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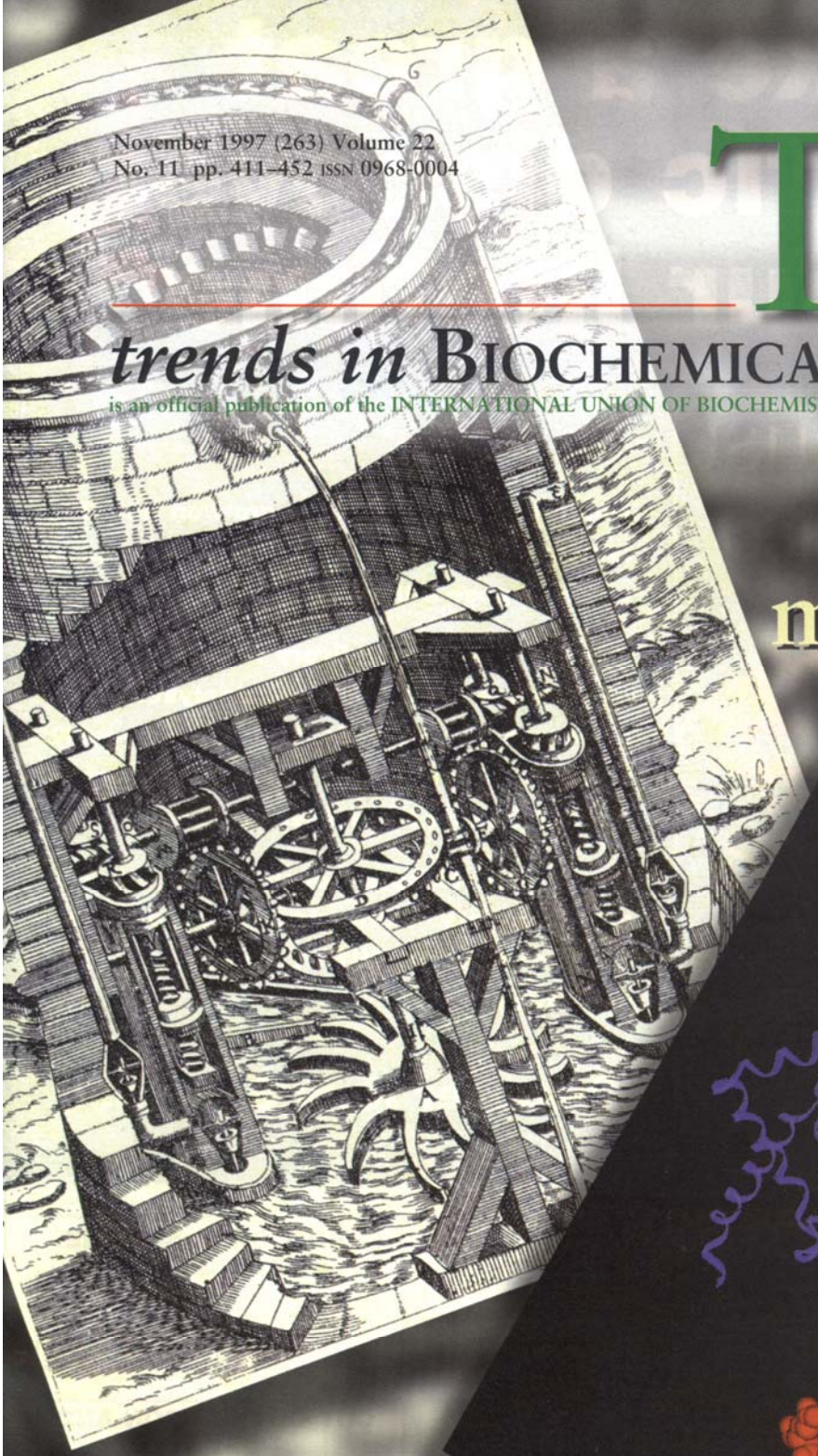
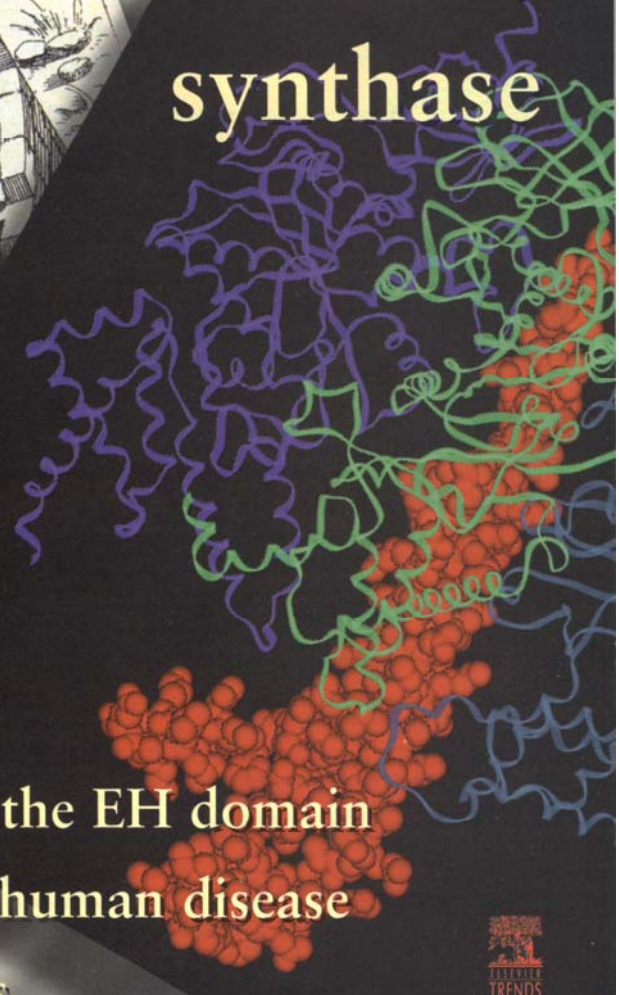


