Annu. Rev. Biochem. 1997. 66:717–49

THE ATP SYNTHASE—A SPLENDID MOLECULAR MACHINE

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KEY WORDS: F_1-ATPase, binding change, ADP, proton translocation, phosphorylation

ABSTRACT

An X-ray structure of the F_1 portion of the mitochondrial ATP synthase shows asymmetry and differences in nucleotide binding of the catalytic β subunits that support the binding change mechanism with an internal rotation of the γ subunit. Other structural and mutational probes of the F_1 and F_0 portions of the ATP synthase are reviewed, together with kinetic and other evaluations of catalytic site occupancy and behavior during hydrolysis or synthesis of ATP. Subunit function as related to proton translocation and rotational catalysis is considered. Physical demonstrations of the γ subunit rotation have been achieved. The findings have implications for other enzymatic catalyses.

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0066-4154/97/0701-0717$08.00

OVERVIEW

All enzymes are beautiful, but the ATP synthase is one of the most beautiful as well as one of the most unusual and important. Its beauty is revealed by the three-dimensional structure of the F_1-ATPase component, the largest asymmetric structure so far solved. It is unusual because of its structural complexity and reaction mechanism. Its importance is illustrated by the estimate that an active graduate student synthesizes more than his or her body weight of ATP in a day.

There is general agreement on several important characteristics of the ATP synthase. Electron microscopy shows a membrane-bound portion connected by a relatively narrow (~45 Å) stalk to a 90–100 Å diameter knob. When exposed to low ionic strength media, the enzyme separates into the membrane-bound F_0 portion, which is involved in proton translocation, and a soluble F_1 portion that catalyzes ATP hydrolysis. The F_1-ATPase has five subunits, designated in order of decreasing size and number of subunits as α_3, β_3, γ, δ, and ε, for the *Escherichia coli* enzyme, with masses of about 55, 50, 31, 19, and 14 kDa, respectively. The simplest F_0 is illustrated by the *E. coli* enzyme, with subunits and stoichiometry designated as a, b_2, and c_{9-12}, with masses of about 30, 17, and 8 kDa, respectively. The stalk region is composed of subunits from both F_1 and F_0. The F_0 in higher organisms is considerably more complex. The enzyme from all sources has multiple copies of a subunit like the small c-subunit in the *E. coli* F_0, and proton translocation by this hydrophobic protein is blocked by a facile reaction of an intramembrane carboxyl group with dicyclohexylcarbodiimide (DCCD).

An ATP synthase of similar structure is found in all organisms that form or cleave ATP coupled to proton translocation. The amino acid sequences of the subunits from a wide variety of sources are known. The α- and β-subunits share considerable homology—about one fifth identical amino acid residues. The β-subunits from different sources show exceptionally strong sequence homology. The minor F_1-ATPase subunits show more sequence and size variation.

Isolated α- and β-subunits have one relatively strong nucleotide binding site, but neither will catalyze significant hydrolysis. ATP hydrolysis can be catalyzed by mixtures of α- and β-subunits, but ATP synthesis by the *E. coli* enzyme requires all F_1 and F_0 subunits.

The F_1-ATPases, as isolated, often have bound ATP or ADP at two to three noncatalytic sites and bound ADP at one catalytic site. There are six potential nucleotide binding sites on the F_1-ATPases: three noncatalytic sites, primarily on the α-subunits, and three catalytic sites primarily on the β-subunits. Nucleotides bound at noncatalytic sites are replaced slowly at different rates during catalysis.

This review focuses on evidence that the subunits of ATP synthase interact in such a way that catalysis is essentially dependent upon the resultant cooperativity. The ATP synthase is the first enzyme for which such strong catalytic cooperativity has been found. This cooperativity is expressed in a binding change mechanism in which energy from the transmembrane proton gradient serves primarily for the release of a tightly bound ATP. The three β-subunits are believed to proceed sequentially through conformational changes that facilitate the binding, interconversion, and release steps. Characteristics of the catalysis and structure of the synthase show that the enzyme has a rotational mechanism in which proton translocation in the F_0 portion drives an internal rotation of the single-copy γ-subunit of F_1, causing sequential conformational changes in the β-subunits. This is the first enzyme for which evidence for such a mechanism has been obtained.

This review focuses on the structural and catalytic properties related to subunit interactions and on controversial aspects of catalytic steps in the binding change mechanism. Useful recent reviews emphasize mutagenic and other approaches to the catalytic site structure and interactions (1, 2), the catalytic mechanism (3), the binding change mechanism (4), the structure and role of minor subunits (5), the structure and mechanism (6–8), the mechanism with emphasis on F_0 participation (9), and F_0 structure (10).

STRUCTURE OF THE F_1-ATPase

X-Ray and Related Structural Probes

An advance of striking importance is the attainment of a structure of the F_1-ATPase from bovine heart mitochondria, first at 6.5-Å resolution (11) and then at 2.8-Å resolution (12). This achievement represented the culmination of years of effort and commendable methodology.

The main portion of the MF_1[1] is a flattened sphere with alternating α- and β-subunits extending from the top to the bottom of the sphere like segments of an orange. The asymmetry of the α- and β-subunits is strikingly evident, confirming evidence of asymmetry from earlier electron microscopic, catalytic,

[1]MF_1, CF_1, TF_1, and EcF_1 refer to the F_1-ATPase from mitochondria, chloroplasts, the thermophilic *Bacillus* PS3, and *E. coli*, respectively.

and chemical derivatization studies. Crystallization was in the presence of Mg^{2+}, 5 μM ADP, and 250 μM AMP-PNP. One catalytic site is filled with ADP, another with AMP-PNP, and a third site is empty. All three noncatalytic sites have bound AMP-PNP. Below a central dimple of about 15 Å, the core is filled with helices formed from the C-terminal and N-terminal portions of the γ-subunit. The helical structures protrude about 30 Å below the main body and are likely a portion of the ~45-Å stalk connecting to the membrane-inserted F_0 portion. Other portions of the γ-subunit and the δ- and ε-subunits are not sufficiently ordered in the crystals for structural definition.

Asymmetric features arise principally from domain shifts. Except for the nucleotide binding domain of the empty catalytic site, the domains of all α- and β-subunits superimpose with root-mean-squared deviations of less than 1 Å between Cα atoms. The marked asymmetry in the overall structure arises from differences in the relative orientations of the domains and in their interactions with the unique γ-subunit. A hydrophobic sleeve with low surface potential formed by interior surfaces of the α- and β-subunits surrounds the C-terminal portion of the coiled γ-subunit. The interactions with the γ-subunit are responsible for the key domain shifts that markedly decrease the ATP binding at one site. The original article gives many additional details and splendid illustrations. Likely the structure will offer an explanation for the differences in chemical reactivity among the three β-subunits, such as that established for the *E. coli* enzyme (13).

An X-ray structure reported for the rat liver MF_1 at 3.6 Å showed a symmetrical arrangement of the α- and β-subunits, with the three β-subunits projecting substantially above the alternating α-subunits (14). In addition, it did not reveal the central helices of the γ-subunit, which are clearly evident in the structure for the heart MF_1 (11, 12). Unlike the bovine heart enzyme, the rat liver enzyme was crystallized in the absence of Mg^{2+} (14). It is possible that in the absence of Mg^{2+} and in the presence of nucleotides, the α- and β-subunits of the liver enzyme assume symmetrical structures. Mg^{2+} has a crucial role in the catalysis, and the structure in the presence of Mg^{2+} seems to have much greater catalytic relevance. However, an alternate view has been advocated (15).

A three-dimensional structure for the TF_1 at about 30-Å resolution (16) obtained by electron crystallography shows a parallel arrangement of the α- and β-subunits similar to that reported for the heart enzyme. In addition, studies of EcF_1 using cryoelectron microscopy (17) and reactivity of mutants containing selected substitutions by Cys (5, 18) provide evidence of structural asymmetry even in the absence of bound nucleotides and Mg^{2+}. Other useful structural analyses are likely to be forthcoming. For example, a β-subunit of a mutant TF_1 has been crystallized and shown to diffract to about 3 Å (20).

Nucleotide Binding Sites

The structure of the heart enzyme provides crucial information about the residues involved in the binding of nucleotides at catalytic and noncatalytic sites. Extensive mutational studies and sequence analogies with other nucleotide binding enzymes provide evidence for residues that are likely involved in binding. For example, the definitive mutational analyses pointing to the presence at a catalytic site of EcF_1 Lys-β162 (analogous to MF_1 Lys-β162), and of Thr-β163 (21, 22) in the glycine-rich loop near the phosphoryl residues, are firmly confirmed. The participation of the glycine-rich loop in nucleotide binding of several enzymes was suggested well over a decade ago as sequence data became available (23).

