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COUPLING H^+ TRANSPORT AND ATP SYNTHESIS IN F_1F_o-ATP SYNTHASES: GLIMPSES OF INTERACTING PARTS IN A DYNAMIC MOLECULAR MACHINE

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Summary

Reversible, F_1F_o-type ATPases (also termed F-ATP synthases) catalyze the synthesis of ATP during oxidative phosphorylation. In animal cells, the enzyme traverses the inner mitochondrial membrane and uses the energy of an H^+ electrochemical gradient, generated by electron transport, in coupling H^+ translocation to ATP formation. Closely related enzymes are found in the plasma membrane of bacteria such as *Escherichia coli*, where the enzymes function reversibly depending upon nutritional circumstance. The F_1F_o-type enzymes are more distantly related to a second family of H^+-translocating ATPases, the vacuolar-type or V-ATPases. Recent structural information has provided important hints as to how these enzymes couple H^+ transport to the chemical work of ATP synthesis. The simplest F_1F_o-type enzymes, e.g. as in *E. coli*, are composed of eight types of subunits in an unusual stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ (F_1) and $a_1b_2c_{12}$ (F_o). F_1 extends from the membrane, with the α and β subunits alternating around a central subunit γ. ATP synthesis occurs alternately in different β subunits, the cooperative tight binding of ADP+P_i at one catalytic site being coupled to ATP release at a second. The differences in binding affinities appear to be caused by rotation of the γ subunit in the center of the $\alpha_3\beta_3$ hexamer. The γ subunit traverses a 4.5 nm stalk connecting the catalytic subunits to the membrane-traversing F_o sector. Subunit *c* is the H^+-translocating subunit of F_o. Protonation/deprotonation of Asp61 in the center of the membrane is coupled to structural changes in an extramembranous loop of subunit *c* which interacts with both the γ and ε subunits. Subunits γ and ε appear to move from one subunit *c* to another as ATP is synthesized. The torque of such movement is proposed to cause the rotation of γ within the $\alpha_3\beta_3$ complex. Four protons are translocated for each ATP synthesized. The movement of γ and ε therefore probably involves a unit of four *c* subunits. The organization of subunits in F_o remains a mystery; it will have to be understood if we are to understand the mechanism of torque generation.

Key words: oxidative phosphorylation, proton translocation, F_1F_o-ATP synthase, molecular mechanism, subunit *c*, membrane and stalk sectors, interacting subunits.

Introduction

Reversible, H^+-transporting F_1F_o-type ATPases (also termed F-ATP synthases) catalyze the synthesis of ATP during oxidative phosphorylation. In animal cells, the enzyme traverses the inner mitochondrial membrane, where it uses the energy of an H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) generated by an H^+-pumping electron transport system. Closely related F_1F_o-ATP synthases are found in the thylakoid membrane of chloroplasts, where the enzyme functions in electron-transport-driven photophosphorylation, and in the plasma membrane of bacteria, such as *Escherichia coli*, where the enzyme can function reversibly depending upon the nutritional circumstance. Under aerobic growth conditions, electron transport systems generate a $\Delta\bar{\mu}_{H^+}$ across the bacterial plasma membrane by pumping of protons from the cytoplasm to the outside of the cell, and the H^+ gradient is then used to drive ATP synthesis. A plasma membrane $\Delta\bar{\mu}_{H^+}$ is also required in other cellular processes such as H^+-coupled nutrient transport and flagellar rotation. Under anaerobic conditions, the required $\Delta\bar{\mu}_{H^+}$ is generated by the hydrolysis of glycolytically derived ATP by the F_1F_o-ATPase, protons being pumped from the cytoplasm to the outside of the cell. The mitochondrial, chloroplast and bacterial F_1F_o-ATP synthases are closely related in structure and mechanism, as I shall review in greater detail below. The enzymes are regulated somewhat differently because of the diversity of physiological circumstances under which they function (Walker, 1994; Harris, 1995; Mills *et al.* 1995). In this essay, I will focus on recent work that is beginning to provide structural insights into how the enzyme works in coupling H^+-transport to ATP synthesis. I will emphasize that the enzyme functions as a molecular machine in utilizing the energy of $\Delta\bar{\mu}_{H^+}$ to make ATP. If we are to understand the mechanism, we will need to define the movements of interacting parts. A few glimpses of the total picture may now be at hand.

Composition and general structure of F_1F_o-ATP synthases

F_1F_o-ATP synthases are composed of two structurally and functionally distinct sectors termed F_1 and F_o. The F_1 portion of the enzyme extends from the surface of the membrane and in electron micrographs projects as a knob-like image. F_1 is

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easily removed from the membrane as a water-soluble complex which hydrolyzes ATP. F_1 is connected to the membrane-traversing F_o sector *via* a narrow stalk. On removal of F_1, the transmembrane F_o sector mediates the passive transport of protons across the membrane. When the two sectors are properly associated, the complex reversibly couples ATP hydrolysis to H^+ transport. The simplest F_1F_o-type enzymes, e.g. as in *E. coli*, are composed of eight types of subunits in an unusual stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ (F_1) and $a_1b_2c_{12}$ (F_o).