Rotatory mechanics of ATP synthase



- Intracellular sorting and the EH domain
- Dynamic mutations and human disease
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ATP synthase: an electrochemical transducer with rotatory mechanics

Wolfgang Junge, Holger Lill and Siegfried Engelbrecht

ATP synthase (F_0F_1-ATPase) uses proton- or sodium-motive force to produce ATP from ADP and P_i. Three lines of experiment have recently demonstrated large-scale intersubunit rotation during ATP hydrolysis by F_1. We discuss how ion flow through the membrane-intrinsic portion, F_0, may generate torque and how this might be transmitted between stator and rotor to finally expel spontaneously formed ATP from F_1 into water.

ATP SYNTHASE uses proton-motive force[1] or, in some organisms, sodium-motive force[2] across the respective coupling membrane to drive the synthesis of ATP from ADP and P_i. The chloroplast enzyme translocates four protons from the exoplasmic to the cytoplasmic side of the thylakoid membrane for each molecule of ATP it produces[3,4]. When hydrolysing ATP the enzyme pumps protons in the opposite direction. Whether the same H^+/ATP-ratio holds for the mitochondrial and bacterial ATP synthases is under debate[5]. The F_0F_1 synthase has a bipartite structure, as its name suggests, with the membrane-intrinsic portion, F_0, responsible for proton transport[6,7] and F_1 performing the task of ATP synthesis/hydrolysis. When F_1 is detached from F_0 and is therefore decoupled from the proton-motive driving force, it catalyses only ATP hydrolysis. Depending on the organism, ATP synthase consists of at least eight different subunits, totalling more than 20 polypeptide chains. The ATP synthase from *Escherichia coli* serves as a prototype with a subunit composition of $(\alpha\beta)_3\gamma\delta\epsilon$ for F_1 and $\mathbf{ab}_2\mathbf{c}_{9-12}$ for F_0, as schematically illustrated in Fig. 1.