The structural data also revealed that Arg-α373 is present at a catalytic site and may participate in catalysis (12). The positions of Tyr-β345 and Tyr-β368 near the adenine rings of the catalytic and noncatalytic nucleotides, respectively, correspond to their labeling by 2-azido-ATP (24); the same tyrosines are labeled by fluorosulfonylbenzoyl derivatives of inosine and adenosine, respectively (25). Both noncatalytic and catalytic sites have α-β-subunit interfacial components.

Catalytic Relationships of the Heart MF_1

Abrahams et al (11, 12) believe their structural findings correlate well with the binding change mechanism suggested for catalysis for the F_1-ATPase and the ATP synthase (4, 26). The three catalytic sites adequately represent the "tight," "open," and "loose" conformations suggested for the binding change mechanism (27). Assignment of the site with bound ADP to the tight conformation is supported by the well-recognized very tight binding of ADP to one catalytic site in the presence of Mg^{2+}. Under conditions for ATP synthesis, such tightly bound ADP is released to the medium in the first turnover (28, 29). Protonmotive force readily causes the tight site to open and release its contents, in this case the ADP. During steady state catalysis, a very tightly bound ATP that had just been formed would be released as the site opens.

In the F_1-ATPase where the γ-phosphate of ATP would be bound, there is density for a water molecule hydrogen-bonded to Glu-β188. The Glu is suggested to activate the water oxygen for attack on the phosphate, with stabilization of the negative charge that develops in the transition state by the guanidinium of Arg-α373 (12). The requirement of a residue from the α-subunit explains why the β-subunit, or a synthetic peptide based on residues β141–190 containing the GX4GKT consensus sequence, binds but does not hydrolyze ATP (30). The presence of a Gln instead of Glu in the α-subunit at the position corresponding to Glu-β188 could account for the lack of catalytic activity of the α-subunit. However, interchange of the Gln and adjacent Lys

with the corresponding Glu-Arg of the β-subunit resulted in an enzyme with weak or no catalytic activity (31).

The importance of Glu-β188 for ATP synthesis is shown by the finding that a replacement of Glu-β204 of the chloroplast synthase (analogous to Glu-β188 of MF_1) by Gln blocked ATP synthesis; but surprisingly, the CF_1 isolated from chloroplasts retained ATPase activity (32). Additional clarification of the role of the chloroplast Glu-β204 is needed.

The availability of the X-ray structure has already allowed elucidation of the action of inhibitory antibiotics. Aurovertin binds to pockets on only two of the β-subunits in a manner consistent with it preventing closure of the catalytic interfaces necessary for interconversion of catalytic sites (33). Efrapeptin binds to a unique site in the central cavity, making hydrophobic contacts with helices in the γ, with the empty β-subunit, and with two adjacent α-subunits, effectively blocking conversion of the empty subunit to a binding conformation (34).

The $\alpha_3\beta_3$ and $\alpha_3\beta_3\gamma$ Structures

The isolated separate α- and β-subunits, although they bind ATP, are essentially devoid of ATPase activity. An $\alpha_3\beta_3$ complex from TF_1 retains some catalytic activity (35). Small-angle X-ray scattering of the $\alpha_3\beta_3$ complex is consistent with six ellipsoids hexagonally arranged, as in F_1-ATPase (36). This means that a hexameric arrangement of α- and β-subunits is not dependent on the presence of the γ-subunit. The $\alpha_3\beta_3$ complex from TF_1 dissociates into an α-β dimer in the presence of Mg^{2+} and ADP or ATP (36–38). A complex with only one TF_1 α- and one β-subunit shows weak and noncooperative ATPase activity, a residual type of uni-site catalysis (39). The catalytic activity of the $\alpha_3\beta_3$ complex from TF_1 is blocked by reaction of a single β-subunit with 7-chloro-nitrobenzofuran (40) or 3′-O-(4-benzoylbenzoyl)-ADP (41). The presence, or more likely the induction, of the β-subunit asymmetry can thus be found without the presence of the γ-subunit. ATP binding might likewise induce asymmetry and catalytic cooperativity. This possibility is uncertain because of dissociation of the complex caused by ATP. With trinitrophenyl-ATP as a substrate, dissociation does not occur and the presence of a high-affinity binding site and cooperative catalysis is induced by the γ-subunit (38).

An active $\alpha_3\beta_3$ complex has also been isolated from chloroplasts. This complex shows only weak ATPase and little cooperative activity unless associated with the γ-subunit (42, 43). Further studies of the $\alpha_3\beta_3$ and $\alpha_3\beta_3\gamma$ complexes may be facilitated by recent reports of the expression of wild-type and mutants of the TF_1 subunits in *E. coli* (44), and by development of systems for overexpression of subunits, expression of subunits fused with the C-terminus of glutathione S-transferase, and purification of *E. coli* α-, β-, and γ-subunits (45). Reported applications indicated that the N-terminal ends of the δ- and

ε-subunits were not involved when an F_1 complex was reconstituted and that the ε-subunit bound strongly to the γ-subunit (45).

Structural Information from Other Approaches

Electron microscopy has played an important role in structural studies of the ATP synthase (see 46 for review), as illustrated by the following examples: 1. Fragments of monoclonal antibodies bound to the α- and ε-subunits of the EcF_1 allowed detection of three types of subunit assemblies in which a central density was closest to a different β-subunit, thus documenting the interesting structural asymmetry (47). 2. Use of monomaleimidogold labeling of -SH groups introduced in the γ-subunit by mutagenesis demonstrated that the central mass contains the N-terminal part of the γ-subunit (48), a feature detailed by the X-ray structural analysis presented above.

Structural change accompanying catalysis by the $\alpha_3\beta_3\gamma$ complex was probed with time-resolved small-angle X-ray scattering, in continuation of extensive studies of TF_1 by Kagawa and associates (49). ATP hydrolysis induced an increase in the radius of gyration, indicative of significant structural change induced by catalysis. Conformational transitions during steady state catalysis may result in a loosened overall structure.

Electron spin resonance spectroscopy with a spin-labeled ATP-analog allowed demonstration that Mg^{2+} not only influenced the binding of the analog but also altered the structure and geometry of the nucleotide binding sites (50). Such results are a reminder that the role of Mg^{2+} in the ATP synthase catalysis is quite inadequately understood.

Chemically induced cross-linking has provided useful information about subunit arrangements. The fact that nucleotide binding sites of TF_1 are at the interface of the α- and β-subunits was confirmed by the use of 2,8-diazido-ATP to cross-link the subunits, the 2-azido reacting with a β-subunit, and the 8-azido reacting with an α-subunit residue (51).

Many interesting studies have provided information about the structure and interaction of the minor subunits. The minor subunits are needed for the net synthesis of ATP. But results so far have not revealed the essential features of their participation in the process. Some illustrative examples of reports are mentioned below.

A range of techniques have been used to probe minor subunit properties. For example, as mentioned above, selected Cys replacements in *E. coli* have been used to demonstrate asymmetry (5, 18). One mutant had a Cys replacement at β-Gly-149, with a conversion of Val to Ala at β198 that suppressed a deleterious effect of the β149 replacement, plus Cys replacements at positions β381 and ε108. Upon $CuCl_2$ treatment, one β-subunit cross-linked to the γ-subunit, a second cross-linked to the ε-subunit, and a third remained mostly free. Reaction

with 2-azido-ATP revealed that the β-subunit remaining free had the highest nucleotide binding affinity (52).

Structural features of the ε-subunit have been revealed by NMR spectroscopy (53). The N-terminal 84 residues form a 10-stranded β-barrel, and the C-terminal 48 amino acids form a domain with two antiparallel α-helices. Dunn provides an interesting analysis of this and related results (54). Surprisingly, related studies (55, 56) showed that the entire C-terminal helical domain, or the 15 N-terminal amino acids, can be deleted and still give a functional ATP synthase.