The atomic resolution structure of the $\alpha_3\beta_3\gamma$ portion of beef heart mitochondrial F_1, recently published by Abrahams *et al.* (1994), confirms many features predicted from decades of study. A representation is given in Fig. 1. The α and β subunits, with approximate molecular masses of 55 kDa each, alternate in an almost symmetrical hexameric arrangement around a central core. The catalytic ATP binding sites are known to lie within the β subunit at αβ subunit interfaces. Extended α-helical segments of the partially resolved 30 kDa γ subunit extend through the center and protrude from the bottom of the $\alpha_3\beta_3$ hexamer as an extended and gently curved coiled coil. The α-helix at the C-terminal end of the γ subunit extends 9 nm from the top of F_1 to the surface of F_o, traversing the entire length of the 4.5 nm stalk (Abrahams *et al.* 1993, 1994; Watts *et al.* 1995; S. D. Watts, C. Tang and R. A. Capaldi, in preparation). The ε subunit is also known to traverse the length of the stalk from the bottom of F_1 to the surface of F_o (Zhang and Fillingame, 1995*b*). The δ and *b* subunits are thought to be the other major components of the stalk in *E. coli* and chloroplasts. The components of the stalk in mitochondria are somewhat different (Collinson *et al.* 1994*a*,*b*; Belogrudov *et al.* 1995; reviewed in Fillingame, 1996).

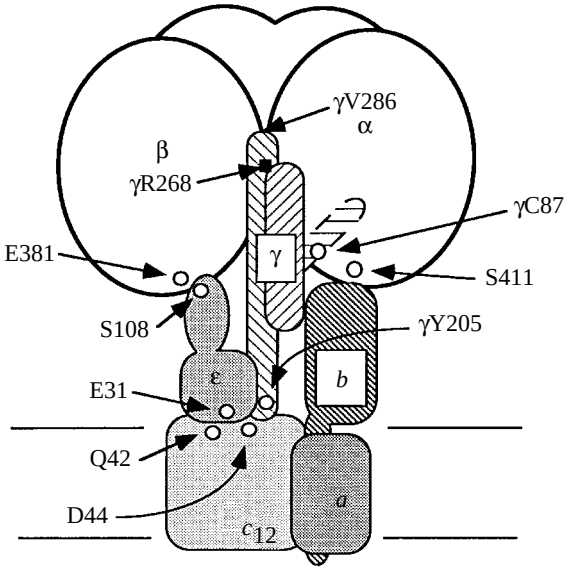

Fig. 1. Cross-sectional view of *Escherichia coli* F_1F_o ATPase emphasizing the stalk subunits. The δ subunit is not depicted in this representation. The three α and three β subunits alternate around a central core formed by the γ subunit. The three extended α-helices of the γ subunit resolved in the X-ray structure of Abrahams *et al.* (1994) are depicted. The C-terminal residue (γV286), at the end of the longest helix, is indicated; the position of the 'catch' formed between the empty β subunit and residues 268–269 in the γ C-terminal helix is indicated by the filled square labeled γR268. The short helix with residue γC87 projects horizontally towards a β subunit at the back of the structure, where it forms a second 'catch' with the β subunit binding the nonhydrolyzable ATP analog AMP-PNP. This 'catch', including residue γC87, is adjacent to residue E381 in the DELSEED sequence of β, and εS108 is adjacent to αS411, when Mg^{2+} AMP-PNP occupy catalytic sites; when Mg^{2+} (ADP+P_i) occupy catalytic sites, εS108 lies close to the β subunit DELSEED sequence (Aggeler and Capaldi, 1996). The sites of Cys substitution used in cross-linking studies are indicated by small circles and by the wild-type residue and number; the numbers used correspond to residues in the *E. coli* enzyme. The subunit pairs formed by cross-linking of these Cys residues are discussed in the text.

