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ATP Synthases in the Year 2000: Defining the Different Levels of Mechanism and Getting a Grip on Each

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ATP synthases are unusually complex molecules, which fractionate most readily into two major units, one a water soluble unit called F_1 and the other a detergent soluble unit called F_0. In almost all known species the F_1 unit consists of 5 subunit types in the stoichiometric ratio $\alpha_3\beta_3\gamma\delta\varepsilon$ while the F_0 unit contains 3 subunit types (a, b, and c) in *E. coli*, and at least 10 subunit types (a, b, c, and others) in higher animals. It is now believed by many investigators that during the synthesis of ATP, protons derived from an electrochemical gradient generated by an electron transport chain are directed through the F_0 unit in such a way as to drive the rotation of the single γ subunit, which extends from an oligomeric ring of at least 10 c subunits in F_0 through the center of F_1. It is further believed by many that the rotating γ subunit, by interacting sequentially with the 3 $\alpha\beta$ pairs of F_1 (360° cycle) in the presence of ADP, P_i, and Mg^{++}, brings about via "power strokes" conformational/binding changes in these subunits that promote the synthesis of ATP and its release on each $\alpha\beta$ pair. In support of these views, studies in several laboratories either suggest or demonstrate that F_0 consists in part of a proton gradient driven motor while F_1 consists of an ATP hydrolysis driven motor, and that the γ subunit does rotate during F_1 function. Therefore, current implications are that during ATP synthesis the former motor drives the latter in reverse via the γ subunit. This would suggest that the process of understanding the mechanism of ATP synthases can be subdivided into three major levels, which include elucidating those chemical and/or biophysical events involved in (1) inducing rotation of the γ subunit, (2) coupling rotation of this subunit to conformational/binding changes in each of the 3 $\alpha\beta$ pairs, and (3) forming ATP and water (from ADP, P_i, and Mg^{++}) and then releasing these products from each of the 3 catalytic sites. Significantly, it is at the final level of mechanism where the bond breaking/making events of ATP synthesis occur in the transition state, with the former two levels of mechanism setting the stage for this critical payoff event. Nevertheless, in order to get a better grip in this new century on how ATP synthases make ATP and then release it, we must take on the difficult challenge of elucidating each of the three levels of mechanism.

KEY WORDS: Bioenergetics; mitochondria; oxidative phosphorylation; ATP synthesis; ATP synthase; F_1-ATPase; molecular motors; rotational catalysis; transition states; enzyme mechanisms; vanadate; nanomotor.

INTRODUCTION

During the last decade of the past century perhaps the two most significant advances in our understanding of ATP synthase (F_0F_1) function were the acquisition of 3-dimensional structural information about the F_1 moiety at atomic resolution (Abrahams *et al.*, 1994; Bianchet *et al.*, 1998; Shirakihara *et al.*, 1997), and the demonstration that the centrally located γ subunit rotates during ATP hydrolysis (Noji *et al.*, 1997; Omote *et al.*, 1999). Both advances added partial support for a long evolving hypothesis for ATP synthesis known as the "binding change mechanism" (Reviewed in Boyer, 1997). One interpretation of this hypothesis as applied to mitochondria is that the proton gradient generated by the electron transport chain drives the rotation of one of the small F_1 subunits located in the center of the three $\alpha\beta$ pairs such that at every 120° its

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differential binding to each pair causes each to assume a different conformational/binding state. Thus, the hypothesis envisioned that at one of the three 120° positions the catalytic site on one $\alpha\beta$ pair would be empty having released newly synthesized ATP, a second site would contain newly synthesized ATP, and a third site would have bound ADP and P_i in preparation for ATP synthesis. Completion of the rotation through 360° of the centrally located subunit, now known from two different X-ray structures of F_1 to be the γ subunit (Abrahams *et al.*, 1994; Bianchet *et al.*, 1998), would result in the catalytic site on each $\alpha\beta$ pair having bound ADP and P_i, synthesized ATP, and released the bound ATP into the medium.

Despite the major advances just noted, there is much more that remains unknown about the overall mechanism of ATP synthesis catalyzed by ATP synthases than is currently known. In fact, several critical features of the binding change mechanism itself remain the subject of debate. Therefore, it is the purpose of this brief introductory article to attempt to put the overall mechanism of ATP synthesis catalyzed by ATP synthases in perspective in order to get a grip on the challenging tasks that lie ahead for investigators working in this field in the future. As illustrated in Fig. 1A, we note that there are three major levels of mechanism that must be understood before we can ever hope to have a solid grip on our understanding of how ATP is made in biological systems. The first level involves the mechanism by which a proton gradient drives the rotation of the γ subunit, most likely together with the ε subunit in bacteria (Kato-Yamada *et al.*, 1998) and its δ subunit counterpart in animals. The second level involves the mechanism by which the rotation of the γ subunit is coupled to conformational/binding changes in the three $\alpha\beta$ pairs, while the final and payoff level involves the mechanism of both ATP formation from ADP, P_i, and Mg^{++} at the three catalytic sites and the release of bound ATP from these sites.