Nucleotide binding to the β-subunit would be expected to cause conformational changes in any subunits transmitting the structural information to the proton-translocating residues in F_0. The demonstration that the γ-subunit of the *E. coli* enzyme extends through the stalk and contacts the c-subunits of the F_0 (57) is important because such contact may couple rotational movement to proton translocation. To explore changes in the γ-subunit, cysteine residues were introduced into the subunit, fluorescent probes were attached, and changes induced by ATP were demonstrated (58). The present status of these and other studies are summarized by Capaldi et al (5). As they state, "Taken together, the evidence reviewed above indicates that with binding of ATP ($+Mg^{2+}$) to EcF_1 and EcF_1F_0, there is a shifting of the ε-subunit, alterations in the interaction of the γ- and ε-subunits with the $\alpha_3\beta_3$ domain, and changes of association of the γ- and ε-subunits with each other" (p. 400).

Extensive mutagenesis studies of the F_1 subunits have also been done. One study, reflecting close β- and γ-subunit interactions, showed that a γ-subunit frameshift mutation that blocks oxidative phosphorylation can be suppressed by either of two single amino acid replacements in the β-subunit (59). Replacements of residues in the γ-subunit, particularly those conserved in comparative sequences, perturbed coupling. Suppressor mutagenesis showed three helical regions, consistent with the X-ray structure, that are likely involved in energy coupling (60). Mutations in other minor subunits impair coupling. For example, mutations in the C-terminal region of the δ-subunit stop proton translocation accompanying ATP hydrolysis in membrane vesicles (61).

Other studies reveal the proximity of subunits by chemical cross-linking. A "zero-length" carbodiimide cross-linker produced an ester linkage between a Glu residue in the highly conserved Asp-Glu-Leu-Ser-Glu-Glu-Asp (DELSEED) sequence of the β-subunit and a specific serine of the ε-subunit of the EcF_1 (62). The location of the DELSEED helix in the lower part of the Walker structure (12), and the prominent affinity of the ε-subunit for the γ-subunit, permits better visualization of possible locations of the ε-subunit in the F_1 complex. The DELSEED sequence contributes importantly to the binding site of a considerable number of amphipathic cations that appear to

block conformational transitions to the catalytic sites; this phenomenon has been studied extensively (see 63 for a review).

The ATP synthase may have other important associations or localizations. Evidence has been presented for an interaction with the adenine nucleotide translocase (64). Proximity could promote the rapid import of ADP and export of ATP formed. The possibilities of some type of localized coupling still merit consideration. Studies of this topic include a discussion of measurements favoring localized proton movements along membrane surfaces (65); a consideration of diffusional interactions between the translocase, redox enzymes, and the synthase (66); a questioning of protonmotive force as an intermediate (67); a review of evidence that some type of localized coupling operates in certain alkaliphilic bacteria (68); and data favoring a Ca^{2+}-controlled localized coupling in chloroplasts (69–71).

STRUCTURE OF THE F_0 COMPLEX

The Escherichia coli F_0

Structural and mechanistic probes have been plentiful and ingenious, but the arrangement of F_0 subunits remains controversial. The understanding of how conformational coupling is achieved is far from satisfactory. A recent study (72) gives references and a good background survey of the *E. coli* F_0 structure. The single copy of the largest subunit a is very hydrophobic. The two copies of subunit b have an elongated hydrophilic and likely largely helical portion that extends toward the F_1 and is anchored in the membrane by a hydrophobic N-terminal region. The small subunit c is present in 9–12 copies with two hydrophobic helices imbedded in the membrane and with the highly conserved more hydrophilic loop of about 18 amino acids extending toward the F_1 subunits. The critical Asp-61 is located near the middle of the hydrophobic region of the C-terminal portion.

A variety of techniques have been applied to structural analysis of the *E. coli* c-subunit (see 10, 73, and references therein). NMR techniques show that the subunit in chloroform-methanol folds as an antiparallel pair of extended helices. The Asp has a pK_a of 7.1, reflecting a more hydrophobic environment than that of the organic solvent. Introduction of a Cys residue and labeling with a paramagnetic nitroxide allowed distance measurements in the region neighboring Asp-61. The structure consists of two gently curved helices, crossing at a 30° angle. The C-terminal helix is disrupted on each side of Asp-61, which is in van der Waals contact with Ala-24. Cross-linking of mutants with introduced Cys residues shows that the polar loop of subunit c is close to residue 31 of the ε-subunit. Mutational analyses led to suggestions of amino acid residues in a transmembrane helix of the a-subunit that interact with the bihelical region

of subunit c during protonation and deprotonation. Such studies permit better visualization of possible conformational changes accompanying proton translocation.

Studies of the labeling of F_0 subunits by a photoactivatable carbene precursor that reacted with membrane-imbedded components led to the proposal that the subunits a and b were outside a cluster of the c-subunits and that the subunit b was anchored to the membrane by a short N-terminal segment that had little interaction with other subunits (74). An alternate proposal is that the membrane helices of the a- and b-subunits are inside a ring of c-subunits (75). Current evidence seems to favor the model with external a- and b-subunits. This conclusion is supported by variational and hydrophobic moment analyses of sequence variations (76), and by recent investigations using electron spectroscopic imaging and immunoelectron microscopy (72), electron cryomicroscopy (77), and atomic force microscopy (78).

The number of c-subunits present has important functional implications. Recent additional information about the number and arrangement of these subunits comes from an unusual source. There is evidence of a common structure among a class of membrane channels including the F_0 subunit c, the 16-kDa proteolipids of the vacuolar H^+-ATPase, and a membrane channel of an arthropod (79). The vacuolar protein appears to be a tandem repeat of the F_0 8-kDa proteolipid (80). The vacuolar ATPase proteolipid in yeast could be functionally replaced by a 16-kDa proteolipid from an arthropod. The 16-kDa proteins have about 20% sequence identity with subunit c, but comparisons of the helical wheels of the putative transmembrane helices show much more striking similarity (79). Such similarities mean that the structure of the arthropod protein—a star-shaped hexamer arranged around a central channel—is likely applicable to subunit c. Such applicability would mean a circular arrangement of 12 c-subunits, possibly in pairs, a structure previously suggested for the analogous subunit III of the chloroplast F_0 (81). The equivalent of 6 double-size c-subunits in the yeast vacuolar ATPase also suggests that there are 12 c-subunits in the ATP synthases (82).

Structural information about the a-subunit is more limited. Mutational studies done several years ago with the 271-amino acid *E. coli* enzyme led to the proposal that Arg-210, Glu-219, and His-245 together with Asp-61 of subunit c functioned in H^+ translocation. Only Arg-210 is absolutely conserved. Recent studies from the same laboratory showed that this essential arginine can be transferred to position 252 with partial retention of activity (75). The a-subunit is highly hydrophobic and likely contributes six transmembrane helices. A recent model for its membrane topology was proposed based on its reaction with antibodies site-specific for the N-terminal, middle loop, or C-terminal segments (83). In contrast to some earlier models, this model shows both the two

terminal and the middle loop regions to be exposed toward the F_1 portion of the synthase.

The possibility that portions of subunits a and b might be close to the α- and β-subunits of F_1 was indicated by early cross-linking studies (84); both a-β and a-α cross-linked products were observed. The interaction of the F_0 b-subunit with the F_1 β-subunit was further characterized by use of a soluble protein lacking the first 24 hydrophobic residues of the b-subunit that anchor the subunit to the membrane (85). The soluble product formed a highly helical, elongated dimer. The dimer bound to F_1 and weakly inhibited the binding of F_1 to F_0. The occurrence of the soluble truncated b-subunit as a dimer supports previous suggestions that the two copies of the b-subunit in F_0 are likewise dimeric. A cooperative cryoelectron microscopy study showed that the truncated b-subunit interacts with a β-subunit different from the β-subunit combined with the ε-subunit (86). Further, the truncated b-subunit does not fill the central cavity, leaving room for extensive conformational changes of the interacting γ- and ε-subunits.

The Mitochondrial F_0 and Stalk

The mitochondrial F_0 is much more complex. Recent studies (87–92) have uncovered new subunits and contributed much essential information. The subunits are designated a, b, c, d, e, f, g, F_6, and A6L (87–89). Presence of the new subunits e, f, and g in highly purified and functional synthase has been shown by immunological techniques (92). The nine F_0 proteins, plus the oligomycin-sensitivity-conferring protein (OSCP), the five F_1 subunits, and the inhibitor protein, make 16 different proteins present in the mitochondrial ATP synthase.

