and proton transfer; a quantitative analysis of the fluxes however proves that the coupling efficiency remains constant in this concentration range. On the other hand at nanomolar concentrations of ADP (a level obtainable only using an enzymatic ATP regenerating system) the efficiency of proton transport drops progressively, while the rate of hydrolysis remains high. Phosphate, at concentrations ≥0.1 mM, inhibits hydrolysis only if ADP is present at sufficiently high concentrations, keeping the coupling efficiency constant. At lower ADP levels phosphate is, however, necessary for an efficiently coupled catalytic cycle. We present a model for a catalytic cycle of ATP hydrolysis uncoupled from the transport of protons. The model is based on the available structures of bovine and yeast F_1 and on the known binding affinities for ADP and P_i of the catalytic sites in their different functional states. The binding site related to the inhibitory effects of P_i (in association with ADP) is identified as the $\alpha_{HC}\beta_{HC}$ site, the pre-release site for the hydrolysis products. We suggest, moreover, that the high affinity site, associated with the operation of an efficient proton transport, could coincide with a conformational state intermediate between the $\alpha_{TP}\beta_{TP}$ and the $\alpha_{DP}\beta_{DP}$ (similar to the transition state of the hydrolysis/synthesis reaction) that does not strongly bind the ligands and can exchange them rather freely with the external medium. The emptying of this site can lead to an unproductive hydrolysis cycle that occurs without a net rotation of the central stalk and, consequently, does not translocate protons.

1. Introduction

ATP synthases are multisubunit enzymes present in the membranes of bacteria, mitochondria and chloroplasts. They catalyze the synthesis of ATP from ADP and phosphate driven by a transmembrane difference of protonic electrochemical potential (in some bacteria by a Na^+ electrochemical potential difference); under some particular physiological conditions they can also pump protons across membranes at the expenses of ATP hydrolysis, acting as generators of an ionic gradient [1–3].

The structures of many ATP synthase have been resolved at atomic resolution, although no structure so far comprises all subunits of the complex. The available structures include several preparations from bovine mitochondria, bound to a variety of substrate analogues or inhibitors [4–8], structures from yeast mitochondria [9] and from chloroplasts [10].

The different subunits of the ATP synthases are either embedded within the membrane (F_o sector of the enzyme) or positioned extrinsically on the high potential side of the membrane (F_1 sector). In its simplest bacterial form F_1 contains five subunits in $\alpha_3\beta_3\gamma\delta\varepsilon$ stoichiometry. The α and β subunits are highly homologous and very similar in their structure; they are arranged in a pseudo hexagonal symmetry with the αβ heterodimers forming three catalytic protomers arranged in a pseudo threefold symmetry. This symmetry is broken by the single copy γδε subunits, two of which (γ and ε) are placed centrally to the $\alpha_3\beta_3$ hexamer and make different contacts with the three αβ heterodimers. These different contacts are related to significant differences in the structure of the αβ catalytic protomers [4].

Adenine nucleotides and phosphate are bound to the α and β subunits in binding sites which are found at the interface between the two types of subunit. ATP, ADP and phosphate can be present in

Abbreviations: $\Delta\psi$, bulk-to-bulk electrical potential difference; ΔpH, transmembrane difference of pH ($pH_{out} - pH_{in}$); $\Delta\tilde{\mu}_{H^+}$, difference of electrochemical potential of protons, ($\tilde{\mu}_{H^+}$, in $\cdot\tilde{\mu}_{H^+}$, out); ACMA, 9-amino-6-chloro-2-methoxyacridine; AMP-PNP, 5-adenylyl-imidodiphosphate; EF_OF_1, ATP synthase of *E. coli*; Phenol Red, 4,4′-(3*H*-2,1-benzoxathiol-3-ylidene) bis-phenol, S,S-dioxide; Tricine, *N*-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine

* Corresponding author. Laboratory of Biochemistry and Biophysics, Department of Biology, University of Bologna, Via Irnerio, 42, I-40126 Bologna, Italy. Tel.: +39 051 2091293, +39 329 1164035 (Mobile); fax: +39 051 242576.
E-mail address: brunoandrea.melandri@unibo.it (B.A. Melandri).

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binding sites located between the β and α subunits, with most of the binding residues provided by β and a fundamental contribution of one residue from α (αArg 373). Since these ligands are exchangeable and interconvertible, these sites are believed to be the active sites for catalysis. Other ATP molecules (and sometime ADP) are bound at the three interfaces between the α and β subunits and are bound to residues of the α subunits. These ligands are not exchangeable and, therefore, these three sites are considered to be as non-catalytic. The non-catalytic sites also are lacking for amino acid residues essential for the catalytic mechanism. The actual role of non-catalytic sites is still unknown.

The high resolution structures of the F_1 sector presently available are quite consistent with the accepted mechanism of catalysis, the so called alternate-binding mechanism [11,12], according to which at a given point in time the three catalytic sites which are present at the αβ interfaces differ from each other both in their catalytic properties and their affinity for substrates and products. Thus the entire catalytic cycle of ATP synthesis/hydrolysis is thought to require the interconversion of one catalytic site into all the three possible functional forms, interconversion that must occur in a strictly sequential fashion [1–3]. The functional differences among the three catalytic sites are a consequence of the different contacts with the central single copy γε subunits (especially with γ). It follows that the interconversion of the catalytic sites from a single functional state to the following one during the catalytic cycle takes place when the central γε subunits modify their position by rotating within the central hollow of the hexamer. These functional concepts are summarized by defining the central γε subunits as a rotor operating inside an external $\alpha_3\beta_3$ stator. The catalytic mechanism of the ATP synthase is therefore defined as a rotational catalytic mechanism.

