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Topical Review

Mechanisms of Active Transport in the F_OF_1 ATP Synthase

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Introduction

The vast majority of ATP synthesis in aerobic metabolism is accomplished by a membrane embedded, multisubunit complex known as the F_OF_1 ATP synthase. The highly conserved complex is found in the inner membrane of mitochondria, the thylakoid membrane of chloroplasts, and the cell membrane of bacteria. In each case, the enzyme utilizes the energy derived from transport of protons down an electrochemical gradient (proton motive force or $\Delta\mu_{H^+}$) to drive the synthesis of ATP from ADP and Pi. In addition, the ATP synthase of many bacteria have physiological roles as true proton-pumping ATPases. In anaerobic metabolism or where electron transport is absent, ATP hydrolysis is used to generate a $\Delta\mu_{H^+}$ which drives secondary transporters that accumulate nutrients and maintain ionic balances.

ATP synthases have complex structures with a total molecular mass of approximately 500,000 daltons. There are at least eight subunits that are divided into two easily dissociated portions, the membranous F_O and the soluble F_1 sectors. The F_O by itself, passively conducts protons through the membrane, while the F_1 catalyzes hydrolysis of ATP. The bacterial complexes have the simplest subunit compositions; F_1 is composed of five different subunits with the stoichiometry of $\alpha_3\beta_3\delta\gamma\epsilon$, while the F_O has three different subunits, $ab_2c_{8\text{-}12}$. The chloroplast enzyme is quite similar to that of bacteria, except that it has one additional F_O subunit [40, 91]. In contrast, the mitochondrial enzyme has several additional subunits in both sectors [71]. (For comparison of subunit compositions, *see* Refs. 55, 94.) Despite these differences, many of the subunits and the mechanism of active transport are highly conserved. This fact has been dramatically demonstrated by the use of mitochondrial and chloroplast subunits as functional replacements in bacterial complexes [11, 18, 29, 66, 82, 92, 119].

The recent structural solutions of bovine F_1 by X-ray crystallography [1] and isolated *E. coli* ϵ [115] and *c* [42–44] subunits by multidimensional NMR have provided a wealth of new information that has contributed greatly to understanding the molecular mechanisms of the three functions of the ATP synthase: (i) catalysis of ATP synthesis/hydrolysis, (ii) translocation of protons across the membrane, and (iii) coupling of catalysis and transport. This review will focus on current understanding of how each function interacts with its substrates.

F_1 Interactions with Nucleotides

The Bovine F_1 Structure Represents the Catalytic Domain

The X-ray crystallographic structure of the bovine F_1 solved at 2.8 Å resolution [1] provided great detail about the portion of the ATP synthase involved in the catalytic mechanism. The arrangement of the homologous α and β subunits had previously been revealed by electron microscopic analysis (reviewed in Ref. 21) and the 3.6 Å X-ray structure of the rat liver F_1 [12]. The three α subunits alternate with the three β subunits like ''the seg-

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ments of an orange'' [1]. Even though all five subunits were in the F_1 crystal, the single copy subunits, δ, ϵ, and most of γ, were not represented in the electron density map. The portions of the γ subunit for which there was electron density were the two termini and a short segment from the middle of the polypeptide. All three segments were α-helical and all three corresponded to the only conserved amino acid sequences of the subunit. The terminal regions (45 residues from the amino terminus and 63 residues from the carboxyl terminus) formed a coiled-coil structure that extended into the core of the β/α ring. Based on reconstitution experiments of the *E. coli* F_1, the minimal complex capable of normal ATPase activity is $\alpha_3\beta_3\gamma$ [28, 41]. At least a portion of the γ subunit appears essential to achieve catalytic properties similar to the holoenzyme. In the cases of F_1 from chloroplast and the thermophilic bacterium PS3, α and β subunits alone will reconstitute ATPase activity; however, the activities of the smaller complexes are lower than when γ subunit is present and are kinetically different [9, 57]. Interestingly, the γ subunit can be clipped by trypsin in the unconserved central regions without affecting activity [76, 107]. Furthermore, mutations in the γ subunit known to affect catalytic properties are only found in the terminal helical regions that appear in the bovine structure (R. Nakamoto, *unpublished observations*). Together, these results suggest that the portion of the F_1 complex modeled in the bovine structure may be considered the domain that contains the catalytic mechanism.

The interaction of this domain with nucleotides and the catalytic mechanism has been extensively discussed elsewhere (e.g., Refs. 16, 90, 94, 97). This review will only touch on some important mechanistic features.

F_1 Interactions with Nucleotides

The bovine F_1 structure contained six nucleotide binding sites; one in each β/α or α/β interface. Six sites have been suspected based on sequence analysis, titrations, and affinity labeling (reviewed in Refs. 23, 90). The three sites found mostly in the α subunits are known as the noncatalytic or nonexchangeable sites, while the three found mostly in the β subunits are catalytic and turnover nucleotides. The physiological role of the noncatalytic sites will not be considered here and has been reviewed elsewhere [16, 90]. At least in the case of *E. coli* F_1, the noncatalytic sites do not appear to have a significant role. Weber et al. [112] demonstrated that an *E. coli* F_1 with the mutation αAsp-261 to Asn[1] does not bind nucleotides in the non-catalytic sites and does not affect catalytic activity.