Descriptions of the F_0 and stalk of various ATP synthases are confusing because of the complicated nomenclature, partly historical in origin. The bovine proteins OSCP and δ are largely equivalent to the bacterial and chloroplast subunits δ and ε, respectively; chloroplasts and bacteria lack the equivalent of the mitochondrial ε-subunit; the multi-copy subunit analogous to the *E. coli* c-subunit is called subunit III in chloroplasts; and chloroplast and mitochondria do not have subunits with amino acid sequences resembling those of subunit b of *E. coli*, although other proteins with distributions of hydrophobic and charge residues similar to the *E. coli* b are present. Thus names and locations of subunits, as well as their functions, are confusing. Fortunately, Nature is not similarly confused.

In addition to identifying F_0 components, Collinson et al accomplished the in vitro assembly of a water-soluble "stalk" that combined with F_1 (87–89). The components of this stalk are one copy each of subunits OSCP, d, F_6, and b′, where b′ is the hydrophilic portion of subunit b. The hydrophobic portion of subunit b is probably critical in anchoring the stalk region to the membrane.

The OSCP is capable of combining with both F_1 and F_0. Binding to F_1 occurs with both the β- and α-subunits, preferentially with α (93). Both the OSCP and the homologous subunit δ from *E. coli* are elongated in shape and highly helical in structure (94, 95). An interesting application of deletion mutagenesis led to a model in which the N-terminus is associated with the F_1 and the C-terminus with F_0, with intervening helices as part of the stalk (96). Binding of OSCP to a central "pit" region of the F_1-ATPase has been suggested (87), although as noted below in the section on Rotational Catalysis, a different binding location might serve a useful function.

HYDROLYSIS BY THE F_1-ATPase

Although the important tenet of the binding change mechanism that the release of products at one catalytic site depends upon the binding of substrates at another catalytic site is well accepted, the number and mode of participation of three potential catalytic sites remain unsettled. My appraisal is that most researchers in the field think it likely that all three sites participate sequentially in an equivalent manner. Some, however, believe the evidence indicates that only two sites participate in catalysis and that the third has a regulatory function.

I believe the latter conclusion is unlikely because of the measurement of ^{18}O isotopomers of P_i or of ATP formed from highly ^{18}O-labeled substrates. The distribution at various substrate concentrations is as expected if all catalytic sites function identically (97, 98). Any differences in the rates of substrate binding or release or in the interconversion rates by participating sites would be expected to give rise to nonhomogenous distributions of isotopomers. If only two sites participated, the β-subunits carrying the sites would need to interchange tight and loose conformations in a manner that can be visualized as interchange of conformations to the right or to the left, as viewed from the vertical axis of the enzyme. The interactions with other groups and subunits, including a regulatory β-subunit, would be expected to be substantially different in the different directions and thus give rise to catalytic differences. For this reason, suggestions that only two sites participate warrant critical evaluation.

Kinetic Evaluation of Catalytic Site Occupancy

The ATP synthase and F_1-ATPase were not designed for ease of kinetic measurement and interpretation. There has been wide agreement that in the hydrolysis of substoichiometric concentrations of ATP by the F_1-ATPase, one catalytic site can be filled and retain tightly bound and interconverting ADP, P_i, and ATP, with the slow hydrolysis of bound ATP and release of products markedly accelerated by the binding of ATP to a second or to a second and third catalytic site. However, the occurrence of a slow uni-site catalysis was questioned in a recent

study using bioluminesence to measure ATP concentration during hydrolysis by MF_1 over a wide range of ATP concentrations (99).

In this study, catalytic activity was measured with nucleotide-depleted MF_1 added to a solution containing ATP in a phosphate buffer. The authors reach the surprising conclusion that under their conditions there is no slow uni-site catalysis at low ATP concentrations. However, the disappearance of medium ATP was used as a measure of hydrolysis even when enzyme was present in excess. The rate of binding of ATP to MF_1 is almost the same under uni-site and multi-site conditions; it is the subsequent hydrolysis and release of products that is slow during uni-site catalysis. Thus it is likely that a slow uni-site catalysis occurred in their experiments when the enzyme concentration exceeded the ATP concentration.

Clarification may be needed, however, on the rate of uni-site catalysis and on how much acceleration occurs with additional ATP binding. The uni-site rate with MF_1 may be as slow as 4×10^{-3} s^{-1} when repeated enzyme turnover does not occur (100). An assessment of uni-site ATPase activity made with bovine submitochondrial particles (101) showed a rate of about $0.1 \times s^{-1}$. This estimate is in the range for steady state uni-site rates reported for MF_1 (102), for submitochondrial particles (103), and for spinach thylakoids (104). Excess ATP has been estimated to produce a 5000-fold rate increase (101), a value that concurs with an estimate made from the extent of oxygen exchange at low substrate concentrations (105).

Heterogeneity of enzyme preparations may interfere with uni-site measurements. Frequently, preparations may have some tightly bound ADP and inhibitory Mg^{2+} present. The Mg^{2+}- and ADP-inhibited enzyme is inactive in uni-site catalysis (106). As noted in an early study, a fair portion of added ATP may be cleaved at a more rapid rate under uni-site conditions (107). Other observations suggesting enzyme heterogeneity have been reported for MF_1 (108), chloroplasts (104), EcF_1 (109, 110), and TF_1 (111).

Unfortunate difficulties arise in assessing apparent K_m values, indicative of additional catalytic site binding, when ATP concentrations are increased above the molarity of the enzyme. Although much effort has been expended, considerable disagreement remains. Enzyme heterogeneity as mentioned above may show some change with exposure to ATP. In addition, there is a high probability of other difficulties due to the inhibitory effects that Mg^{2+} can produce. The inhibition by Mg^{2+} arises by a complex interaction with the F_1-ATPase, which has a tightly bound ADP at one catalytic site.

The extensive studies of the Mg^{2+} inhibition were initiated by the observation that Mg^{2+} induced a decrease in velocity to a near steady state level after MF_1 was exposed to Mg^{2+} and ATP (112). Later studies (113) showed that the Mg^{2+}-induced inhibition requires the presence of a tightly bound ADP, that

azide stabilized the Mg^{2+}- and ADP-inhibited complex, and that activating anions promoted reactivation accompanying ATP hydrolysis. The tightly bound ADP was subsequently shown to be at a catalytic site without accompanying P_i (114–116). The first inhibited complex formed is slowly converted to a second complex that binds Mg^{2+} more tightly (117, 118). With time, and in the absence of ATP, other sluggish forms may arise. Prior exposure of CF_1 to Mg^{2+} may increase the rate of ADP binding (119). Catalytic site ADP required for the inhibition of the MF_1 can arise from the MgATP being hydrolyzed, but the inhibitory Mg^{2+} is bound from the medium (120). Whether the inhibitory Mg^{2+} is liganded in part to the tightly bound ADP or is bound elsewhere on the enzyme remains uncertain. Mutational replacements of *E. coli* βGlu-185 gave evidence that this residue, near the catalytic site, was essential for cooperative catalysis and may participate in Mg^{2+} binding (121). The ATPase activity of CF_1 and of chloroplast thylakoids is more readily inhibited than the mitochondrial enzyme. Bicarbonate, sulfite, and other anions can help prevent inactivation or promote reactivation. Reactivation of the Mg^{2+}-inhibited chloroplast ATPase by sulfite parallels the replacement of the ADP bound tightly at a catalytic site without P_i (122).

Whether the EcF_1 is susceptible to the Mg^{2+}-ADP inhibition has been questioned. However, recent reports give convincing evidence that the EcF_1 shows the strong Mg^{2+}- and ADP-induced inhibition (123, 124). It seems likely that the strong Mg^{2+}-ADP-induced inhibition is a property of all F_1-ATPases. Under most assay conditions, when a near-steady-state activity is attained, a fair fraction of the F_1-ATPase will be in the Mg^{2+}-ADP inhibited form. The enzyme is slowly being inactivated and reactivated (113, 120). The fraction in the inactive form can be assessed by the portion of the total activity that is quickly quenched by azide (125).

The properties of the Mg^{2+}-induced inhibition mentioned above could understandably complicate kinetic measurements. The kinetic evaluations became even more murky with the recognition that the presence of ATP at noncatalytic sites was necessary for the CF_1 ATPase to overcome or prevent the Mg^{2+}-ADP-induced inhibition (126). In addition, with the MF_1 ATPase exposed to low concentrations of ATP, a relatively slow binding of ATP at a noncatalytic site can even accelerate the onset of the Mg^{2+}- and ADP-dependent inactivation (120, 127). Indeed, with nucleotide-depleted MF_1, three kinetic phases were detected over a 2–3 min time span during hydrolysis of 50 μM ATP (127). The $\alpha_3\beta_3\gamma$ complex, but not the $\alpha_3\beta_3$ complex, shows the Mg^{2+}- and ADP-induced inhibition, and an $\alpha D_{261}N$ substitution reduced the ability of noncatalytic site ATP to overcome the inhibition (128).