E. coli F_o is composed of three subunits in an experimentally determined stoichiometry of $a_1b_2c_{10\pm1}$ (Foster and Fillingame, 1982). The content of subunit *c* is sufficiently uncertain that we now favor a number of 12 for reasons that are discussed below. The organization of subunits in F_o remains to be determined, although recent electron microscopic imaging experiments suggest that subunits *a* and *b* may associate at the periphery of a complex of *c* subunits (Birkenhäger *et al.* 1995), as is suggested in the cartoon of Fig. 1. This arrangement contrasts with previous suggestions that subunits *a* and *b* may be centrally located and rotate within a ring of *c* subunits (Hatch *et al.* 1995). Some information is available on the folding of individual subunits (Fillingame, 1990). The 79-residue subunit *c* spans the membrane as a hairpin of two extended hydrophobic α-helices, with a more polar loop region which directly contacts subunits γ and ε at the bottom of the stalk (see Fig. 2). Asp61, centered in the second transmembrane helix of subunit *c*, is known to be the site of H^+ binding, as will be discussed below. The 156-residue subunit *b* is anchored to the membrane by a single hydrophobic α-helix with the charged, protease-sensitive bulk of the protein extending from the membrane to form part of the stalk interacting with F_1. Proteolytic cleavage of the exposed region of subunit *b* abolishes F_1 binding. The 271-residue subunit *a* is generally hydrophobic and is predicted to fold through the membrane with five or six transmembrane helices. A compelling, experimentally based model for the folding of subunit *a* in the membrane is still lacking. The composition of the chloroplast enzyme is easily related to that of *E. coli* with apparent substitution of *b* and *b′* subunits for the b_2 dimer of *E. coli*. The bovine heart mitochondrial F_o is considerably more complex, with at least nine polypeptides present (Collinson *et al.* 1994*a*). Homologs of the *E. coli a* and *c* subunits are clearly present in bovine F_o; the relationship of the other bovine F_o subunits with *E. coli* subunit *b* is less clear (Fillingame, 1996).

Relationship b etween F-ATP synthases and V-ATPases

The F_1F_o-type ATP synthases are distantly related to a second family of H^+-translocating ATPases, termed V (for vacuolar-type) or V_1V_o-ATPases (Harvey and Nelson, 1992). These enzymes are found in a variety of intracellular vesicles including plant and fungal vacuoles, clathrin-coated vesicles, secretory vesicles, Golgi bodies and lysosomes. The vesicles share the common feature of having an interior whose acidity is controlled by the H^+-pumping ATPase. V-ATPases are also widely distributed in the plasma membrane of cells specialized in H^+

secretion, such as osteoclasts, where the enzyme plays a role in bone resorption (Chatterjee *et al.* 1992; Mattsson *et al.* 1994), and in kidney intercalated cells, where it functions in urinary acidification (Gluck and Nelson, 1992; Gluck *et al.* 1992). The diversity of distribution is exemplified by the tobacco hornworm midgut plasma membrane H^+-ATPase, which energizes membrane potential formation *via* K^+/H^+ antiport (Wieczorek *et al.* 1991; see also Merzendorfer *et al.* 1997), and the distribution in cells specialized in alkalization (Harvey, 1992). V_1V_o-ATPases are composed of three types of subunits that bear obvious sequence similarity with the α, β and *c* subunits of F_1F_o and 5–6 other types of subunits bearing no obvious relationship. The stoichiometric ratio of subunits also appears to be similar, i.e. $A_3B_3...c_6$ (Forgac, 1992). The vacuolar subunit *c* is predicted to fold through the membrane with four transmembrane helices, with the first and second halves of the molecule bearing some sequence relationship to each other. The V-type gene is thought to have arisen by duplication and fusion of a primitive, bacterial-like ancestral gene (Nelson, 1992; Kibak *et al.* 1992). The *c* subunits of F_o and V_o could be organized similarly if there are 12 *c* subunits per bacterial F_o and 6 vacuolar-type *c* subunits per V_o. Each unit would then be composed of 24 transmembrane helices. Closely related ATPases are also found in the archeabacterial kingdom. In at least some cases, the *A* and *B* subunits most closely resemble the V-type enzyme, whereas the *c* subunit resembles the F-type subunit, folding with two transmembrane helices (Denda *et al.* 1989; Nelson, 1992).

Mechanism of coupling ATP hydrolysis with H^+ transport

Mg^{2+}-ATP substrate binds with strong negative cooperativity to three potentially catalytic sites in F_1- and F_1F_o-ATPase complexes (Weber *et al.* 1994). Binding of the second and third nucleotide strongly promotes catalysis. Evidence from a direct binding study (Weber *et al.* 1993) and from an independent hybridization approach with mutant β subunits (Amano *et al.* 1996) now strongly indicates that all three catalytic sites must be occupied and catalytically competent for optimal catalysis. Boyer's postulated 'binding change mechanism' for the enzyme was based upon these assumed and other properties (Boyer, 1993; Cross, 1981). The proposed ATPase mechanism envisages three alternating and cooperatively interacting sites with varying affinities for ATP and the [ADP+P_i] products, i.e. tight (T), loose (L) and open (O). The binding of ATP at an open site would lead to an O→T transition, with subsequent formation of tightly bound [ADP+P_i]; simultaneously, at a second site with bound [ADP+P_i], a T→L transition would occur; and simultaneously, at a third site, [ADP+P_i] would be released following an L→O transition. The energy released with the changes in binding affinity would be used to drive H^+ translocation. ATP synthesis driven by $\Delta\bar{\mu}_{H^+}$ would occur in the reverse order, i.e. binding of [ADP+P_i] substrate at an open site followed by sequential O→L→T transitions and ultimately release of ATP from the tight site. Although cooperative interactions between catalytic sites in the binding of substrates appear to be essential to the function of the enzyme, the $\Delta\bar{\mu}_{H^+}$ generated by electron transport is the primary driving force leading to release of ATP (Souid and Penefsky, 1995).