Rate Constants of Elementary Reaction Steps are Measured in Unisite Conditions

The three catalytic sites show both strong positive catalytic cooperativity and negative cooperativity for Mg · nucleotide binding. Because of the complicated interaction among sites, kinetic analysis of the reaction in the steady state is difficult; however, most of the elementary reaction steps have been analyzed by limiting occupancy to a single site. In unisite conditions, ATP concentrations are kept low and F_1 is used at 10-fold concentration over substrate to assure that ATP binds to only the first site. Affinity for ATP in this site is very high and the rate constants for ADP and Pi release are very slow [48]. Rate constants for each of the partial reactions have been determined: ATP binding and release, ATP hydrolysis and synthesis, ADP binding and release, and Pi release (7, 48; reviewed in Refs. 90, 95, 97). There is essentially no binding of Pi (K_d > 2 M) to soluble F_1 or to F_OF_1 in the absence of $\Delta\mu_{H^+}$.

Catalytic Sites Optimize Conformation of Substrates for Chemistry

Unisite conditions represent a portion of the cycle where reactants are coordinated in a hydrophobic environment that is optimized to carry out the chemistry of ATP hydrolysis/synthesis. The F_1 ATPase has no covalent interaction with substrates or products. The stereochemistry of isotopic phosphate oxygen is inverted after hydrolysis of ATP indicating an in-line nucleophilic attack of a water molecule on the terminal phosphate [111]. The equilibrium constant of hydrolysis/synthesis (ATP ↔ ADP + Pi) is close to one indicating that the step does not involve a large free energy change [48]. Phosphate-water ^{18}O exchange experiments demonstrated that the hydrolysis/synthesis reaction occurs several times during occupancy and the number of exchanges depends on the overall turnover rate [87, 116].

The above results indicate an important mechanistic feature of this enzyme—the use of binding energy to achieve a stabilized catalytic transition state, and subsequently, to disrupt tight binding of products so that they are released. By analyses of thermodynamic and kinetic properties of F_1 mutants, Al-Shawi et al. [7] derived linear free energy relationship [30, 31] to demonstrate

[1] Because the vast majority of mutagenesis studies have been done in the *Escherichia coli* system, the subunit nomenclature and residue numbering used in this article will be that of the *E. coli* F_OF_1 complex unless otherwise mentioned.

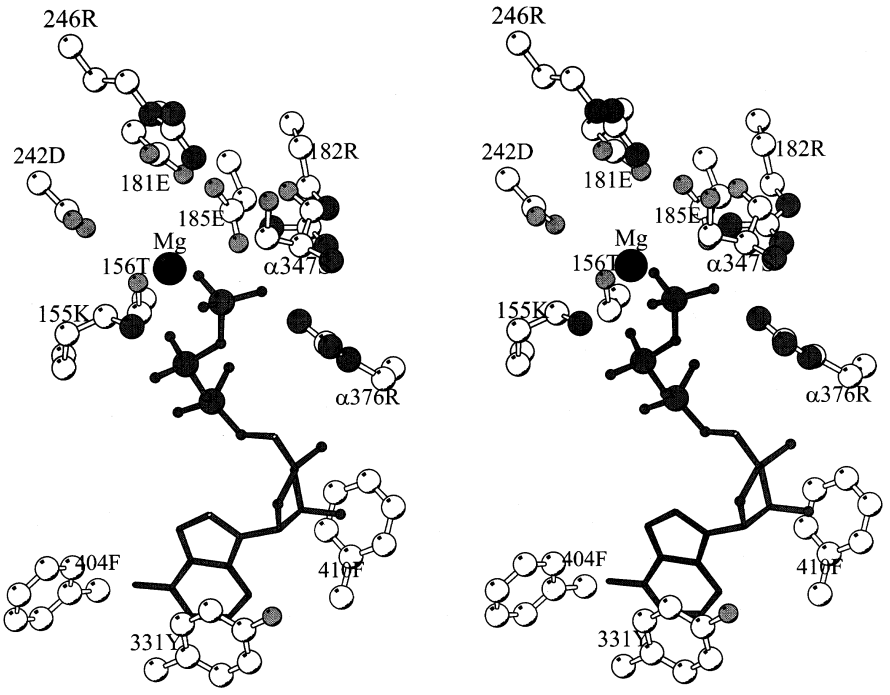

Fig. 1. Stereo view of amino acid residues in the ATP-bound catalytic site (*see* Ref. 1). Residue numbers are those of the *E. coli* complex. ATP is in dark grey. Protein carbons are white, oxygens are light grey, and nitrogens are dark grey. The Mg^{2+} ion is black. All amino acids are from the β subunit except αSer-347 and αArg-376.

that binding energy is the major driving force for catalysis (for a review, *see* Ref. 97 and references therein).