The various complex interactions governing the Mg^{2+}- and ADP-induced inactivation of MF_1 ATPase are suggested to be responsible for the appearance

of two or more apparent K_m values above the μM range, as noted over two decades ago (129). A similar explanation has been advanced for multiple K_m values observed with the EcF_1 (124). Many other kinetic evaluations need to be reconsidered in the light of possible Mg^{2+} and ADP inhibitory effects. For example, extensive kinetic probes interpreted to indicate that free ATP and not MgATP may be the substrate (130–132) appear deficient in this regard. Also, it now seems likely that the Mg^{2+} and ADP inhibitory effects were responsible for a report from my group of two K_m values above the ATP concentration required for saturation of uni-site catalysis (133). Unfortunately, a kinetic model was presented to explain these values and the concentration dependency of oxygen exchanges. This model now joins others in a kinetic graveyard.

Assays at higher ATP concentrations can be initiated by adding MF_1 without inhibitory Mg^{2+} to ATP solutions without a large excess of Mg^{2+}. With adequate initial velocity measurements, a single K_m for beef heart mitochondrial F_1-ATPase of about 120 μM is observed (see 134, 135). Such a single K_m above the μM range has the important implication that only two sites need to be filled for ATP hydrolysis by F_1-ATPase to attain a high velocity.

Another way of avoiding complications of the Mg^{2+}-ADP inhibition is suggested by the valuable observation that a yeast F_1-ATPase βT197S mutation does not show the inhibition and thus has lost sensitivity to azide (136, 137). Allison et al (138) found similar properties with the corresponding replacement in the TF_1 ATPase, and with the $\alpha_3\beta_3\gamma$ complex from the mutant found K_m values of 1.4 and 110 μM. On this basis they suggest that three sites need to be filled for rapid catalysis. However, other factors need to be considered. The TF_1 ATPase does not readily retain nucleotides under uni-site conditions, and this raised doubt as to whether alternating site cooperativity occurred with TF (139). However, the fact that such cooperativity occurs with the TF_1 ATPase was demonstrated by ATP modulation of the oxygen exchange (140). These properties of the TF_1 ATPase suggest that the lower K_m for the mutant $\alpha_3\beta_3\gamma$ (138) may represent a filling of the first catalytic site, with rapid catalysis when the second site is filled.

Evidence from kinetic characteristics of TNP-ATP hydrolysis in the presence or absence of ATP was interpreted as favoring participation of three catalytic sites, with rapid ATP hydrolysis attainable when only two sites are filled (141). The behavior was consistent with TNP-ATP in the presence of 500 μM ATP being able to enter the catalytic cycle by binding to a third catalytic site that bound ATP weakly but, interestingly, preferred ADP to ATP.

Other Evaluations of Site Occupancy

A suitable quantitative measure of the number of catalytic sites filled during hydrolysis of about 1–200 μM ATP by F_1-ATPase is not readily accessible

experimentally and has not yet been reported. Such an evaluation for TNP-ATP hydrolysis by MF_1-ATPase was made some years ago (142). A tight initial binding of one TNP-ATP per enzyme was shown, with hydrolysis and product release increased to a near maximum rate by binding of TNP-ATP at a second site.

A more recent approach to site occupancy during ATP hydrolysis is based on mutational replacement of the tyrosine of EcF_1 that interacts with the adenine ring at the catalytic site with tryptophan, mutant βY331W (143, 144). The mutant retained the ability to grow on succinate, the isolated F_1-ATPase had good activity, and the additional fluorescence contributed by the catalytic site tryptophan was strongly quenched by binding of ATP, ATP-PNP, or ADP with or without added Mg^{2+}. The use of this method to monitor nucleotide binding has been confirmed elsewhere (145). However, correlating the extent of binding with catalytic activity is difficult. Based on the concentration dependency of fluorescence quenching upon ATP addition, it has been suggested that three catalytic sites must be filled for near maximal hydrolytic activity to be attained (143, 144). The approach is ingenious, but unfortunately properties of the EcF_1 ATPase interfere with interpretation of these results.

The activity of the EcF_1 ATPase is strongly inhibited by the ε-subunit and by the ease with which the Mg^{2+}- and ADP-inhibited form arises. Under conditions of the fluorescence measurements (143, 144), a considerable portion of the enzyme will be in these inhibited forms, with much or most of the measured ATPase activity due to the uninhibited enzyme present. Thus, the fluorescence quenching undoubtedly results at least appreciably from binding to inhibited forms. The inhibited forms may have considerably different nucleotide affinities than the active form. Correlation of binding with activity from these studies thus becomes uncertain. Other factors not considered here may also interfere with the correlation.

The fluorescence quenching approach has been extended to distinguish the binding of ADP or ATP (146) and to measure the binding and hydrolysis with trinitrophenyl-ATP (147). Difficulties similar to those mentioned above may interfere with the correlations of binding and catalysis.

Results from other research groups have been interpreted as evidence for models in which only two sites participate in catalysis and the third site has some other function (148, 149). Alternate explanations for these results (4) have been strengthened by more recent observations. The finding that binding of two ADP-fluroaluminate complexes is necessary for complete inactivation (148) is explained by the binding of the first strongly promoting the binding of a second complex to the same enzyme (150). The supposition that the ADP causing the interesting hysteretic inhibition of the MF_1-ATPase (151) is at a catalytic site (148) has been corrected by the demonstration that it is at a noncatalytic site (152).

Some Other Catalytic Properties

The question of whether catalysis of covalent bond change can occur at more than one site at a time has yet to be resolved. Such catalysis is known to occur at the tight site and likely does not occur at the open site. The loose site in the X-ray structure with bound AMP-PNP present is considered to have groups present that are suitable for catalyzing hydrolysis (12). Studies have shown that when the tight site contains inhibitory ADP and Mg^{2+}, the other sites lack hydrolytic capacity (106). Most likely hydrolysis of ATP bound at the loose site can occur only when accompanied by the conformational changes associated with formation of the tight site.

Slow ATP hydrolysis by the CF_1-ATPase can occur when one site contains an ADP moiety from reaction with 2-azido-ADP (153). The modified β-subunit may still be able to go through conformational changes, unlike the Mg^{2+}- and ADP-inhibited form. Interestingly, ^{18}O probes showed that when one catalytic site was modified, more than one catalytic pathway was operative. This result is as expected if remaining catalytic sites can no longer have identical interactions with adjacent moieties.

The α-subunits, in addition to providing some of the catalytic site residues, can modulate catalytic events through binding of nucleotides. ATP binding to noncatalytic sites enhances GTPase activity of the CF_1-ATPase fivefold (154), and if noncatalytic sites are originally empty, the ATPase activity appears as ATP becomes bound at noncatalytic sites (126). In the presence of sulfite, CF_1 can hydrolyze ATP without ATP binding at noncatalytic sites, but the steady state rate is increased by such binding (125). Similarly, with MF_1, the slow binding of ATP to noncatalytic sites accelerates ATP hydrolysis (127). The amount and composition of noncatalytic-site nucleotides affects both the formation and the ATP-dependent discharge of the Mg^{2+} and ADP inhibition (126).

Synthesis as well as hydrolysis of ATP may be modulated by noncatalytic-site nucleotides. With the synthase from thermophilic *Bacillus PS3* co-reconstituted in liposomes with bacteriorhodopsin, binding of ATP at a noncatalytic site doubled the rate of ATP synthesis (155).

SYNTHESIS BY THE ATP SYNTHASE

The designation ATP synthase is used in this review in preference to the synonymous term F_1F_0-ATPase that often appears in the literature because the term synthase better indicates the function of the enzyme.

There appears to be general agreement that the coupling of proton translocation to the formation of ATP occurs through indirect conformational changes in the subunits. This concept was recently further substantiated by a demonstration that under uni-site conditions the equilibrium between ATP and ADP $+ P_i$ at the catalytic site is independent of the membrane energization whether

the reaction runs in the synthesis or hydrolysis direction (156). It also seems probable that ATP hydrolysis and ATP synthesis by the ATP synthase proceed through the same intermediate steps, but this probability was questioned in a recent paper on the basis of the observation that even with the presence of protonmotive force, submitochondrial particles retain a Mg^{2+} and tight ADP inhibition of ATPase activity (157). However, a different explanation of this result seems likely because, under the experimental conditions used, medium ADP would be lacking. Without both ADP and P_i binding at the open site, the proton translocation and the accompanying binding changes cannot occur. The results (157) actually demonstrate this type of requirement for the coupled ATP synthesis.