The atomic resolution model of bovine F_1 ($\alpha_3\beta_3\gamma$) provides an obvious structural scenario for the binding change mechanism of ATP synthesis. The enzyme was crystallized in the presence of the nonhydrolyzable ATP analog AMP-PNP, along with ADP and Mg^{2+}. Nucleotide occupancy at each of the three catalytic sites differs, i.e. AMP-PNP is bound at one site (the triphosphate or βTP site), ADP is bound at the second site (βDP) and the third site is empty (βE). The structures of each of the sites differ, as does the juxtaposition of each of the three β subunits to the asymmetrical γ subunit. The nucleotide binding site is literally opened in the empty β subunit by movements of β-strands proximal to the binding site and a more global movement of the largely α-helical, C-terminal third of the subunit by distances of up to 2 nm. Hydrogen-bonding rearrangements in the displaced loop of βE, near the nucleotide binding site, result in the formation of a 'catch' with the long C-terminal helix of the γ subunit (see Fig. 1). A second 'catch' is formed between the short, horizontally inclined helix of γ and the conserved DELSEED loop sequence in the C-terminal helical domain of the βTP subunit. Abrahams *et al.* (1994) propose that the conformational changes predicted in the binding change mechanism occur as a consequence of the rotation of the γ subunit relative to the three structurally asymmetrical β subunits. Subunit γ is proposed to rotate within a hydrophobic sleeve which surrounds the C-terminal end of the long F_1-traversing α-helix. During oxidative phosphorylation, rotation would be driven by $\Delta\bar{\mu}_{H^+}$ to cause a conformational change at a tight ATP site, thus opening that site and releasing ATP. Direct evidence for rotation between all three catalytic sites is still minimal. In experiments designed to test the rotational model, Duncan *et al.* (1995) demonstrated that the β subunit neighboring Cys87 of subunit γ moved during catalytic turnover. The extent of subunit repositioning was consistent with the alternating three-site model. Sabbert *et al.* (1996) have concluded that eosin-labeled subunit γ rotates by more than 200 ° within an immobilized $\alpha_3\beta_3$ core during the hydrolysis of ATP, based upon polarized absorption relaxation measurements following photobleaching.

In the F_1F_o complex, the α and β subunits lie well above the plane of the lipid bilayer, while the γ subunit extends from the crown of F_1 to the membrane surface. In further considering the mechanism of coupling H^+ translocation to ATP synthesis, I will first review the evidence defining the H^+-binding site in F_o and then return to consider the subunit–subunit interactions which couple events in F_o to those in F_1.

H^+-translocating unit of F_o

Asp61 in the second transmembrane helix of subunit *c* has long been thought to be the H^+-binding site in F_o and to undergo protonation–deprotonation as each H^+ is transported (Fillingame, 1990), although compelling kinetic evidence for this hypothesis was lacking prior to the recent work of Dimroth and coworkers with a structurally related, Na^+-transporting F_1F_o-ATPase in the bacterium *Propionigenium modestum* (Dimroth, 1995). Asp61 is the site of reaction with

dicyclohexylcarbodiimide (DCCD), which covalently modifies the carboxyl side-chain in a very specific reaction that blocks H^+ translocation. Substitution of Gly or Asn for Asp61 abolishes H^+ translocation, suggesting a requirement for an ionizable group at this position. Surprisingly, the essential carboxyl can be moved from position 61 in helix-2 to position 24 in transmembrane helix-1 with retention of function (Miller *et al.* 1990; Zhang and Fillingame, 1994). These findings suggest that the essential carboxyl can be anchored in essentially the same position in the center of the membrane from either of the two transmembrane helices and, further, that the two helices may act together as a structural unit during the protonation–deprotonation cycle.