Three important catalytic residues have been identified, βLys-155, βGlu-181, and βAsp-242 (see Fig. 1). The critical interaction of the protonated ϵ-amino group of βLys-155 with the γ-phosphate enhances Mg · ATP binding and probably Pi binding as well [86, 98]. This lysine residue in the common nucleotide binding motif known as the P-loop has a similar role in many nucleotide-utilizing proteins such as adenylate kinase and p21*ras* [93, 110]. Based on the structure, Abrahams et al. [1] suggested that Mg^{2+} was likely coordinated by βThr-156, two or three phosphate oxygens, and two or three water molecules. In addition, Al-Shawi et al. [6] found strong evidence from mutagenesis experiments that βAsp-242 also participated in coordinating Mg^{2+}. Analysis of the structure also suggested that βGlu-181 is the likely catalytic base that activates a water molecule for the in-line nucleophilic attack on the γ phosphate of ATP [1]. In agreement with this assessment, replacement of βGlu-181 with Gln strongly affects the transition state of the hydrolysis/synthesis reaction [88, 96]. α subunit residues also participate in the catalytic sites; the guanidinium group of αArg-376 is in the correct position to stabilize the negative charge that develops when the γ phosphate passes through a pentacoordinate transition state [1].

Site-site Interactions Promote Catalysis

The promotion of catalysis in multisite activity is a manifestation of strong positive catalytic cooperativity between sites. The mechanism of site-site interactions was first proposed by Boyer [13–15] and referred to as the ''binding-change mechanism.'' Senior and coworkers [97] have argued that there are two major conformations of each catalytic site: the ATP (or ADP + Pi)-binding site which is hydrophobic, constrained, and optimized to carry out chemistry, and the ADP-binding site which is less hydrophobic and less constrained.

The differences between the two conformations involve coordination of Mg^{2+} and the phosphoryl moiety of the nucleotide. Replacements of βGlu-185, which is close to Mg^{2+} in the catalytic site, disrupted cooperativity and altered interactions with Mg^{2+}, while unisite rates were not strongly affected [85]. These results suggest that, at least in part, cooperative interactions act through βGlu-185 and coordination of Mg^{2+} to alter affinity for nucleotide and Pi. In contrast, the degree of fluorescence quenching of a Trp mutation near the adenine moiety is constant during the course of the catalytic cycle [113]. The ability of this mutation, βTyr-331 to Trp, to act as a specific reporter of nucleotide occupancy, is easily understood because of its proximity to the adenine ring (Fig. 1). These data indicate that conformational

changes that cause affinity changes do not involve this portion of the nucleotide binding site.

Conversion between the conformations requires input of energy. In both unisite and multisite catalysis, the energy requiring steps are Pi binding and ATP release. During net synthesis, energy from $\Delta\mu_{H^+}$ is used to induce conversion from the ADP-binding conformation to the ATP conformation which has an increased affinity for Pi of about seven orders of magnitude [47, 87, 103]. Energy from $\Delta\mu_{H^+}$ is also used to convert a site in the ATP-binding conformation to the ADP conformation which has a greatly increased rate of ATP release. These conformational changes occur in different sites in a coordinated manner.

All Three Sites Participate in Catalysis

Even though the two major conformations may imply that only two interacting sites (or bi-site catalysis) are required, evidence suggests that all three sites must participate to achieve maximal rates of catalysis. Weber et al. [114] used the βTyr-331 to Trp mutation described above as a direct monitor of nucleotide binding during turnover conditions. They found that the rate of hydrolysis was slow when only two catalytic sites were occupied. Occupancy of the third site was required for V_{max} rates, and all three sites appeared to be filled during V_{max} conditions suggesting that ATP enters a site as soon as ADP is released. An important feature of the bovine F_1 structure [1] was that each site had a different bound nucleotide; one had AMPPNP, the second had ADP, and the third was devoid of nucleotide. The asymmetry of the three β subunit active sites is also observed in different rates of protein modifications occurring on otherwise identical α or β subunits (reviewed in Ref. 23). For example, lucifer yellow labels only one of the chloroplast α subunits [81]. Similarly, the specific modification by N,N′-dicyclohexylcarbodiimide (DCCD) of βGlu-192 [118] will not occur on a β subunit that is cross-linked to ϵ [69]. The same cross-linked β subunit was also resistant to reaction with various thiol reagents [104]. Haughton and Capaldi [49] have been able to distinguish each of the three β subunits by differences in chemical modification and cross-linking patterns. Significantly, the differences between β subunits was retained even in the nucleotide-free enzyme suggesting that the single copy subunits contribute to site asymmetry.

Taken together, these results are consistent with a sequential catalytic mechanism such as that proposed by Boyer (discussed in Refs. 15, 16). At any one time, the three catalytic sites are in different conformations, but all pass sequentially through the same conformations. The rotating mechanism will be discussed later in relationship to coupling catalysis to proton transport.

F_O Interactions with Transported Protons

All *E. coli* F_O Subunits are Required for Function

The F_O of all ATP synthases passively conducts protons after F_1 has been stripped off. Bacterial ATP synthases have the simplest F_O sectors which contain only three different subunits: one *a*, two *b* and 8–12 copies of *c* (most likely 9 or 10; Refs. 36, 52). The possible arrangement of the F_O subunits and their topological models have been discussed elsewhere [33].

The Transport Mechanism is in F_O

Several authors have argued that the transported proton does not participate in the chemistry of ATP synthesis. Instead, energy derived from the downhill passage of the proton through the F_O is indirectly coupled to catalytic activity via kinetically linked conformational or electrostatic effects (*see* Ref. 32). The most compelling of these arguments are as follows:

(1) The equilibrium constant for the ATP + H_2O $\leftrightarrow$ ADP + Pi + H^+ reaction step is independent of proton motive force [46, 87] or pH [8].