ATP synthase is present in the plasma membrane of eubacteria, the thylakoid membrane of chloroplasts and the cristae membrane of mitochondria. The enzyme is surprisingly well conserved considering its structural complexity and the early evolutionary divergence of bacteria, plants and animals[8]. Some chimeric constructs from evolutionarily remote organisms are even functional. This holds true for constructs combining subunits from chloroplasts or cyanobacteria with those from *E. coli*[9] and *Rhodospirillum rubrum*[10] or combining the sodium-conducting F_0 portion from *Propionigenium modestum* with the F_1 portion from *E. coli*[11]. Such evolutionary conservation justifies the use of data so far collected to construct a unified model of ATP synthase.

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Nucleotide-binding sites, their cooperativity and the structure of F_1

The membrane-peripheral component, F_1, carries three catalytic nucleotide-binding sites. Three other sites are present, which apparently have no direct involvement in the catalytic function, their deletion by site-directed mutagenesis does not inhibit the activity[12]. The conversion of ADP + P_i into ATP occurs spontaneously on the enzyme and without major input of free energy[13]. However, ATP remains tightly bound to its binding site. The release of ATP from this site into the aqueous medium is the major energy-requiring step of the catalytic reaction cycle[14]. The hydrolysing activity by a single site is low[15]. The rate of hydrolysis increases by several orders of magnitude when three sites participate. That these sites are strongly coupled is evident from the negative cooperativity of sequential nucleotide binding[16]. According to Boyer's concept[17] of a binding change mechanism, three reaction sites might cycle concertedly through the partial reactions of ATP synthesis/hydrolysis.

A major portion of the structure of F_1 from bovine heart mitochondria has been disclosed at 0.28 nm resolution by the groups of Walker and Leslie[18]. Crystals were grown at nucleotide concentrations that enforced asymmetry between the three catalytic sites. One site was filled with AMP–PNP (a non-hydrolysable ATP analogue), another site with ADP and the third site was empty. Crystal structure analysis reveals that the three copies of subunit α and the three copies of β are arranged alternately in a hexagonal ring. Two twisted α-helices of subunit γ (comprising the 44 N-terminal and the 64 C-terminal residues) are located axially in the centre of this ring. The N-terminal portions of subunits α and β are β-barrels, distal to the membrane, and arranged as if to provide a bearing for the rotating γ-subunit[18]. Although this structure represents the static situation of the *inhibited* enzyme (because of bound AMP–PNP), it is a snapshot of a rotatory mechanism of catalysis, where the three catalytic sites cycle through the three catalytic stages of hydrolysis, namely loaded with ATP,

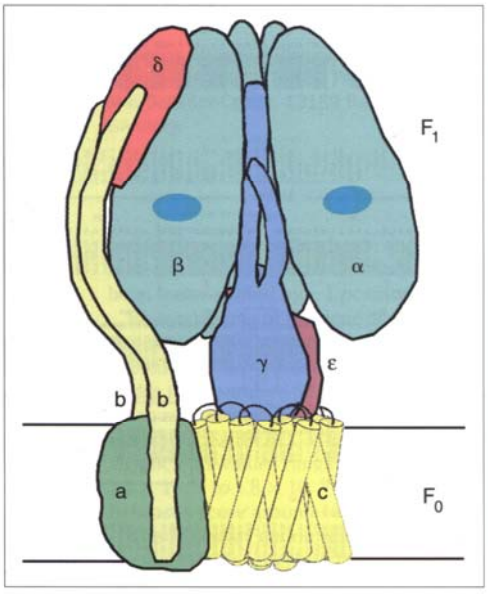

Figure 1
Artist's view of the gross structure of ATP synthase, which is bipartite both structurally and functionally. On the one hand, it is divided into the membrane-intrinsic and ion-conducting F_0 portion, and the peripheral F_1 portion, which carries the nucleotide-binding sites. On the other hand, it is divided into a stator portion comprising subunits **a**, $\mathbf{b}_2$, δ and $(\alpha\beta)_3$, and a rotor portion comprising subunits $\mathbf{c}_{12}$, ε and γ (see text).