Kinetic Evaluation of Catalytic Site Occupancy

Although rate measurements of ATP synthesis in the presence of adequate protonmotive force are free of complications by Mg^{2+} and ADP inhibition, present information is not adequate to determine whether rapid catalysis is achieved with only two or with three catalytic sites filled. Many laboratories have determined K_m values for ADP and P_i during ATP synthesis, with the synthase from various sources. Only one K_m has been noted with more than about 5 μM ADP and about 100 μM P_i and with high protonmotive force. Whether there are one or two K_m values indicative of one or two catalytic sites filled at ADP concentrations below about 1–2 μM is unclear.

With the synthase from *Paracoccus denitrificans* (158), from submitochondrial particles (159), and from chloroplasts (160, 161), two kinetically distinguishable K_m values, one near and one considerably above 1 μM, were detected. A reasonable interpretation of these findings is that these values represent the filling of a first and a second catalytic site with ADP and P_i. With de-energized submitochondrial particles, a higher affinity and exchangeable site for ADP was also detected (159). This finding called attention to the possibility that three catalytic sites need to be filled for rapid ATP synthesis. Alternatively, the very tight ADP binding, measured with 2 mM Mg^{2+} present, may arise from the formation of the inhibitory Mg^{2+}-ADP complex.

In a more recent evaluation, the rate of ATP synthesis and hydrolysis by the chloroplast synthase was measured as a function of concentrations of all substrates, and of protonmotive force (162). A simulation analysis of experimental rate and equilibrium constants based on the binding change mechanism was presented. The model does not discern whether filling of two or three catalytic sites suffices for rapid catalysis. An interesting feature of the model is a shift of the rate-limiting step for ATP synthesis, from a binding-change with small transmembrane ΔpH to an ATP release at higher ΔpH. The model includes the assumption of a fast interconversion at the tight site. This feature seems

unlikely because the rate of interconversion is only 2–3 times the maximum rate of net ATP synthesis with mitochondria (163). This interconversion rate is accommodated by the model if the calculations are generalized (B Rumberg, personal communication).

Other Evaluations of Site Occupancy

Suitable methods for measuring catalytic site occupancy directly are very limited. One approach is based on the separation of chloroplast thylakoids from the surrounding medium by rapid filtration as photophosphorylation is occurring, with added ADP not far above the enzyme molarity. Concentrations of synthase in the μM range can be obtained, and the amount of free ADP in the filtrate tells how much was bound (164). The K_m for ADP during rapid photophosphorylation is about 30 μM, and thus with about 2 μM ADP present, the site that fills for rapid catalysis should be largely empty. If more than one site with K_m values of less than about 2 μM are already mostly filled, slightly more than two sites should be filled. Only close to one filled catalytic site was detected, giving strong evidence that the rapid increase in the photophosphorylation rate as the ADP concentration is increased toward 30 μM or above results from the filling of a second catalytic site. These studies were not extensive, and this experimental approach merits more attention.

Being able to achieve rapid catalysis with only two sites filled means that even though ATP may have departed from a loose site, ADP and P_i can fill another site and allow binding changes so that a tightly bound ATP can become loosely bound. When a tightly bound ATP is present, only the site that will become tight in a binding change needs to be filled with ADP and P_i for rapid catalysis to proceed.

Coupling to H^+ and Na^+ Translocation

Coupling to H^+ and Na^+ translocation is an area of intense interest and activity. Most information about coupling to H^+ translocation has come from studies of the *E. coli* F_0 (see 9, 10, 165, 166, and references therein). In addition to the carboxyl group of Asp-61 blocked by DCCD in subunit c, mutational probes have identified polar residues from the a- and c-subunits, but none from the b-subunit, of F_0 that are essential for proton conduction. A particularly striking mutation, Asp-c61 to Gly and Ala-c24 to Asp, is evidence that the essential DCCD-reactive carboxyl can be moved to an adjacent helix and retain activity (166). As to the path for protons, one suggestion (167) is that a pathway involving five c- and three a-subunit residues is involved. But some of the suggested residues may serve a function other than proton binding, and a path as short as possible that gives conformational coupling but does not allow uncoupled proton leakage seems advantageous.

Studies of an Na^+-specific ATP synthase from *Propionigenium modestum* bacterial ATP synthase have had a constructive impact on the field (168, 168a). The structural and functional similarity is dramatically illustrated by reconstitution of a functional complex from *E. coli* F_1 and F_0 from *P. modestum*. Na^+ protects the F_0 from DCCD inhibition and competes with H^+ for binding. The site of the essential carboxylate has the saturable binding properties of a classical carrier rather than a channel like gramicidin. The possibility that minor differences in amino acid composition may govern cation specificities is shown by the demonstration that a double mutation in subunit c of the ATP synthase of *P. modestum* causes a switch from Na^+- to H^+-coupled ATP synthesis. Evidence for a carrier instead of a channel mechanism also comes from the observation that the F_0 of *P. modestum* will catalyze $^{22}Na^+$ counterflow. As might be anticipated, other specialized anaerobic bacteria also have Na^+-linked ATP synthases (169). Replacement of four residues in the *E. coli* subunit c conferred Li^+ but not Na^+ binding capacity and led to suggestions of types of residues that may be involved (170). Small changes in the nature and positions of ligands may change specificity as observed in binding by crown ethers (171).

Theoretical and experimental insight into how protons may be translocated has been provided by Zundel and associates (167). They report that infrared spectra allow detection of hydrogen-bonded chains with large proton polarizabilities owing to collective proton tunneling, and that such chains can rapidly transport protons. They further report that such a pathway is present in the F_0 from *E. coli* and that the indicative infrared continuum is no longer observed if the F_0 is inhibited by DCCD. Recently they showed that Na^+ could have similar collective motions and cation polarizabilities, and they suggest that this similarity underlies the coupling to Na^+ transport. The requisite properties might be shown by a water-containing channel essentially filled with hydronium ions or hydrated Na^+ ions, with the positive charges neutralized by close association with or transfer of a proton to O or N atoms of amino acid side chains. At a key position a cluster of liganding groups with preference for bonding of a hydronium or Na^+ ion could confer the specificity and give a binding site whose alternate exposure might account for the $^{22}Na^+$ counter-transport mentioned above.

The striking cooperativity of the c-subunits in the F_0 complex must be accommodated by any transport scheme. The ability to reconstitute a functional F_0 complex from isolated subunits has allowed an assessment of effects of the presence of functionally defective D61N or D61G mutant subunits. The presence of only one mutant subunit blocked proton translocation, confirming an earlier demonstration of the cooperativity among the 9–12 c-subunits present (165). Every c-subunit knows what the other c-subunits are doing.

The light-activated chloroplast ATP synthase has allowed clever spectrophotometric probes of events accompanying proton translocation. Transmembrane

voltage steps induced on a background of pH difference allowed the kinetic resolving of proton intake, transfer, and release steps. ADP at a catalytic site blocked the transfer step, and dequalinium at the nearby DELSEED sequence blocked the release of protons (172). Such results not only extend previous findings showing long-range conformational interactions but also suggest specific stages of binding changes that may be affected by the progress of proton translocation.

Other experiments measured the dependence of the rate of ATP formation on the pH on both sides of the coupling membrane, using the chloroplast synthase reconstituted into liposomes, with an acid-base transition for energization (173). The dependence of phosphorylation on internal H^+ concentration indicated protonation of three groups inside with a pK of 5.8 and release of protons inside from outside groups with a pK of 7.8. The change in exposure of proton binding groups was attributed to the binding of ADP and P_i. A related assessment of nucleotide ability to stop proton flow across the membrane also indicated that substrate binding controls the orientation of proton translocating groups, as well as their participation as alternating catalytic sites (174).

Steps Promoted by H^+ Translocation

Translocation of at least three, and likely four, protons is necessary for each net formation of ATP (see 175). Steps that must be modulated by H^+ movement in the same binding change sequence are the binding of ADP and P_i, the conversion to bound ATP, and the release of ATP. Such coordinated binding changes account for the fact that the apparent on-constant for ADP binding for phosphorylation is sharply increased upon energization (176, 177).