(2) The F_OF_1 variant from *Propionigenium modestum* is capable of utilizing $\Delta\mu_{Na^+}$ in place of $\Delta\mu_{H^+}$ to drive ATP synthesis [61–63]; therefore, participation of the transported proton in the chemistry of ATP synthesis is unlikely. Extensive characterization of Na^+ transport strongly suggests that the *P. modestum* F_O has the kinetics of a facilitated carrier and not those of an open channel [58]. This behavior is expected for a transport mechanism that includes a cation binding site that alternates access to either side of the membrane (*see below*). Laubinger et al. [60] demonstrated that cation specificity resides in the F_O by reconstituting the F_O sector from *P. modestum* with the F_1 from *E. coli*. The hybrid complex had the characteristics of the *P. modestum* complex: the hybrid carried out ATP-dependent Na^+ or H^+ translocation, and H^+ pumping was abolished at [Na^+] greater than 1 mM.

Subunit *c* is Essential for Proton Transport and Coupling

The extremely hydrophobic subunit *c* has a simple hairpin conformation. Its structure in chloroform-methanol-water was modeled based on distance measurements obtained by multidimensional NMR (see Fig. 2; Refs.

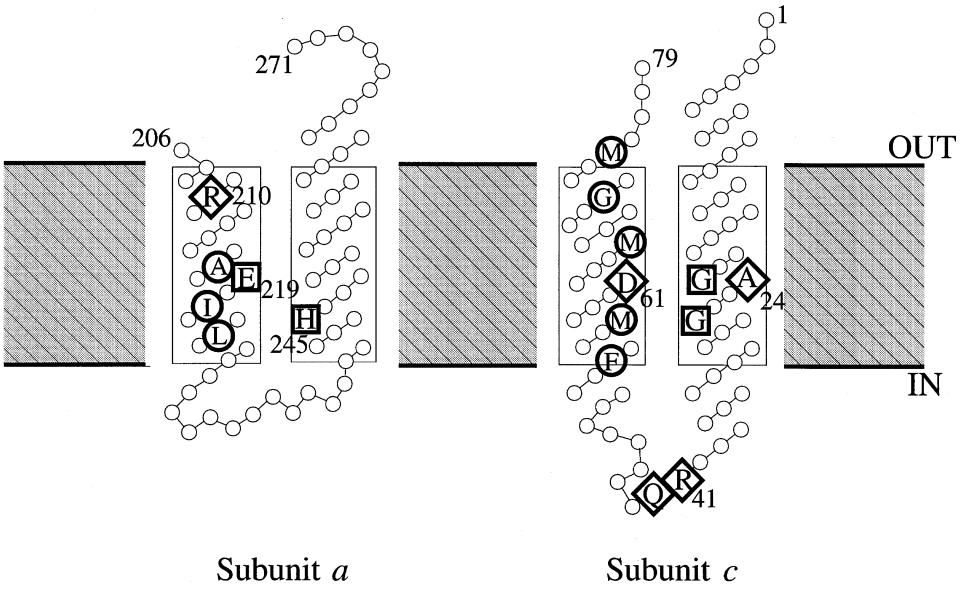

Fig. 2. Potential sites of interactions between subunit *c* and the carboxyl terminal region of subunit *a*. The structure of subunit *c* is taken from Girvin & Fillingame [42–44]. Note that *c*Ala-24 is in van der Waals contact with *c*Asp-61 [44]. The arrangement of subunit *a* is conjectural. Residues in diamonds are believed to be essential and the residues in boxes are functionally important (*see* text). Residues in the large circles are sites of third-site mutations identified by Fraga et al. [38] that optimized activity of the double mutant, *c*Asp-24/*c*Gly-61. Modified from Ref. 38 with permission.

42–44). Even though the polypeptide was in organic solvent and only the helical segments modeled, the agreement of the structure with genetic and biochemical evidence was quite satisfying. Two antiparallel α-helices that span the membrane are connected by a polar stretch of seven amino acids. This loop extends from the membrane on the side that binds F_1 (*see below* and Ref. 25).

In the carboxyl terminal helix resides one of the most studied amino acids of the F_OF_1, *c*Asp-61 (Asp in *E. coli* and Glu in all others). Several lines of evidence suggest that the acidic residue plays an essential role in proton translocation activity. ^{1}H-NMR suggests that this residue has an extremely high pK which is expected of an acidic residue that participates in hydrogen bonding and movement of a proton [75, 84]. A hallmark of this residue is its reactivity with DCCD. Only one of the *c* subunits need react with DCCD to completely inhibit proton translocation activity [52]. The same modification inhibits ATPase activity as well. Changing this residue by mutagenesis to Asn has similar effects [35]. Significantly, when changed to Gly, proton translocation is again blocked, but ATPase activity is unaffected. These results suggest that modification with DCCD or replacement with Asn allows the transport mechanism to maintain the conformation required for coupling to catalysis. In contrast, a Gly replacement likely alters conformation and perturbs communication to the catalytic sites.