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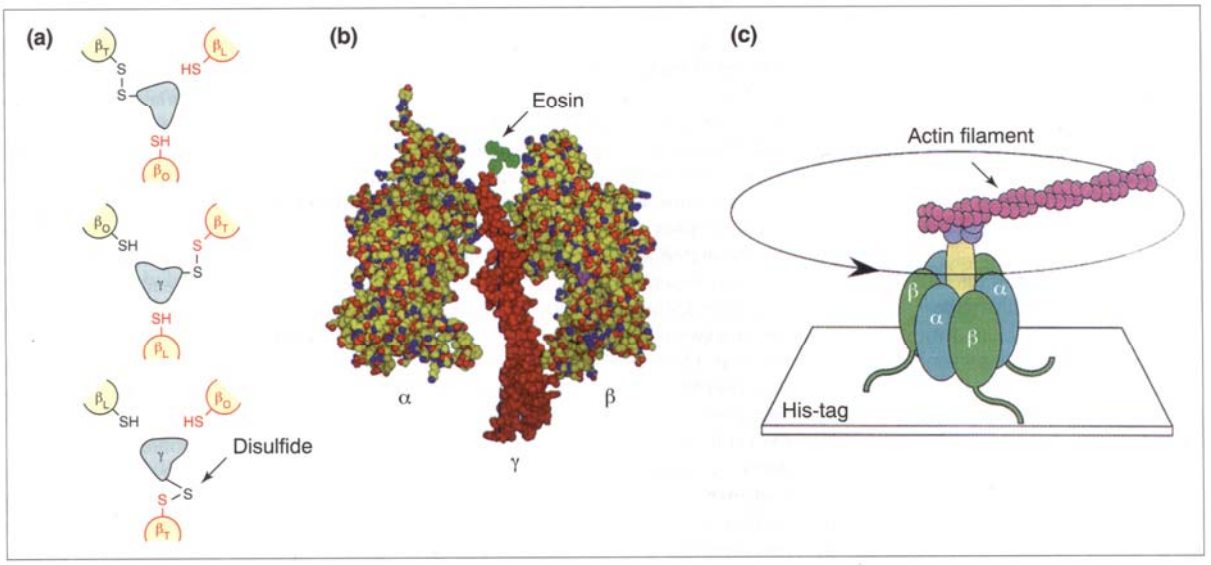

Figure 2

Three techniques used to detect the rotation of subunit γ relative to $(\alpha\beta)_3$ during the hydrolysis of ATP by F_1. **(a)** Schematic diagram showing the cleavage and reformation of disulfide bridges between engineered γ and β[19,20] during ATP hydrolysis produces new γ–β pairings, thus providing evidence for rotational mobility. **(b)** The technique of polarized absorption recovery after photobleaching (PARAP) of a probe (eosin), attached to the γ-subunit under immobilization of $(\alpha\beta)_3$ on anion-exchange resin[22,23], provides evidence for large-scale rotation (>280°) of γ in the time range of enzyme turnover, owing to the relaxation of the polarization anisotropy of the probe under conditions of ATP hydrolysis. **(c)** Fluorescence microscopy with a fluorescent actin filament attached to γ under immobilization of $(\alpha\beta)_3$ by His-tags[26]. The rotation of the macroscopic filament (length ≈ 2 μm) has been directly videographed.

being empty or loaded with ADP and P_i. It has been inferred that the γ-subunit is driven around during enzymatic activity like a crankshaft that is coupled to the opening and the closing of the catalytic nucleotide-binding sites[18].

Intersubunit rotation

Three separate lines of investigation increased confidence in the hypothesis of a rotation of subunit γ relative to $(\alpha\beta)_3$ under conditions of ATP hydrolysis. The principles of these experiments are illustrated in Fig. 2.

(1) Cross and co-workers relied on cleavable (disulfide) crosslinks between engineered β- and γ-subunits (see Fig. 2a). Starting from a closed disulfide bridge between a particular γ–β pair, they showed that cleavage and subsequent reformation of disulfide bonds produces new γ–β pairings if F_1 hydrolyses ATP, but the same pairing if the enzyme is inactive. By quantifying this result they stated that the motion of γ during activity of the enzyme caused it to hit all three copies of β during ATP hydrolysis by F_1 (Ref. 19) and by F_OF_1 [Refs 20, 21 (ATP synthesis)]. Whether the rotational motion was kinetically related to the activity and whether it was random or directed was not to be decided.