Recent experiments show that the dissociation of ATP from the synthase requires an energy input about equivalent to that obtainable from hydrolysis of ATP (178). The concomitant change of a second site with bound ADP and P_i to the tight conformation may, likely because of tight ADP binding, provide part of the driving force for ATP release. The balance of the energy input from the proton translocation may be needed to change the conformation of the third site and to provide for modulation of the covalent bond formation step.

If ADP is already bound tightly to the catalytic site, P_i does not bind readily. This is evident from the lack of exchange of P_i oxygens by submitochondrial particles when protonmotive force is absent (179) and from the high P_i concentrations needed for bound ATP formation by CF_1 (180) and ECF_1 (181). Gräber has considered this and other features in an analysis of the energetics of the catalytic cycle (182).

As noted above, under uni-site conditions the rate of bound ATP synthesis from the bound ADP and P_i is less than 1/100 the maximum possible rate of net ATP synthesis. This means that for rapid net ATP synthesis the rate of formation of bound ATP must be drastically increased and the quasi-equilibrium of

bound substrates must be shifted so that essentially only ATP is present. When conditions are optimal, probable events are as follows: The binding of ADP and P_i is necessary before protonmotive force can be used. Then, as the substrates undergo the binding change from being loosely to tightly bound, covalent catalysis starts, the rate of ATP formation is markedly increased, and essentially only tightly bound ATP appears at the tight site. Another binding change rapidly takes place, and the tightly bound ATP becomes loosely bound and dissociates (164). Another possibility (which seems less likely) is that when conditions are favorable for net ATP synthesis, the rate of substrate interconversion at the tight site is sharply increased without appreciable shift in the quasi-equilibrium, and a binding change can only occur when ATP alone is present at the tight site.

Even in rapid photophosphorylation or oxidative phosphorylation, about 2–3 reversals of ATP formation occur before ATP is released to the media (163). This rapid reversal continues even when the rate of net synthesis is reduced by uncouplers. Such results led to a model in which rapid formation and cleavage of ATP occurs only in the occluded active site (183). Whether the appearance of water oxygens into ATP results from reversal of both ATP formation and proton translocation or from reversal of ATP formation not accompanied by reversal of proton translocation remains an important question.

OTHER MODULATIONS OF CATALYSIS AND CONFORMATION

A host of mutational studies have provided information about the role of various amino acid residues. Prominent among mutational studies are those concerned with residues that might be at the catalytic sites. For example, the definitive studies by Senior et al (184) showing the critical participation of *E. coli* Lys-β155 ($\sim MF_1$ βLys β162), a conserved residue in the "glycine-rich" or "P-" loop, are amply explained by MF_1 X-ray structure. The structure is also consistent with other mutational analyses that suggested a lack of participation of *E. coli* Asp-β242 in catalysis (185), and with a fluorescence and mutational study that suggested that residue *E. coli* β331 is part of the adenine binding subdomain (186). The Asp-β242 is in a sequence analogous to one participating in the adenylate kinase catalysis. The coordinates for the MF_1 X-ray structure have recently been made available, and many mutational studies can now be further evaluated. These include extensive studies about arrangements near the catalytic site (187) and in the center region of the β-subunit (188). Revertants of *E. coli* Met-β209, analogous to a conserved Met in all known F_1 ATPases, include only wild type and Ser-β209 (189). Interestingly, Ser is found in the equivalent position in homologous vacuolar and archaebacterial ATPases. Other replacements of the Met-β209 have a severe impact on

catalysis. The Met-β209 lies only 3.1 Å from an essential Glu at the catalytic site, emphasizing stereo-chemical constraints that are critical for catalysis.

The importance of interactions between the β- and γ-subunits is emphasized by the somewhat surprising finding that β-subunit amino acid replacements would suppress a γ-subunit frameshift mutation with a long unrelated C-terminus (190). Mutations in the N-terminal region of the EcF_1 γ-subunit had limited effect, except replacement of Met-γ23 by Arg or Lys strongly impaired oxidative phosphorylation (191). Second-site mutations in the C-terminal region largely reversed the effect (192).

The ability of a mutation in an *E. coli* α-subunit to restore coupling efficiency to a deleterious β-subunit mutant indicates the importance of interaction between regions of the α- and β-subunits in energy coupling (193). Changing *E. coli* Asp-β261 to Asn blocked binding of Mg-nucleotides to the noncatalytic sites but not ATPase activity, showing that ATP hydrolysis does not depend on nucleotide binding to noncatalytic sites (194). Mutations of TF_1 Tyr-β341 (analogous to MF_1 Tyr-β345) had parallel effects on the K_d for ATP for the isolated β-subunit and on the K_m for ATP hydrolysis by the $\alpha_3\beta_3\gamma$ complex, showing that the Tyr residue is a major K_m-determining residue (195). A replacement of 19 amino acid residues of the *E. coli* ε-subunit by alanine identified residues important for binding to F_1 or for catalytic inhibition and led to a model for the secondary structure of the subunit (196). The gene encoding for the chloroplast ε-subunit was expressed in *E. coli*, and the product recombined with ε-deficient CF_1 (197). Mutations that weakened ATPase inhibition also lessened the ability to restore proton impermeability. As with *E. coli*, N-terminal truncations had a more profound effect than C-terminal deletions. A mutational study was made of βHis-211 of yeast, analogous to His-β177 of MF_1 and to His-36 of adenylate kinase, which are near the nucleotide binding domains (198). Major effects of substitutions were on the stability and assembly of the ATPase.

A variety of other interesting techniques have been applied to gain insight into the ATP synthase. With ^{1}H NMR, signals from 12 His residues of the TF_1 β-subunit were separately observed (199). Nucleotide binding changed signals from His-179 and His-200, but not from His-119, although substitution of His-119, located at a long loop opposite the catalytic site, suppressed ATPase activity and thermostability. In an unusual approach it was shown that changes in conformation of the ε-subunit of MF_1 can be observed by the phosphorescence emission of Trp (200). An interesting cross-reconstitution of F_1 from MF_1 or EcF_1 with F_0-containing but F_1-stripped membranes of *E. coli* or heart mitochondria was achieved (201). Several interesting observations were made, including the fact that subunits of mammalian F_0 can confer oligomycin sensitivity to MF_1 or EcF_1 attached to the *E. coli* membranes. The introduction

of a chloroplast β-subunit into a bacterial synthase allowed probes of the effect of the interfacial Cys-β63 on catalysis (202). In the chloroplast, this Cys is known to conformationally interact with the nucleotide binding site over 40 Å away. Replacement of the Cys by Trp blocked ATP synthesis with little effect on the ATPase activity, showing a conformational uncoupling. In a continuation of valuable probes of amphipathic cations that inhibit by binding near the conserved DELSEED sequence of the β-subunit, [^{14}C]dequalinium was synthesized and upon photoactivation cross-linked conserved Phe-α403 or Phe-α406 to sites within residues β440–459 (203). Conformational changes in the ε-subunit of the chloroplast ATP synthase induced by light and nucleotides were demonstrated by use of the reactivity of Lys-ε109 to modification by pyridoxal 5′-phosphate (204). The alkylation of Cys-γ89 of the chloroplast synthase is known to inhibit ATP synthesis and hydrolysis. Rates of release of bound nucleotides were monitored by stopped-flow fluorescence (205). The maleimide attachment increased rates of nucleotide exchange by over two orders of magnitude, although nucleotide binding sites are more than 50 Å from Cys-γ89. A demonstration of variable proton translocation stoichiometry for the ATP synthase of two phototropic prokaryotes (206), and earlier related observations, suggests that the H^+/ATP ratio can be higher than 3 or 4 under some conditions. A variable stoichiometry may need to be accommodated in mechanistic considerations.

ROTATIONAL CATALYSIS

Catalytic and Structural Assessments

The concept of rotational catalysis, first outlined in the early 1980s (207, 208), was based on three facets of experimental observation (4). One was the evidence, principally from ^{32}P and ^{18}O exchange studies, for strong catalytic cooperativity with sequential participation of catalytic sites. The second was evidence from distribution of ^{18}O isotopomers of P_i and ATP formed from ^{18}O-labeled substrates that catalysis proceeds in an identical manner at all participating sites. The third was the recognition that catalytic events are markedly influenced by the γ-subunit whose amino acid sequence made unlikely the presence of structurally similar portions for interaction with different β-subunits. Other studies suggested that some type of rotational movement might occur in F_0. It was proposed on the basis of chemical derivatization studies that a core of c-subunits might rotate against the a-subunit (209). A similar proposal was made on the basis of structural and mutational studies (210).