The spatial arrangement and environment of the acidic group are also critical. Replacement of Asp with Glu greatly reduces activity [74]. Moreover, changes of the surrounding residues also affect function. Changing *c*Ala-24 in the amino-terminal helix to Ser or *c*Ile-28 to Thr or Val reduces the sensitivity to DCCD but still allows proton translocation activity [34]. In contrast, changing *c*Gly-23 or *c*Ala-24 to Leu reduced activity enough to eliminate oxidative phosphorylation [83]. These residues of the amino terminal helix are included in a glycine-rich sequence between residues *c*Gly-23 to *c*Gly-20 which forms a pocket around *c*Asp-61 and creates a site of high affinity for the cyclohexyl rings of DCCD [44]. Introduction of a spin label on a Cys replacement of *c*Ala-67 allowed Girvin and Fillingame [44] to make a complete set of assignments for the residues around *c*Asp-61. These measurements established that *c*Asp-61 is in van der Waals contact with *c*Ala-24. The close interaction of the two residues was predicted by the F_OF_1 double mutation, *c*Asp-61 to Gly and *c*Ala-24 to Asp, which retained some function [74].

Changes adjacent to *c*Asp-61 also result in interesting effects. Zhang & Fillingame [120] observed dramatic differences in sequence near the carboxylic acid between the Na^+ transporting *P. modestum* F_OF_1 (*see above*) and the H^+ transporting *E. coli* enzyme. Assuming that the equivalent of *c*Asp-61 is the Na^+ binding site for transport, they hypothesized that the sequence difference may indicate accommodations made for binding Na^+. By making multiple changes to match the *P. modestum* sequence, they created an *E. coli* en-

zyme that had some of the characteristics of the *P. modestum* complex, i.e., sensitivity to Li^+ and competition between Li^+ and protons for the transport site.

Subunit *a* Participates in Proton Transport

The participation of subunit *a* (271 amino acids in *E. coli*) in proton translocation was first realized when Cain & Simoni [19] discovered two missense mutations, *a*Ser-206 to Leu and *a*His-245 to Tyr, that greatly reduced ATP-driven proton translocation and F_O-mediated protein conduction but did not affect binding of F_1 to F_O. Subsequently, a large number of site-directed mutations have been introduced in subunit *a* which have surveyed residues in the relatively conserved carboxyl-terminal region for involvement in proton transport (reviewed in Refs. 32, 33). From this work, only one residue, *a*Arg-210, has emerged as essential as even replacement with Lys is not acceptable [20, 64]. Two other residues appear to be important but not essential, *a*Glu-219 and *a*His-245. *a*Glu-219 to Leu or *a*His-245 to Tyr or Leu render the F_O inactive, whereas *a*Glu-219 to Gln or His, or *a*His-245 to Glu retain some function [19, 64].

Interactions Between F_O Subunits are Important for Proton Transport

Genetic studies demonstrated that subunits *a* and *c* functionally interact with each other. Fraga et al. [38] identified several third site mutations in subunit *a* that resulted in enhanced activity of the double mutant, *c*Asp-24/*c*Gly-61 (*see* Fig. 2). Most of the changes mapped to *a*Ala-217, *a*Ile-221, or *a*Leu-224, all of which are proposed to lie on one face of the membrane spanning α-helix which includes the essential *a*Arg-210. Third site mutations were also found in subunit *c;* *c*Phe-53, *c*Met-57, and *c*Met-65, residues believed to form a hydrophobic face along the carboxyl-terminal helix. The simplest interpretation of these results suggests that the subunits *a* and *c* helices physically interact and both contribute residues to the proton translocating mechanism.

F_O May Conduct Protons via a Hydrogen-bonded Chain

Physical techniques have been employed to characterize the mechanism of proton transport. Measurement of F_O unit conductance have been most successful with the chloroplast complex because the proton motive force is easily manipulated in thylakoid membranes by light intensity. Other attempts to determine the conductance of reconstituted F_O resulted in grossly underestimated values probably because most of the complexes were inactive [68]. With corrections, conductance rates of the chloroplast F_O were estimated to be at least three orders of magnitude greater than the minimum required for overall turnover of the F_OF_1 [65].

The rather fast conductance rate can be accommodated by a hydrogen-bonded chain with an adequate number of components. Using FT-IR spectroscopy, Bartl et al. [10] observed an infrared continuum in *E. coli* F_O reconstituted in cardiolipin liposomes. The continuum vanished when the preparation was reacted with DCCD or when the liposomes were dehydrated. These results indicate a proton pathway in which the protons shift in a hydrogen-bonded chain with large proton polarizability. Protons can move along these highly polarized hydrogen-bonded chains in the picosecond time domain [123].

If the transport mechanism is in the F_O as suggested by studies of the *P. modestum* sodium transporter, then the hydrogen-bonded chain must contain a mechanism for vectorial transport. A possible mechanism, which involves alternating access of *c*Asp-61, has been described by Fillingame [32]. Two key features which are common to any transport mechanism are (i) the proton binding group is accessible from only one side of the membrane at a time, and (ii) the group shifts in its binding affinity (i.e., pK) in a manner that is energetically appropriate for the transport function [56, 106]. Whatever the mechanism of proton transport, it must be energetically and kinetically coupled to the catalytic cycle.