(2) Our group recorded the rotation of γ relative to $(\alpha\beta)_3$ in real time[21]. A photoselection technique termed polarized absorption recovery after photobleaching (PARAP) was applied to isolated spinach CF_1 immobilized through $(\alpha\beta)_3$ on an anion-exchange resin. The photobleachable dye eosin was covalently linked to the penultimate C-terminal residue (Cys322) of γ as shown in Fig. 2b. Under conditions of ATP hydrolysis, the polarization anisotropy relaxed, which was indicative of the rotation of γ relative to $(\alpha\beta)_3$. This relaxation was absent in the presence of AMP–PNP, as expected. The rotational relaxation time (≈ 100 ms) was compatible with the catalytic turnover time under these conditions (≈ 70 ms; Ref. 22). The angular range of the rotation of γ was larger than 280° (Ref. 23). We expected a unidirectional rotation. In this case, a theory of rotational drift predicted an oscillation of the polarization anisotropy[24], by contrast to the observed monotonous relaxation[22]. The cited theory was, however, based on an angular continuum. Our theory of molecular stepping motors[25] solved the apparent discrepancy. It predicted the monotonous decay of the polarization anisotropy for two and a quasi-monotonous decay for three steps. More steps, however, were expected to cause a damped oscillation that has not been observed[23]. These data proved the kinetic competence of the rotation, and they pointed to a three-step motion, if it was unidirectional.

(3) Recently, the groups of Yoshida and Kinosita videographed the unidirectional rotation directly. They used a macroscopic fluorescent label attached to the γ-subunit[26]. Recombinant F_1 from a thermophilic bacterium was immobilized head-down via engineered His-tags to a Ni-coated support. A fluorescent actin filament served as reporter (see Fig. 2c). Filaments were up to 2.5 μm long, ~200 times longer than the diameter of F_1. In the presence of Mg^{2+}–ATP, counterclockwise rotation of γ was observed (viewed from the 'membrane side' of the enzyme). This directionality conformed with the crystal structure, because the cyclic reaction was expected to progress from the site filled with ATP via the empty one to the site filled with ADP. The speed of rotation depended on the length of the attached filament. That the rotation, although directed, showed stochastic elements and was observed only in a small fraction of immobilized molecules was not surprising in view of the large viscous drag, accidental stop-and-go and the supposed elasticity of the attachment region on γ.

The data described above on isolated F_1 prove that the hydrolysis of ATP, cycling through the three catalytic sites on $(\alpha\beta)_3$, drives γ around, probably in three steps. It is likely that the reversal of this rotation in the F_OF_1 holoenzyme drives the

synthesis of ATP, but it is not yet experimentally established (1) how ion flow generates torque, (2) how the 20 polypeptide chains are organized in a rotor/stator structure that transiently stores elastic energy, and (3) how precisely the rotation is geared to the opening and closing of the catalytic sites. Below, we discuss possible clues to these questions.

F_0 and the generation of torque at the expense of proton-motive force

Proton translocation through the F_0 portion requires the presence of three types of subunits in *E. coli*, $\mathbf{ab_2c_{9-12}}$ (Ref. 27). The proteolipid subunit **c** is folded like a hairpin, with two transmembrane helices connected by a short loop facing the cytoplasmic side of the membrane (see Ref. 28 for its structure in a mixed solvent). Its presence in many copies has led several authors to postulate a ring-like structure of **c** in the membrane. Images of F_0 by electron[29] and atomic force microscopy[30], support this view. Subunit **c** carries one essential amino acid residue, Asp61 in *E. coli*, which is placed roughly in the middle of the membrane. Deletion of this residue abolishes enzyme activity, but its displacement by mutation to the corresponding position on the other branch of the hairpin (residue 24) revives it[31]. Subunit **a** consists of at least five transmembrane helices and subunit **b** comprises a single transmembrane stretch and a long hydrophilic head[32].