Each level of mechanism as it pertains to what we know and what remains to be established is discussed hereafter.

Mechanism of Proton Gradient Driven Rotation of the γ Subunit

This first level of mechanism that ATP synthases must deal with in making ATP is illustrated very simply in Fig. 1A. Certainly, it has received the greatest notoriety in recent years and generated the most excitement among those investigators working on ATP synthases. Significantly, two different laboratories, one working with the F_1 moiety of the thermophilic *Bacillus* enzyme (Noji *et al.*, 1997) and the other with the F_1 moiety of the *E. coli* enzyme (Omote *et al.*, 1999), provided direct evidence that the γ subunit rotates during ATP hydrolysis. These experiments were carried out by first engineering onto the γ subunit a fluorescently labeled actin probe and then monitoring its rotation with a video camera (Cover figure and legend). Subsequently, similar experiments were carried out with the complete *E. coli* ATP synthase (F_0F_1) with the fluorescent actin probe attached to the c-subunit oligomer rather than to the γ subunit (Sambongi *et al.*, 1999; Pänke *et al.*, 2000). The results of these latter experiments, also conducted under ATP hydrolysis conditions, were interpreted as direct evidence for rotation of the c-subunit oligomeric ring. The two types of experiments have been taken by many as proof that, during ATP synthesis by ATP synthases, the electrochemical gradient of protons drives rotation of the c-subunit oligomer and this in turn drives rotation of the γ subunit. In other words, it is currently being assumed that because the γ subunit and the c-subunit oligomer rotate during ATP hydrolysis catalyzed by F_1 and F_0F_1 respectively, they will also rotate in the reverse direction during proton gradient driven synthesis of ATP. Although this reverse logic is reasonable, it is important to directly demonstrate that a proton gradient can drive the rotation of the c-subunit oligomer and that this in turn can drive the rotation of the γ subunit. In fact, a well-designed experiment that can test this possibility is perhaps the most important experiment that one can do at this time as it relates to elucidating the first level of mechanism defined earlier and in Fig. 1A. One approach to performing this experiment would be to impose an electrochemical proton gradient across a liposomal system reconstituted with subunits a, and c, the membrane part of subunit b, and the γ subunit (or truncated form thereof) to which is covalently attached an appropriate "arm" labeled with a fluorescent probe. The rotation of the probe, if it occurs, could be monitored visually as was done in earlier studies.

Other points that need to be clarified in order to understand the first level of mechanism involved in ATP synthase function are as follows: First, the issue of the subunit stoichiometry of the c-subunit oligomer needs to be clarified. Recent values in the literature for ATP synthases from various sources range from 10–14 (Dimitriev *et al.*, 1999; Seelert *et al.*, 2000; Stock *et al.*, 1999). Although the recent structure at 3.9 Å of a yeast F_1-subunit c complex implicates a stoichiometry of 10 c subunits/F_1 (Stock *et al.*, 1999), it should be noted that this complex was derived from the complete F_0F_1 from yeast that apparently had lost all other F_0 subunits during the crystallization process, presumably because of the detergent used. Therefore, it cannot be ruled out that one or more subunit c monomers were also removed under these conditions. Second, the position in the membrane of the proton or sodium ion binding site within c-subunit oligomers needs

to be established with certainty. In the case of the *E. coli* ATP synthase, the proton binding site (aspartic acid 61) is interpreted from available data to be located near the center of the membrane (Reviewed in Fillingame *et al.*, 2000), whereas in the *Propionigenium modestum* ATP synthase the comparable sodium ion binding site is interpreted from available data to be located near the cytoplasmic boundary of the membrane (Dimroth *et al.*, 1999). Finally, much more is needed to be learned about subunit a, as it is believed to serve at least three different roles, that is, as a stator for the c-subunit oligomer when acting as a rotor, as the point of entry for protons from the electrochemical proton gradient into this oligomer, and as the point of exit of protons from the same (Fig. 1A). Presumably, the a subunit contains two half channels, one for directing protons from one side of the membrane to the subunit c oligomer within the membrane thus facilitating its rotation, and the other for directing protons to the other side of the membrane into the medium where they react with hydroxyl ions to form water, thus providing the driving force for the rotation (Fig. 1A; reviewed in Altendorf *et al.*, 2000; Vik *et al.*, 2000). These purported multiple roles of subunit a in the overall process of proton gradient-driven ATP synthesis by ATP synthases make this subunit potentially as important or even more important than some of the other subunits.