Subunits of the *E. coli* and *P. modestum* enzymes show high degrees of sequence homology, and hybrid enzymes have been constructed both biochemically (Laubinger *et al.* 1990) and genetically (Kaim and Dimroth, 1994). The *P. modestum* enzyme transports Na^+ but, at low concentrations of Na^+, it has also been shown to transport H^+ (Laubinger and Dimroth, 1989). Na^+ and H^+ apparently compete for the same site. The homologous, DCCD-reactive residue in the *P. modestum* subunit *c* is Glu65. Na^+ protects Glu65 from reaction with DCCD, providing further evidence that the Glu65 carboxylate is the Na^+- and H^+-binding site (Kluge and Dimroth, 1993, 1994). On the basis of sequence comparisons of the *E. coli* and *P. modestum* subunits, we constructed mutants of the *E. coli* subunit *c* in the hope that the ion binding specificity could be changed (Zhang and Fillingame, 1995*a*). One combination of four mutations, Val-Asp61-Ala-Ile→Ala-Glu61-Ser-Thr did generate an F_o that binds Li^+, as evidenced by Li^+ inhibition of both H^+ transport and F_1F_o-ATPase activity. Thr at position 63 could be substituted by Ala or Gly with retention of the Li^+-sensitive phenotype, which suggested a requirement for a flexible but not necessarily polar residue at this position. We have suggested that the Glu61 carboxylate together with the Ser62 hydroxyl and perhaps a peptide carbonyl may provide the oxygens of the Li^+-liganding pocket. We predict that the Ser residue will be essential for Na^+ binding and translocation in the *P. modestum* enzyme. In combination, these studies provide compelling evidence that Asp61 in *E. coli* and Glu65 in *P. modestum* are the site of H^+ binding.

Nuclear magnetic resonance (NMR) studies of purified subunit *c* have recently provided information about its folding and the structure around Asp61. The protein was shown to fold as a hairpin of two extended α-helices in a single-phase solvent mixture of chloroform–methanol–water (4:4:1) made 50 mmol l^{-1} in NaCl (Girvin and Fillingame, 1993). The protein can be purified in this solvent and reconstituted with other F_o subunits with full retention of activity (Dmitriev *et al.* 1995). Further, while in this solvent, it retains some of the properties and structural features expected of the protein in native F_o (Girvin and Fillingame, 1993, 1994). As indicated in Fig. 2, a high-resolution NMR structure has been proposed for the interacting helices from the region around Asp61 to the N- and C-terminal ends (Girvin and Fillingame, 1995). One of the most interesting features of this model is the close proximity of Ala24 in helix-1 to Asp61 in helix-2. The side-chains are within Van der Waals contact with a distance of 0.32 nm between β-carbons. This proximity might, of course, be predicted from the genetic experiments discussed above in which the essential carboxyl is exchanged from position 61 to position 24 with retention of function. In looking at the model, it is easy to see how the carboxyl could be moved from one residue to the other with little effect on the structure of the bihelical unit.

Asp61 exhibits another remarkable property in the solvent system being used for NMR. The carboxyl side-chain has a pKa of 7.1, which is 1.5 units higher than that of any of the other carboxyls in the protein (Assadi-Porter and Fillingame, 1995). The other carboxyls exhibit pKa values close to that expected for solvent-exposed residues. These results suggest that the protein must be folded with the Asp61 carboxyl inaccessible to solvent and, indeed, the structural model predicts that the group should lie buried in a pocket of hydrophobic side-chains

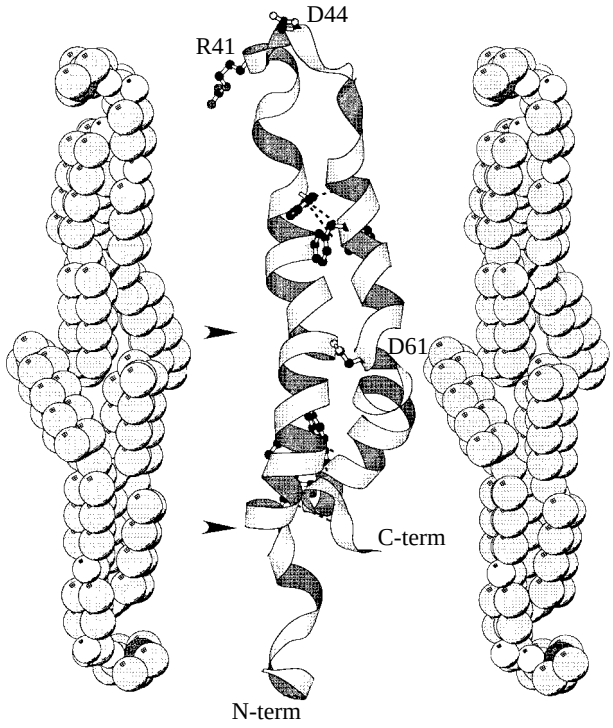

Fig. 2. Suggested folding of subunit *c* based upon current distance contraints between transmembrane α-helices (drawing modified from Fillingame *et al.* 1995). The arrowheads indicate the well-defined region of helical–helical interaction (Girvin and Fillingame, 1995), i.e. from residues Tyr10 to Ala25 in helix-1 and Val60 to Ala77 in helix-2. The side-chain of Ala24 is shown juxtaposed to the Asp61 side-chain. The distance restraints from Girvin and Fillingame (1993) were combined with those in the well-defined region (Girvin and Fillingame, 1995) and used as input for modeling the whole subunit. The model was generated by distance geometry and dynamic simulated annealing using the program XPLOR. The restraints between aromatic clusters at the top and bottom of the structure are emphasized since they limit possible folding regimens. The loop region in the model shown is very similar to that in the other low-energy structures generated in this exercise, but all structural details in this region are speculative. The protein model has been placed in a bilayer of dioleoylphosphatidylethanolamine, modeled according to Peitzsch *et al.* (1995), to emphasize that the polar loop region of subunit *c* (at the top) cannot extend much beyond the plane of the membrane.