Figure 3 illustrates a ring of 12 proteolipid molecules that faces the large transmembrane **a**-subunit. How torque might be generated has been proposed by one of us (W. Junge, 1993) and discussed since[19,33]. Three properties required for this function of F_0 are, in principle, shared with most models for the generation of torque by the flagellar motor[34,35]. (1) An *electrostatical constraint* implies that carboxyl groups on the ring are always protonated and electroneutral when facing the lipid core, whereas carboxyl groups facing protein (here subunit **a**) can be deprotonated and charged. (2) The symmetrical ensemble of subunit **a** and the ring of **c** is endowed with clockwise or anticlockwise *handedness* by the noncolinear placement of the two access channels for protons, which contact subunit **a** from either side of the membrane. (3) Under the influence of frequent impacts by neighbouring (lipid and water) molecules, the ring carries out Brownian *rotational fluctuations* relative to **a**. These non-directed thermal fluctuations are the basis for the directed rotation as induced through properties (1) and (2).

When a proteolipid molecule on the ring that carries a negative charge hits the boundary of **a**, it is reflected back into **a**. Therefore, the range of rotational fluctuations is limited to a narrow angular domain. Only if the electrostatical constraint is removed by binding of a proton may the ring move by one step further. Whether it progresses in the clockwise or counterclockwise direction is solely determined by the respective probability of protonation through the lower or the upper access channel. If the lower phase is more acidic than the upper one, the ring will turn clockwise (viewed from the bottom). It reverses direction if the pH-difference changes sign. The generated torque can be stored for useful work if the rotor (the ring of **c**) and the stator (**a**) are connected by a spring. This entropic machine counts and compares the numbers of (non-interacting) particles at both sides. It is driven by the chemical component of the transmembrane electrochemical potential difference, namely $\Delta(RT \ln c)$ and for protons $-2.3RT\Delta pH$, where c denotes the concentration of the transported ion. Because ATP synthase can work on electric driving force alone[36], one has to assume that the rate-limiting step is located between the access channels, which are strictly selective for one particular ionic species, e.g. protons (Mitchell's concept of a proton well[37]). If other ions are excluded from a given channel in Fig. 3, the partial voltage drop across this channel (about one half of the transmembrane voltage) is compensated by an appropriate concentration difference of the particular ion. The electrochemical potential, $\tilde{\mu}$, at any position of the channel is constant and the same as in the adjacent aqueous phase. Both the pH and voltage vary concertedly over the channel length as given by the definition of the electrochemical potential of the proton, $\tilde{\mu}_{H^+} = -2.3RT\text{pH} + F\psi$, where F denotes the Faraday constant and ψ, the electric potential. As a consequence of such a construct the interior of the access channel at the electropositive side of the membrane becomes more acidic.

It is obvious that such a rotatory engine (Fig. 3) would also work with sodium ions instead of protons, if the specificity of the access channels and the binding pocket around the acidic residue of the proteolipid are modified accordingly. It is also obvious that it pumps protons if torque is applied between the ring of **c** and **a**.

The linear dimensions of the rotor in F-ATPase are one order of magnitude smaller than those of the flagellar motor. Thus, the stochastic character of the motion is even more pronounced in the former, as shown in recent theories of molecular stepper motors with few steps (F-ATPase) and those with a few hundred steps (flagellar motor)[25,38]. It is a common feature of molecular motors that the inertial force is negligible compared with the frictional one. The stop distance is of atomic length[39]. In other words, a molecular motor is *not* spinning like a top. Nevertheless, at large driving forces, its motion is practically unidirectional.

The proposed torque generator could also work in another gear, as in V-ATPase[40], if the proteolipid has only one mid-membrane acid residue per four transmembrane helices and if one assumes the same total number of transmembrane helices per ring, the ratio of protons over ATP molecules ration will be halved (e.g. from 4:1 to 2:1). This appears a useful gearshift for an enzyme designed to acidify intracellular compartments. It could then generate twice the pH difference (e.g. across the vacuolar membrane) that was used (in the thylakoid membrane of chloroplasts) to sustain the given intracellular level of ATP.