Despite identification of many amino acids important for transport and the solution of the subunit *c* structure, the nature of the transport mechanism is not understood. Progress has been limited primarily because of the difficulty of obtaining structural information. Many key questions remain unresolved including the quaternary structure of the F_O subunits, and in particular, the relationship between the many copies of subunit *c* and the single *a*. One possibility suggests an intimate interaction between subunit *a* and a unique *c* which has strong reactivity with DCCD and dictates the conformation of the other *c* subunits. A different model suggests that the *c* subunits are arranged in a ring or in groups of three or four, and rotate past subunit *a* at specific points of the transport cycle (*see* Ref. 32). Corollaries to this question are how many proton pathways are present within one F_O complex, what are the components of each pathway, and what is the relationship of each pathway to coupling.

Coupling Interactions between Catalysis and Transport

The mechanism of energy coupling links transport and catalytic mechanisms kinetically so that one cannot proceed without the other [53]. The previous sections have

discussed the location of the catalytic sites in the β/α complex and the transport mechanism within the membranous F_O. Matsuno-Yagi et al. [72] have shown that the conformation of the β subunits is linked to the presence of a $\Delta\mu_{H^+}$ or other perturbations in the F_O. Clearly, conformational effects must be efficiently transmitted between the catalytic and transport sites which are separated by approximately 100 Å (45; reviewed in Ref. 21). Investigators have hypothesized that the single copy F_1 subunits, γ, δ, and ϵ, are involved in the long distance linkage between transport to catalysis. Recently, new structural information has stimulated a number of studies on the single copy subunits that have demonstrated a series of subunit-subunit interactions and conformational changes that appear to be relevant to the catalytic and transport states of the enzyme. The following sections will present an overview of results that have contributed to the understanding of the function of coupling.

Subunit *c* Interactions with ϵ Subunit are Important for Coupling

F_OF_1 with mutations in the hydrophilic loop of F_O subunit *c*, *c*Arg-41 to Lys or *c*Gln-42 to Glu, are unable to carry out ATP-dependent pumping, but the F_O still conducts protons [37, 39, 77]. Furthermore, both mutations disrupt the ability of F_1 to prevent passive proton flux without affecting the ability of F_1 to bind to the membrane. These results established a role of the subunit *c* hydrophilic loop in coupling. In a very exciting result, Zhang et al. [122] found that the uncoupling phenotype of the *c*Gln-42 to Glu mutant could be suppressed by three different replacements of ϵGlu-31. This result demonstrated a functional interaction between subunits *c* and ϵ which was later found to be a physical one. A cysteine in place of ϵGlu-31 formed spontaneous disulfide bridges with cysteines in three positions of the subunit *c* hydrophilic loop, including *c*Gln-42 [121]. In agreement with these results, various amino acid replacements for nearby ϵGlu-32 and ϵHis-39 disrupted interactions between F_O and F_1 [59]. In addition, a cysteine replacement for ϵHis-38 was modified by N-ethylmaleimide (NEM) when in the F_1 complex, but could not react when F_1 was bound to F_O [5]. Skakoon and Dunn [100] found that ϵCys-38 cross-linked to γ subunit with bifunctional reagents suggesting that a part of the γ subunit may also be close.

Wilkens et al. [115] recently determined the tertiary fold of the isolated *E. coli* ϵ subunit by multidimensional NMR. The amino-terminal 84 residues formed a 10-stranded β-barrel and the carboxyl-terminal 48 residues formed a hairpin with two anti-parallel α-helices. ϵGlu-31 and ϵHis-38 lie along one side of the β barrel suggesting that this surface is the *c*-ϵ interface.

ϵ Subunit Binds Tightly to γ Subunit

On a different side of the β-barrel, cross-linking studies identified two residues, ϵSer-10 [2] and ϵThr-43 [115], that closely interact with the γ subunit. Both residues are close to a conspicuous patch of hydrophobic residues made up of the side chains of residues ϵ-9, 15, 42, 68, 77 and 79. The ϵ subunit is known as the inhibitory subunit because it inhibits F_1 ATPase activity with a $K_I \sim 10^{-9}$ M; the same value as the dissociation constant of ϵ subunit binding to F_1 and to the γ subunit [27, 102]. Purified γ subunit is rather insoluble [27]; however, it becomes quite soluble when bound to ϵ subunit to the extent that the γ-ϵ dimer can be crystallized [22]. It is likely that the hydrophobic patch on ϵ interacts with a hydrophobic patch on γ and exerts its inhibitory effect through this interaction.

The hairpin segment of the ϵ subunit interacts with the α and β subunits. A zero-length cross link could be induced by 1-ethyl-3-[(3-dimethylamino)propyl]-carbodiimide (EDC) between ϵSer-108 in the turn of the hairpin and βGlu-381 in the conserved β subunit sequence D**E**$^{\mathbf{381}}$ LSEED [24, 70]. A disulfide bridge was induced with cysteine replacements at both positions [3]. Significantly, the yield of this disulfide was different in the presence of ATP vs. ADP. Inhibition of ATPase activity correlated to formation of the disulfide and the block was relieved in reducing conditions. Furthermore, a cysteine replacement for ϵSer-108 could form a disulfide bridge with a cysteine replacement for αGlu-441, the homologous position of βGlu-381 [115].