(Girvin and Fillingame, 1995). In the double mutant Ala24→Asp/Asp61→Asn, the functional Asp24 carboxyl shows a similarly elevated pKa of 6.9, a finding consistent with the structural model which shows that a carboxyl from either residue 24 or residue 61 could extend into the same hydrophobic pocket. A mutant with aspartyls at both position 24 and position 61 has also been examined and both carboxyls show pKa values close to 7.0. The function of this mutant is pH-sensitive, with one of the carboxyls predicted to have a pKa in the range 7–7.4 *in situ* (Zhang and Fillingame, 1994). The environment around Asp61 in the isolated protein may thus correlate very well with that in native F_o. During an active proton-pumping cycle, the structure around Asp61 of subunit *c* is predicted to change from a high-pKa form to a low-pKa form as protons are pumped from low concentration to high concentration (Fillingame, 1990). The structure of subunit *c* being determined by NMR may correspond to the high-pKa form of the protein.

The stoichiometry of H^+ pumped per ATP hydrolyzed/synthesized is generally estimated to be at least three and more probably four (Fillingame, 1990; van Walraven *et al.* 1996). The H^+/ATP stoichiometry may explain the presence of multiple subunits *c* in the F_o complex, i.e. four subunits *c* may undergo protonation and deprotonation per ATP synthesized. At a stoichiometry of four, a change in pKa for Asp61 of 2–3 pH units would provide changes in binding energy of 40–60 kJ mol^{-1}, which is within the range of the free energy change (ΔG) estimated as necessary for ATP synthesis under varying conditions (Souid and Penefsky, 1995). If three different sets of four subunits *c* protonate and deprotonate as the three β subunits alternate in ATP synthesis, a functional explanation for the stoichiometry of 12 subunit *c* per F_o would be at hand.

Subunit *a* is also likely to play a role in H^+ translocation and perhaps in the coupling of H^+ translocation to ATP synthesis. A number of subunit *a* mutants have been described, but the phenotypes are still difficult to interpret owing to the lack of a structural model (Fillingame, 1990). The number of transmembrane helices and the sidedness of the protein in the membrane are still not defined with certainty. Arg210 is generally thought to play a critical role in H^+ transport, but that role is not clear. In its protonated form, it could form a transient salt bridge with Asp61 of subunit *c* to lower the pKa during the part of the H^+ translocation cycle involving interaction of a single copy of subunit *c* with the single copy of subunit *a*, as I have suggested previously (Fillingame, 1990). If this hypothesis were correct, a class of Arg210 mutants might be found that retain the capacity to function in passive H^+ transport not involving pKa changes, while being inactive in active H^+ transport cycles where the pKa changes are required. Indeed the Arg210→Ala mutant shows hints of such a phenotype, i.e. the mutant lacks ATP synthase and ATPase-coupled H^+ transport activities but shows significant indications of passive H^+ conductance activity (Hatch *et al.* 1995). Arg210 could also serve in either more or less direct roles.

Hatch *et al.* (1995) have now generated functional pseudorevertants to the Arg210→Gln mutant where the essential Arg is replaced by a Gln252→Arg mutation. This functional double mutant is unusual in that oxidative phosphorylation clearly takes place, whereas ATP-driven H^+ transport was not demonstrable. These results are difficult to rationalize since the two activities are presumed to represent a reversal of the same process. The studies of Hatch *et al.* (1995) most simply suggest a proximity of residues 210 and 252 in the membrane, which would occur with Arg210 on transmembrane helix-4 and Gln252 on transmembrane helix-5 in the five-helix model they propose. Such folding would also bring Gly218 and Glu219 into close proximity of His245, as predicted by other genetic studies (Cain and Simoni, 1988; Hartzog and Cain, 1994). The idea of an extended transmembrane helix with a continuous functional face from residues 212 to 222 is supported by Ala insertion mutagenesis experiments (Wang and Vik, 1994). Other genetic studies suggest that a face of this putative helix, including residues 217, 221 and 224, may interact with the essential carboxyl group of subunit *c* during ATPase-coupled H^+ transport (Fraga *et al.* 1994*a*). In these studies, the function of the Ala24→Asp/Asp61→Gly double mutant was shown to be optimized by third-site mutations in residues 217, 221 or 224. Since passive proton conductance is not disrupted in the Asp24Gly61 double mutant (Zhang and Fillingame, 1994), the optimizing mutations may facilitate the movements between subunits which lead to changes in pKa or which couple to conformational changes linked to ATP synthesis.