Functional counterparts to the stator and rotor in F_1 are also present in the F_o sector: in the simplest bacterial enzymes the former is composed by an *a* subunit and a homodimer of *b* subunits, connected to the $\alpha_3\beta_3$ hexamer through the δ subunit. The F_o rotor consist of a bundle of *c* subunits helical hairpins, forming a transmembrane cylindrical structure [13,14]. The copy number of *c* subunit is variable in different ATP synthases, from 10 copies in yeast to 14 in chloroplasts [15,16]. Proton transfer across the membrane is thought to occur at the interface of the *a* subunit with one or two *c* subunits, with a stoichiometry of one proton per each *c* subunit involved. The *c* subunit bundle is attached to the basis of the γε F_1 rotor. The rotation of the γε rotor in F_1, as well as of the complete $\gamma\varepsilon c_n$ rotor has been demonstrated experimentally in very detailed studies using single molecule epifluorescence [3,17,18 and references therein] or fluorescence energy transfer [19].

ATP hydrolysis catalyzed in F_1 according to the sequence of the alternate binding mechanism produces a sequential displacement of the γ subunit, that is forced to rotate in a unidirectional way (anticlockwise when observed from the membrane surface) and generates a torque transmitted to the *c* subunit bundle in the membrane [17]. The rotation of the rotor in F_o is coupled the translocation of protons from the F_1 face of the membrane to the opposite one. Thus the entire enzyme acts as an ATP driven proton pump. Conversely, when a transmembrane protonic potential difference is present, the protons flowing spontaneously across F_o from the high potential side of the membrane (the distal side with respect to F_1) to the low potential side cause a clockwise rotation of the rotor that forces the three catalytic sites in F_1 to produce and release ATP from ADP and phosphate [20,21]. In this magnificent molecular machine, therefore, the coupling between ATP hydrolysis/synthesis and proton translocation is obtained through a mechanical transmission of rotational energy, a unique example, so far, of such type of energy transduction in the biological world. Since it is believed that the whole rotor rotates as a single solid body [22], the number of protons translocated per ATP hydrolyzed should be fixed and solely determined by the structure of the enzyme (i.e. by the number of *c* subunits), and, conversely, the number of ATP molecules synthesized should be proportional to the number of protons driving its synthesis. More precisely, since three catalytic sites are present in F_1, the number of protons translocated per ATP hydrolyzed or synthesized should be constant and equal to the copy number of *c* subunit divided by three.

2. A survey of the experimental results

2.1. The efficiency of proton transport coupled to ATP hydrolysis can vary

At odds with above conclusions data have been obtained in our laboratory indicating that the number of protons translocated per ATP hydrolyzed can change depending on the experimental conditions. Two ligands of the enzyme, ADP and phosphate, products of the hydrolysis reaction, have positive effects on the efficiency of proton transfer. These studies have been performed in membrane fragments obtained from the photosynthetic bacterium *Rhodobacter capsulatus* [23], grown photoheterotrophically, and from *Escherichia coli*, grown aerobically [24]. Only the main results of these studies, which have already been reviewed [25], will be here succinctly summarized:

1) ADP, at submicromolar concentrations, must be present in the assay mixture in order to observe an efficient proton transport. Trapping of ADP by a very active ATP regenerating system (pyruvate kinase and phospho-enol-pyruvate) results in a decrease of the amount of protons translocated, while ATP hydrolysis is strongly stimulated.
2) ADP at higher micromolar concentrations inhibits the hydrolysis, presumably generating the well known ADP inhibited state.
3) Also phosphate, at concentrations ≥100 μM, must be present for an efficient proton transfer.
4) Phosphate also affects the rate of ATP hydrolysis, but its effects are different in *E. coli* with respect to *Rb. capsulatus*. In the former case phosphate inhibits the hydrolysis with an apparent K_d of about 200 μM, K_d that increases above 1 mM when ADP is trapped. In the photosynthetic bacterium such inhibition cannot be observed, but rather the hydrolysis is stimulated by phosphate (the apparent K_d for this effect is about 0.1 mM) and no inhibition at higher concentrations takes place.

2.2. Results on purified ATP synthase reconstituted into liposomes

This type of experiments has been repeated using proteoliposome containing a partially purified ATP synthase from *E. coli*, thereby eliminating any possible interference from other proton translocating enzymes which may be present in the membrane (e.g. protons synporters or antiporters). This approach also allows a more quantitative analysis of these phenomena, since the low permeability to protons of liposomes, as compared with that of intact membranes, allows an empirical calibration of the response of ΔpH probe ACMA to transmembrane pH differences [manuscript in preparation,27]. Proton transport could therefore be analyzed in terms of actual ΔpH (a measure of the whole $\Delta\tilde{\mu}_{H^+}$ since in all experiments the formation of a significant transmembrane electric potential difference [$\Delta\psi$] was prevented by the addition of 50 mM KCl and valinomycin). The results of this work [manuscript in preparation] have fully confirmed the qualitative data obtained in *E. coli* membrane fragments, in support of the conclusion that all phenomena observed are intrinsic to the function of the ATP synthase.

The quantitative analysis of proton pumping has also allowed a more precise definition of the proton pumping efficiency. In fact the rate of hydrolysis at different phosphate or ADP concentrations is a measure of the amount of ATP hydrolyzed per unit time, while the rate of internal acidification is a function of the number of protons translocated and of the buffering capacity inside the vesicle lumen (itself a function of the internal pH). At pumping time close to zero, when the internal pH is very