Subsequently, other evidence relevant to possible rotational catalysis accumulated. Differences in chemical reactivity were used to show that catalysis causes change in the relative positions of the β-subunits of MF_1 (212) and CF_1

(213). Cryoelectron microscopy of EcF_1 labeled with fragments of antibodies to the α- and ε-subunits revealed that a central mass or a domain including the ε-subunits moves in response to MgATP addition (214). Similarly, electron microscopic studies of the CF_1 showed movement of a central mass (215).

A cross-linking of major and minor subunits of EcF_1 (216) or of the γ- and β-subunits (217) can reversibly inhibit catalysis. But a cross-link between the γ- and β-subunit was reported to still permit catalysis (218). The long cross-linker used in the latter studies may have attached in a manner such that its flexibility allowed the positional interchanges to continue; other possibilities are considered elsewhere (4).

The structure found for the MF_1 (12) dramatically supports rotational catalysis. The biological novelty of the mechanism and the interest it generated is indicated by the range of perspectives published about this finding (219–222). The structural asymmetry of the β-subunits correlates nicely with the functional asymmetry predicted for sequential binding changes and nucleotide affinities. The asymmetries arise from interactions with different portions of the γ-subunit that fills the central cavity. The hydrophobic sleeve surrounding the C-terminus of the γ-subunit appears to be designed to allow rotation.

Subsequent to the structure report, some excellent experiments supporting rotational catalysis appeared from Capaldi's laboratory. Specific cross-links between mutationally introduced cysteines in the β- or ε-subunits allow disulfide formation with the adjacent γ-subunit. Bonds between either β and γ or β and ε inhibit activity in proportion to the extent of cross-linking, and the activity is regained upon cleavage of the disulfides (223). Movement of the ε between α- and β-subunits was also demonstrated (224). Related studies further characterized the interface between γ- and ε-subunits (225) and monitored these by nucleotide-dependent conformational changes of the γ- and β-subunits (226).

Experiments in Cross's laboratory provide an elegant demonstration of rotational movement of subunits (227). Based on the MF_1 structure, they introduced cysteine by mutation and induced a specific disulfide bond between a β- and γ-subunit. Activity loss was restored by reduction of the disulfide. Radiolabeled β-subunits were incorporated into the two non-cross-linked β-subunits. Catalytic turnover was shown to randomize the position of the γ-subunit relative to the radiolabeled β-subunits as expected for rotational catalysis. The approach has been extended to show similar rotational movement with the intact synthase and, further, that reaction of the F_0 component with DCCD blocks rotation (228). These and other related experiments relevant to rotational catalysis have been reviewed recently (229).

Although the preceding evidence for rotational catalysis seems convincing, widespread acceptance of such a novel behavior in enzymology likely requires physical demonstration of rotation. As noted in a recent short review,

attachment of probes to the C-terminus of the γ-subunit that protrudes from the central portion of the F_1 is one possible approach (230). Such a procedure has quite recently been used with the CF_1, by labeling the penultimate cysteine residue of the γ-subunit with eosin-5-maleimide. With application of polarized absorption relaxation after photobleaching, an ATP-dependent rotation of the γ-subunit relative to immobilized α- and β-subunits was observed (231, 232). An even more striking example comes from a report that may become a classic in the field. An $\alpha_3\beta_3\gamma$ complex was fixed to a glass surface through the β-subunits, and a fluorescent actin filament was attached to the γ-subunit. ATP addition resulted in more than 100 rotations, as viewed under a microscope. An estimate was that the torque led to a nearly 100% energy conversion from ATP to motion (233). Only one other well-documented rotary motor is known, that of the much larger bacterial flagella, a complex assembly of some 100 protein molecules. With the globular F_1 ATPase, the rotary motor has a central rotor with a radius of only about l nm in a stator barrel with a radius of about 5 nm. These results justify the statement that the ATP synthase is a splendid molecular machine, hence the title of this review.

The physical demonstration of rotation also answered a question that arose whenever I sketched a rotational model. This question is: As net ATP formation is proceeding, and the synthase is viewed from above the coupling membrane with the ring of α- and β-subunits considered stationary, is the central γ-subunit rotating in a clockwise or counterclockwise direction? The results show that as ATP is synthesized, the rotation is counterclockwise (233). This means that in bi-site ATP hydrolysis the ATP adds to the "loose" site, and for bi-site ATP synthesis the ADP adds to the "open" site of the Walker (12) structure.

Two Interactions Between F_0 and F_1

For rotational catalysis, one obvious requirement is that a rotatory movement in F_0 must be coupled to the γ-subunit. A second and less recognized requirement is also likely. If a rotation of a central core of c-subunits directly drives the γ-subunit rotation, something must keep the α-β-subunit ring from rotating. The situation is analogous to a motor doing work—the outer casing needs to be anchored or it will spin with the rotor. The b-subunit of *E. coli* F_0 merits consideration for such a role, as does the δ-subunit and the analogous OSCP of MF_1. Of interest in this regard is the observation that a readily formed disulfide link between the δ- and α-subunit does not inhibit ATPase activity (234, 235) but does stop proton pumping coupled to ATP hydrolysis (235). In addition, removal of about 20 amino acids from the C-terminal portion of the δ-subunit resulted in loss of the DCCD-sensitivity of the ATPase (236), and mutations in the δ-subunit of the *E. coli* synthase uncoupled the ATP hydrolysis from proton pumping (62), as would be anticipated if the subunit has an anchoring

role. A similar role for the OSCP protein of the mitochondrial F_0 is consistent with its role in conferring oligomycin sensitivity; without the anchor between the α-β-subunits and the α- and β-subunits of the F_0, ATP hydrolysis would proceed uncoupled from proton translocation.

The attachment of a restraining bridge could be governed by or promote the heterogeneity of nucleotide binding by the α-subunit. The differences between ATP and ADP binding to α-subunits are retained during catalysis (237). This structural asymmetry does not seem to be expressed as functional asymmetry. In analogy with a motor, the function of the rotor is not changed because the external casing is anchored at its base.

The α-subunit may have a function in addition to nucleotide binding and participation in a nonrotating anchor. The α-subunit has been shown to have sequence homology with a chaperone (238), suggesting that it might help keep the β-subunit properly folded as it undergoes the rather major conformational shifts during catalysis.

Coupling to Proton Translocation

A suggestion for the coupling of proton translocation to the required rotational movements is as follows: (*a*) The orientation of the DCCD-reactive carboxyl group so that it can accept a proton from one or the other side of the membrane could be determined by whether catalytic sites are filled predominantly with ADP and P_i or with ATP. All c-subunits might shift in unison in response to the conformational signaling, in agreement with the pronounced cooperativity. As a proton moves through the contact region of a c-subunit and the a-subunit, there is circular movement of an internal c-subunit ring with respect to the a-subunit. Depending upon the required stoichiometry, either 4 protons or 3 protons would need to be transported in sequence for a group of 12 or 9 c-subunits to move 120° and promote release of one ATP. Aspects of this suggestion have been made by others (for example, see 229, 239).

RELATED CATALYSES

The most closely related enzymes are the vacuolar ATPases that have marked structural, sequential, and functional homology to the F_1-ATPases (see 240, 241, and references therein). The similarity extends to the archaebacteria ATPases (242). The proteins needed for flagella rotation include one that has antibody reactivity like and extensive sequence homology with the ATP synthase β-subunit (243). Proteins involved in bacterial virulence and invasion show sequence homology with the β-subunit (244, 245). The ATP synthase F_0 component and membrane channel proteins show structural homology, as mentioned above (79).

Recent data show that an RNA/DNA helicase (termination factor rho) has structural and sequence homology with the F_1-ATPase (245). The hexameric T7-phage DNA helicase has structural resemblance to the F_1-ATPase and has been postulated to act with a similar cooperative mechanism (246). The recent attainment of a crystal structure of a helicase has been interpreted as evidence that strengthens this analogy (247). Nature appears to use a ring structure of ATP-hydrolyzing proteins to drive conformational changes or movements of centrally located macromolecules.

Energy-requiring domain shifts linked to ATP binding are common features of other enzymes. Molecular motors are good examples. The myosin-, kinesin-, and dynein-driven systems show burst kinetics of ATP hydrolysis with rate-limiting product release (248). Binding of the products complex to the filament accelerates product release and completes the ATPase cycle (248). As noted recently (249), these and other similar systems all depend upon the ability, well demonstrated by allosteric enzymes, of ligand to bind to one site on proteins to drive energy-requiring conformational changes at other locations.

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