Coupling of H^+ transport to ATP synthesis

The protonation/deprotonation of Asp61 in subunit *c* was initially proposed to be linked with conformational changes in the polar loop region on the basis of the phenotype of an 'uncoupled' mutant (Mosher *et al.* 1985). Mutation of Gln42→Glu in the conserved Arg-Gln-Pro sequence of the polar loop gave rise to an F_1F_o complex with normal ATPase activity that was uncoupled from H^+ translocation. The F_1 moiety was bound by the mutant F_o with normal affinity, and the mutant F_o was shown to promote normal passive proton conductance. However, in contrast to the case of wild-type F_1F_o, when F_1 was bound it did not block the intrinsic proton conductance of *c*Gln42→Glu F_o. We have more recently described a very similar phenotype for the Arg41→Lys polar loop mutant (Fraga *et al.* 1994*b*). Other mutations in Gln42 and Pro43 give rise to F_1F_o showing varying degrees of uncoupling (Fraga and Fillingame, 1989; Miller *et al.* 1989).

In an attempt to define the subunit in F_1 that interacted with the polar loop to couple ATPase and H^+ translocation, we selected suppressor mutations to the *c*Gln42→Glu mutation. All four of the extragenic suppressors isolated proved to lie in the Glu31 codon of subunit ε; the suppressor mutations being Glu31→Lys (found twice), Glu31→Gly and Glu31→Val (Zhang *et al.* 1994). To test whether there was a direct interaction between residue 31 of subunit ε and the polar loop of subunit *c*, Cys residues were introduced into both regions and crosslinking attempted by oxidation. Cross-links were found with the εCys31/*c*Cys40, εCys31/*c*Cys42 and εCys31/*c*Cys43 pairs, but not with the εCys31/*c*Cys39 pair (Zhang and Fillingame, 1995*b*).

A *c*Asp44→Cys mutant was also generated. The Cys in this mutant proved to be hyper-reactive, crosslinking with other subunits by mechanisms other than Cys–Cys oxidation. In collaboration with R. Capaldi's laboratory (University of Oregon, Eugene), the region of crosslinking in subunit γ was defined by peptide mapping (Watts *et al.* 1995). S. D. Watts, C. Tang and R. A. Capaldi (in preparation) have now introduced Cys at position 205 in subunit γ and shown crosslinking with Cys42, Cys43 and Cys44 of subunit *c*. In sum, these results suggest that the conformational coupling between the loop region of subunit *c* and the stalk of F_1 is likely to occur *via* direct interactions between loop residues of subunit *c* and residues in regions surrounding Glu31 of subunit ε and Tyr205 of subunit γ.

The structure of the loop region of subunit *c* is not yet known but, on the basis of known NMR distance constraints, it is not likely to extend beyond the head group region of the phospholipid bilayer (Fig. 2; Fillingame *et al.* 1995). We do know that the structure of the loop region around Pro43 changes as Asp61 is titrated in the chloroform–methanol–water solvent used for NMR (Assadi-Porter and Fillingame, 1995), adding further support for the idea of H^+ binding causing global changes in loop structure. The atomic resolution X-ray model of F_1 does not resolve the region of subunit γ around Tyr205, but it can be assumed that the C-terminal α-helical structure protruding from the bottom of F_1 extends to the surface of the membrane. Crosslinking studies indicate that subunit ε must also extend the entire length of the stalk as it can be crosslinked to both the F_o and F_1 domains (Zhang and Fillingame, 1995*b*; Aggeler and Capaldi, 1996). Wilkens *et al.* (1995) have recently reported an NMR-derived structure for isolated subunit ε (Fig. 3). It is a protein of two distinct domains, where the N-terminal domain of 84 residues forms a flattened 10-stranded β-barrel and the C-terminal 48 residues fold as an antiparallel hairpin of two extended α-helices. Although the C-terminal domain interacts with important regions of the β or α subunits, the interaction depending upon the nucleotide bound at catalytic sites (Capaldi *et al.* 1995; Aggeler and Capaldi, 1996), this interaction appears to be unimportant in coupling since the entire domain can be deleted without loss of function (Kuki *et al.* 1988). Subunits ε and γ are known to bind to each other *in vitro* (Dunn, 1982), and regions of interaction have been defined by crosslinking, including interactions around residue Tyr205 of γ and within the N-terminal β-barrel domain of subunit ε (Tang and Capaldi, 1996; see Fig. 3). As protons are translocated, the γε complex is hypothesized to move as a unit from one subunit *c* to another to generate torque on γ that would cause it to rotate as an axle within the $\alpha_3\beta_3$ hexamer of F_1 (Tang and Capaldi, 1996).