Finally, in animal systems where it is known that the F_0 unit of ATP synthases contains, in addition to subunits a, b, and c, at least seven additional subunit types (d, e, f, g, OSCP, F_6, and A6L; reviewed in Pedersen, 1996), the question arises about the roles of these extra subunits. F_6 may play a "stator" role, that is, to stabilize the F_1 headpiece while energy coupling and ATP synthesis are taking place (Ko *et al.*, 2000), a role that has been implicated also for OSCP because of some homology to the *E.coli* δ subunit recently found at the top of *E. coli* F_1 (Wilkens *et al.*, 2000). However, there are few clues about the roles of the other five F_0 subunit types that are found in animal systems but not in bacteria. Understanding why animal ATP synthases, and yeast as well, have many more "bells and whistles" associated with their proton translocating units than their bacterial cousins will be a challenging area of research in this new century. If the primary role of the F_0 unit is to drive the rotation of the γ subunit and to stabilize the headpiece from excessive wobble while ATP synthesis is taking place, then how do the extra subunits participate in these roles?

Mechanism of Coupling Rotation of the γ Subunit to Conformational/Binding Changes in F_1 $\alpha\beta$ Pairs

This second level of ATP synthase mechanism noted in Fig. 1A is likely to be the most difficult and therefore the most challenging to unravel in future studies because, at every 120° position during its 360° rotation, the γ subunit will contact each of the three $\alpha\beta$ pairs differently. Therefore, there are three different "coupling events" that must be elucidated, one that favors substrate (ADP and P_i) and Mg^{++} binding to the catalytic site; a second that favors ATP synthesis from ADP, P_i, and Mg^{++} at this site; and a third that favors product (MgATP) release. In each case, the correct contact amino acids between the γ subunit and the three different $\alpha\beta$ conformers must be known both because these contacts determine the ligand specificity of each conformer and because the torque derived from the rotating γ subunit is likely to focus on one or more of these locations, providing the "power strokes" essential for driving ATP synthesis.

Surprisingly, attempts to clearly identify $\gamma\beta$ contact amino acids related in some way to the rotation of the γ subunit have stifled investigators. Although elucidation of the atomic resolution structure of bovine heart F_1 provided several attractive candidates (Abrahams *et al.*, 1994), those tested to date have not panned out. Specifically, predicted regions of interaction (or near interaction) between γ subunit residues 73–90 and β subunit residues 391–395 (DELSEED) in the bovine heart F_1, when subjected to mutagenesis in the corresponding region of the thermophilic *Bacillus* F_1, are without effect on either rotation of the γ subunit or torque generation (Hara *et al.*, 2000). Here, it is interesting to note that in an earlier study conducted with *E. coli* F_1, suppressor mutagenesis studies identified three helical regions of the γ subunit involved in energy coupling (18–35, 236–246, and 269–280; Nakamoto *et al.*, 1995). However, the region corresponding to bovine residues γ-73–90 was not among them. In a more recent study of the N-terminal region of the *E. coli* γ subunit, the amino acid (methionine 23) was identified as one of the most promising candidates for a role in rotation (Reviewed in Futai *et al.*, 2000). However, mutagenesis of this residue had no effect on the ATP hydrolysis dependent rotational properties of the γ subunit within *E. coli* F_1 (Omote *et al.*, 1999). Finally, in another study with thermophilic *Bacillus* F_1 related to coupling and rotation, it was shown that mutagenesis of several residues (Histidine 179, Glycine 180, and Glycine 181) in the purported "hinge" region located near the center of each β subunit has no effect on the ATP hydrolysis dependent rotational properties of the γ subunit (Masake *et al.*, 2000). Similar to the preceding observations, this was somewhat surprising as one of the implications about coupling derived from the three-dimensional structure of bovine heart F_1 (Abrahams *et al.*, 1994) is that changes in the hinge region may impart a strain on the γ subunit promoting its rotation (Oster and Wang, 2000).