Both ϵ and γ subunits change conformation in response to catalytic states of the enzyme. In the chloroplast enzyme, these subunits change conformation when the enzyme becomes activated in light (reviewed in Ref. 55). Capaldi and coworkers used a number of approaches to demonstrate that ϵ and γ subunits change conformation whether $Mg^{2+} \cdot ADP$ or $Mg^{2+} \cdot ATP$ is bound (reviewed in Ref. 21). Using a fluorescent indicator covalently attached to the γ subunit, Turina and Capaldi [109] observed fluorescence changes which they interpreted as occupancy of the catalytic site with ATP. Significantly, the γ subunit conformational changes were only observed when the ϵ subunit was present suggesting that the absence of the ϵ subunit breaks the linkage between catalytic site conformation and the coupling mechanism [21].

δ Subunit Participates in Coupling

Circular dichroism and sedimentation analysis of the δ subunit suggests a highly helical and elongated conformation [105]. It is required in *E. coli* for F_1 binding to F_O and reconstitution of coupled transport [101], and

some anti-δ monoclonal antibodies bind to F_1 but not to F_OF_1 [4]. For these reasons, δ subunit is believed to make up a portion of the connection between F_O and the β/α complex. Its interactions with other subunits is not well understood. δCys-140 will form a disulfide with α subunit under mild oxidizing conditions [17, 73, 108]. The cross link does not affect enzyme properties, but cross-linked F_1 fails to reconstitute coupled transport. Interestingly, δ and ϵ subunits can be extracted from chloroplast F_OF_1 and F_1 remains bound to F_O [89, 117]; however, protons freely pass through the complex without coupling to catalytic activity [67]. Hazard & Senior [50, 51] found that several replacements for δAla-149 and δGly-150, in particular δGly-150 to Asn, caused uncoupling. These results point to the carboxyl terminal region interacting with α subunit in a manner that is important for coupling.

γ Subunit Presents a Different Interface to Each β Subunit

The α-helices of the γ subunit termini contact the β and α subunits in two major regions (*see* Fig. 3 and Ref. 1). The first region of interaction was referred to as the ''molecular bearing'' which was proposed to provide a sleeve where the γ subunit could rotate. The other region was between the conserved $_{380}$DELSEED$_{386}$ sequences of the β subunits (*see above*) and all three helices of the γ subunit. The γ subunit does not have an obvious three-fold symmetry and presents a different face to each of the three β subunits. It is likely that the different β subunit conformations observed in the bovine F_1 crystal are determined at least in part by the orientation relative to the γ subunit. An important question is whether the γ subunit maintains its asymmetric orientation to create unique β subunits or changes orientation during turnover.

γCys-87 has Close Contact to Different β Subunits During Turnover

In an elegant series of experiments, Duncan et al. [26] used an intersubunit disulfide bridge to demonstrate that different β subunits interact with the γ subunit. They dissociated unlabeled F_1 containing a disulfide bridge between γCys-87 and βCys-380, mixed with radiolabeled subunits, and then allowed the complex to reconstitute. Following this, the disulfide bridge was reduced, ATP + Mg^{2+} was added, and finally, formation of the disulfide was again induced. They found that the amount of γ subunit attached to radiolabeled β subunits was that predicted if the γ subunit randomized its orientation to β subunits. The results suggest that the γ subunit interacts with different β subunits during turnover and is therefore rotating relative to the β subunits.

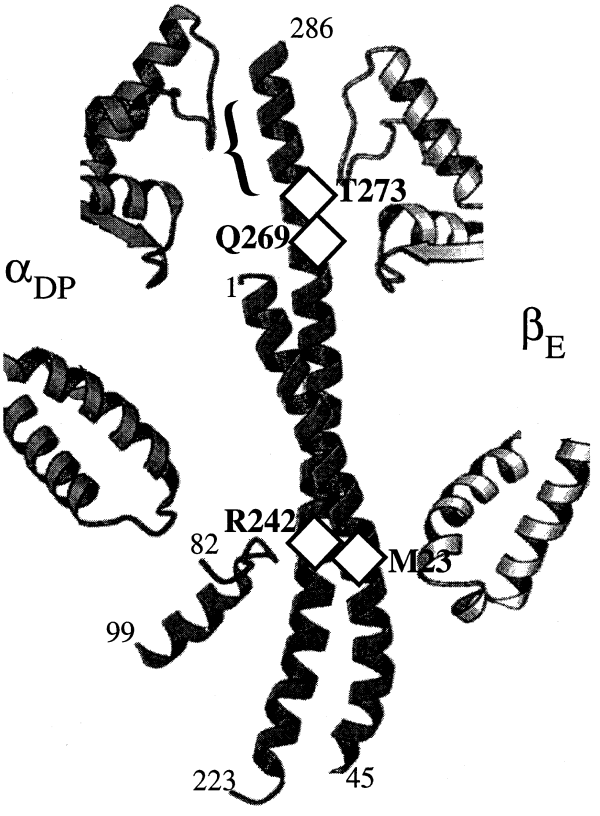

Fig. 3. Partial structure of the bovine γ subunit with sections of one of the α and β subunits that are in close contact. Taken from the crystallographic F_1 structure of Abrahams et al. [1]. α and β subunits are designated as in Ref. 1. The upper region of close contact is the hydrophobic sleeve and the lower is the ''DELSEED'' sequence (*see* text). γ subunit residues (*E. coli* numbering) found to be important for promotion of catalysis and coupling to transport are identified by the diamonds (*see* text, Refs. 54, 78–80, 99). Second-site mutations at γArg-242 and the bracketed section near the γ carboxyl terminus suppressed the uncoupling mutation γMet-23 to Lys.