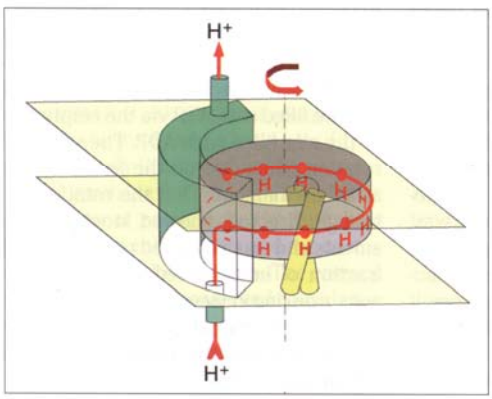

Figure 3
Model for the generation of torque in F_0 as driven by proton-motive force. The graph shows a ring of proteolipid molecules (subunit **c**) and subunit **a** that carries two access channels for protons from either side of the membrane (see text)

Subunits γε and δb$_2$ at the interface between F_0 and F_1

In a common view of the structure of F_0F_1, subunits γ, ε, δ and $\mathbf{b_2}$ are represented as elements of a single, narrow stalk between the two portions of the

holoenzyme. However, a rotatory function seems to call for separate 'stalk' segments, which are associated with the rotor and the stator function, respectively. Recent crosslinking data (see below) attributes the subunits γ and ε to the former, and δ and $\mathbf{b}_2$ to the latter (see Fig. 1).

The structure of soluble subunit ε, as shown by NMR, consists of two domains, a ten-stranded β-barrel joined to an α-helix/turn/α-helix motif[41]. The location of subunit ε relative to the other subunits of F_0F_1 has been studied, mainly by Capaldi and co-workers, by crosslinking both the native and the mutated protein from *E. coli*[42]. The results allow definition of four interfaces of subunit ε with subunits α, β, γ and **c** that leave few possibilities for the insertion of ε into F_0F_1. The observation that crosslinks between ε and γ do not impair the enzyme's activity justifies the tentative representation of ε, plus a portion of γ, as a spoke on the wheel of proteolipid molecules. It functions to clamp the rotation of the latter to the shaft of γ in $(\alpha\beta)_3$.

Recent crosslinking data from two laboratories, both for chloroplasts[43] and *E. coli*[44] show that the major portion of δ is located on the outside of the upper half of F_1. That the covalent attachment of δ to $(\alpha\beta)_3$, by bifunctional and photolabile crosslinkers, does not inhibit the activity of the chloroplast F_1 qualifies δ as an element of the stator[43]. Two lines of evidence suggest that δ is linked to $\mathbf{b}_2$: a crosslink product between subunits δ and **b** in the chloroplast enzyme[45] and the necessity to derive δ and **b** from the same source when aiming at the construction of functional chimeric enzymes from *P. modestum* and *E. coli*[11]. It is conceivable that the two copies of subunit **b** serve to hold and link the stator elements of both the headpiece and the membrane-intrinsic portion of the enzyme; at the one end, the hydrophilic stretches of $\mathbf{b}_2$ may clamp the δ-subunit that is attached to $(\alpha\beta)_3$, and at the other end, the hydrophobic stretches may hold **a**. The parallelogram of two **b**-subunits clamped together by δ on one side and by subunit **a** on the other could serve as one of the elastic elements that would transiently store the free energy gained from the translocation of the first to the fourth proton until the reaction proceeds further from one catalytic site to the next under the liberation of spontaneously formed and sequestered ATP.

Conclusions

It has been established experimentally that the F_1 portion of ATP synthase behaves as a rotatory engine, the smallest so far known in nature, and probably operates in three steps. It is likely that the synthesis of ATP by the F_0F_1 holoenzyme proceeds by a similar mechanism in the sequence: ion flow → intersubunit rotation → extrusion of spontaneously formed ATP. According to such a mechanism and supported by recent crosslinking data, previously favoured model structures, in particular those with a single, narrow stalk between F_0 and F_1 have to be qualified. Instead, the enzyme with its >20 polypeptide chains is likely to be conceived as bipartite. A central rotor is formed by a ring of subunits **c** plus the subunits γ and ε, which runs in a stator comprising **a**, $\mathbf{b}_2$, δ and $(\alpha\beta)_3$ (Ref. 46). Functionally, their relative rotation matters, and therefore the terms rotor and stator have only relative meaning. The concept of an 'electrochemical → mechanical → chemical' nanoengine is a challenge for critical examination concerning both its static structure and its intramolecular dynamics.

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