How might ATP synthesis be driven by such a molecular machine? The γ subunit inside the $\alpha_3\beta_3$ complex would be predicted to rotate by 120° as each tightly bound ATP is released, and each 120° rotation would be expected to break and establish new 'catches' with the βE and βTP subunits. The 120° rotation would be driven by the translocation of 4 H^+ and the unidirectional movement of the γε complex across the loop regions of four subunits *c*. Such a molecular machine could be

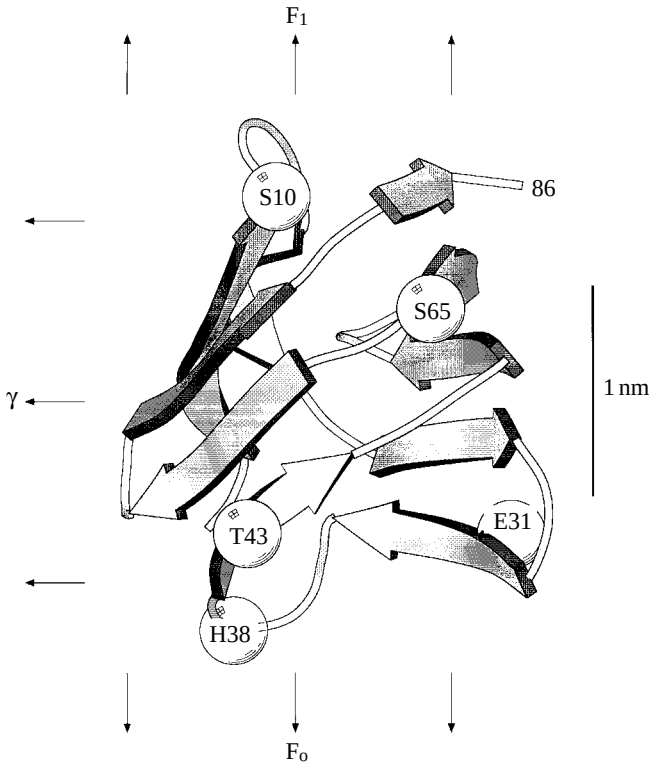

Fig. 3. Structure of the β-barrel domain of subunit ε, determined by nuclear magnetic resonance, emphasizing the sites of interaction with F_o and with subunit γ of F_1 (Wilkens *et al.* 1995). The distance between the α-carbons of His38 and Glu31 at the bottom the structure is 1.8 nm. The coordinates and figure were provided by Dr S. Wilkens and Dr R. Capaldi (University of Oregon).

designed by the following rules. Formation of the γε–*c* complex would require a loop structure, as seen only in the low-pKa form of subunit *c*, i.e. the form that accepts H^+ from the high H^+ activity side (outer surface) of the membrane. The association of the single-copy, subunit *a* with subunit *c* might be required to generate the low-pKa Asp61, as was discussed above. Movement of subunit *a* away from subunit *c* would be coupled to a pKa shift and translocation of the carboxyl group to the low H^+ activity side (inner surface) of the membrane. The release of H^+ from the high-pKa form of subunit *c*, or the conformational change accompanying the pKa shift, would result in changes in subunit *c* loop structure causing disassociation of the γε complex. An asymmetry in association of the γε pair with F_o subunits *a* or *b* could unidirectionally restrict movement of γε to the loop of the next low-pKa subunit *c*, i.e. force movement in a clockwise or counter-clockwise direction. As the fourth H^+ was translocated on completion of the 120° rotation, sufficient torque would be generated to drive the 120° rotation of subunit γ within the $\alpha_3\beta_3$ hexamer, by the breaking and reformation of the βE and βTP 'catches', with a cooperative alteration of nucleotide binding site structure in the three β subunits and release of ATP from the 'tight' site.

In evaluating such a model, one needs to know whether the γ subunit moves smoothly in four isoenergetic steps over each 120° of rotation, as it moves from one β to the next, or in contrast,

whether most of the energy is spent in a single event such as driving 'catch' breaking and/or 'catch' reformation and the associated conformational changes. On the basis of the X-ray crystal structure, the latter seems more likely. If the energy is consumed in a single event, then the sequential release of four protons in isoenergetic translocations steps, as suggested in the paragraph above, is problematic. That is, how would the energy be stored over the interval between the first and fourth translocation event? A solution to the problem is to make the release of H^+ from the high-pKa form of subunit *c* dependent upon the position of γ within the $\alpha_3\beta_3$ core, e.g. to permit H^+ release only when γ is in position to form a new catch. In such a model, the association of γε with the loop of subunit *c* would still be dependent upon formation of the low-pKa form of the protein, and transition to the high-pKa form would still cause γε disassociation, but H^+ would not be released from the high-pKa sites before simultaneous completion of the 120° movement of subunit γ.

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