It remains to be seen whether the γ subunit rotates in a circular fashion or shifts back and forth in different directions. Certainly, circular rotation would be an attractive part of the sequential binding-change mechanism described above. Another issue is what moves with the γ subunit. γCys-87 is in the part of the γ subunit that was observed in the bovine F_1 crystal structure [1], and the above results suggest the entire subunit is moving relative to the β subunits. However, δ and ϵ, and most of the γ subunit were not represented in the electron density map suggesting that they may have a movement separate from the helical portion of the γ subunit.

The γ Subunit Transmits Coupling to the Catalytic Sites by Interaction with the β/α Hexamer

The interactions between the γ and β subunits appear to have two functions. First, residues near the carboxyl terminus are involved in catalysis. Abrahams et al. [1] specifically pointed out that γGln-269 forms a hydrogen

bond with a β subunit loop close to the catalytic site. Mutagenesis proved the significance of this residue. Replacement with Glu resulted in greatly reduced turnover and stability of the F_1 complex [54, 78]. Enzymes with changes of γThr-273 to Val, one turn of the helix away, had similar properties.

Second, other portions of the conserved regions of the γ subunit are involved in coupling. Replacement of the conserved γMet-23 with Arg or Lys caused extremely inefficient coupling without effects on the transport mechanism and only slight effects on turnover rate [99]. Interestingly, the enzyme could accomplish a small amount of net ATP synthesis suggesting that the mutation caused a slip or bypass pathway in the kinetic regulation of catalytic steps that are controlled by transport. Characterization of the γMet-23 to Arg and Lys mutants was the first demonstration that the γ subunit participates in coupling.

Other regions of the γ subunit were implicated when several second-site mutations were identified that suppressed the effects of the γMet-23 to Lys mutation [79]. Each of the second-site mutations, which mapped to the carboxyl terminal region, restored efficient coupling. One change was γArg-242 to Cys and seven others were between γGln-269 and γVal-280, including γGln-269 to Arg and γThr-273 to Ser (*see* Fig. 3). All were replacements of conserved residues. Most importantly, all the changes were within the contact areas between the γ subunit and the α and β subunits. In turn, the effects of the deleterious mutations mentioned above, γGln-269 to Glu and γThr-273 to Val, were suppressed by second-site mutations that mapped near γMet-23 (changes at residues 18, 34, and 35) and near γArg-242 (changes at residues 236, 238, 242, and 246) [78]. These results demonstrated a functional interaction among three sections of the γ subunit: γ-18-35, γ-236-246, and γ-269-280. From analysis of the bovine F_1 structure [1], γ-269-280 is not in contact with the other two sections; however, all of the above changes, including γMet-23 to Lys, appear to alter interactions between γ and β subunits. In support of this observation, thermodynamic analysis of the γMet-23 to Lys and γGln-269 to Glu mutant enzymes indicated that the energy of interactions between these subunits were altered (M.K. Al-Shawi & R.K. Nakamoto, *unpublished results*). These results suggest that the suppression effects by second-site mutations are not through direct interactions within the γ subunit, but by countering the altered inter-subunit interactions caused by the primary mutations.

The above results emphasize the importance in considering the energy of interactions between γ and β subunits. Two points may be made about mechanistic implications on promotion of catalysis and coupling. First, there appears to be a balance in the energy of interaction: too many or too few bonds between γ and β subunits will result in large and insurmountable transitions state energies, or energy wells that are too stable. Either will cause kinetic blocks and affect the efficiency of coupling. Second, because there are three β subunits, the differences in energy of interactions caused by changes in the γ subunit sequence are potentially multiplied by three if the β subunits rotate relative to γ subunits. The differences may be even greater if similar interactions occur between γ and α subunits.

The effects of the γ subunit mutations are consistent with the γ subunit rotating relative to the β subunits. Subunit asymmetry is an essential part of catalytic cooperativity and coupling. Imposition of the γ subunit asymmetry on β/α hexamer in a cyclic fashion may be a plausible mechanism to promote catalytic turnover and to couple conformational effects to and from transport.

The Coupling Mechanism Links Functions

From the above discussions, it is easy to imagine a series of conformational and/or electrostatic interactions that mediate linkage. A conformational perturbation caused by a protonation in the transport site involving *c*Asp-61 is transmitted through the hydrophilic loop of subunit *c*, through ε and γ subunits, and into the β subunits to the catalytic sites. Through a combination of structural, mutagenesis, and biochemical data, two key regions of the F_OF_1 complex that are critical in coupling have been revealed. One is the *c*-ε interface (*see* Ref. 121), and the other is the β subunit $_{380}$DELSEED$_{386}$ segment which interacts with different parts of the γ subunit as well as with the hairpin domain of ε subunit. Obtaining structural, thermodynamic, and kinetic data on the nature of the interactions that occur in these regions as well as those occurring throughout the enzyme complex will be crucial for understanding how the ATP synthase interacts and manipulates its substrates during active transport.

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