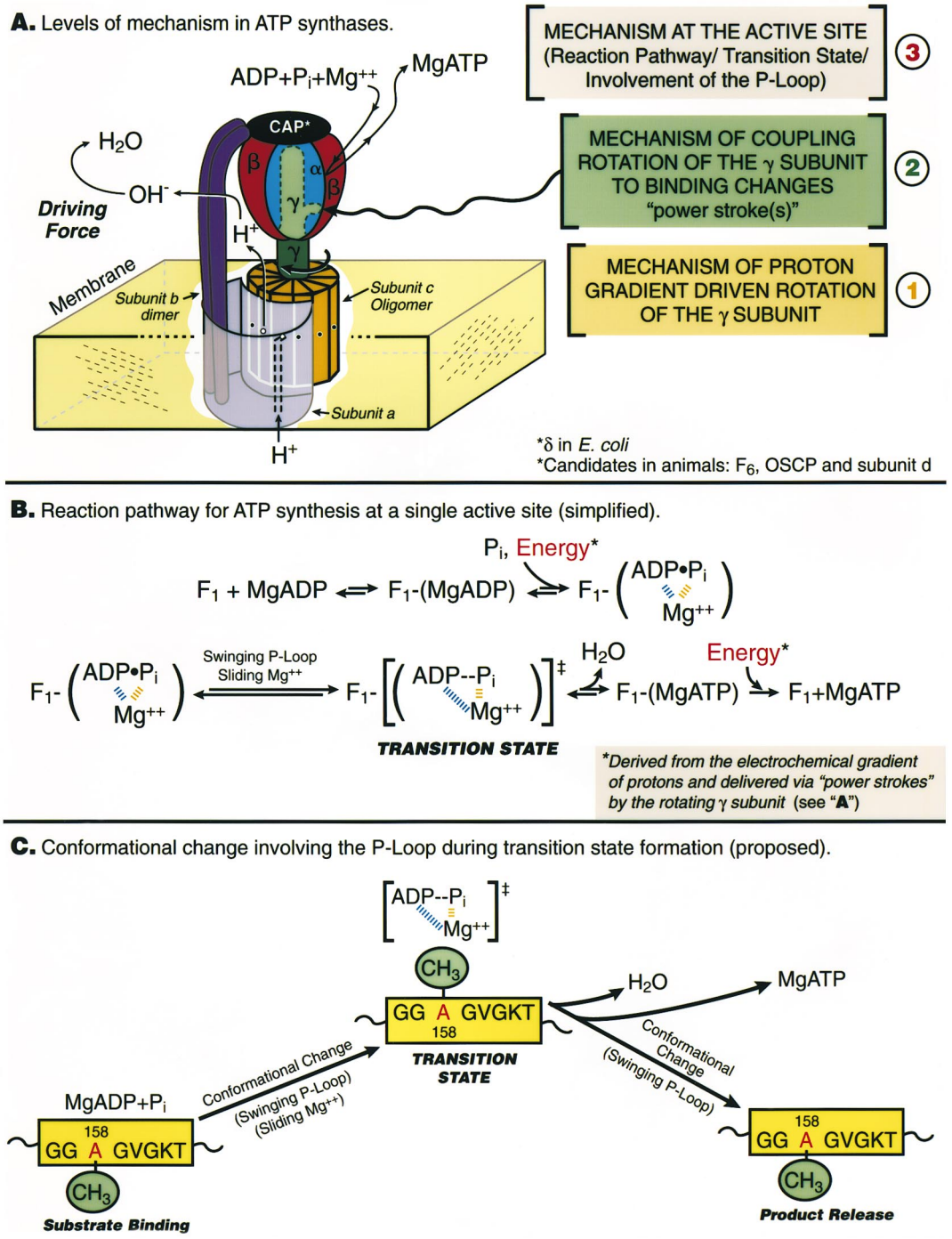

Fig. 1. (A) Diagramatic illustration of the three major levels of mechanism that must be elucidated to fully understand how ATP synthases work. The projection used for the ATP synthase drawing is patterned after that of Fillingame *et al.* (2000). The small subunits that reside at the bottom of F_1, that is, ε (*E. coli*) and $\delta + \varepsilon$ (animals) are not shown. (B) A simplified version of the reaction pathway for an ATP synthase at a single catalytic site. The reaction pathway proceeds in the ATP synthesis direction via a series of steps that are very similar to the reverse steps in the myosin ATPase catalyzed pathway. Moreover, studies using vanadate (Ko *et al.*, 1997, 1999) implicate a transition state for the ATP synthase catalyzed reaction with the expected trigonal bipyrimidal geometry observed in the X-ray derived structure of myosin (Smith and Rayment, 1996). (C) Proposed conformational change involving the P-loop during transition state formation in the ATP synthase catalyzed reaction. Studies with vanadate implicate alanine 158 within the P-loop as "swinging" into the catalytic site during transition state formation and "taking a seat" within contact distance of the P that will become the γ-P of ATP. The hydrophobic methyl group of alanine at this position is suggested to help facilitate the formation/release of water during ATP formation.

The inability of investigators to clearly identify to date either $\gamma\beta$ subunit contact sites or purported β subunit hinge region sites involved in coupling the proton gradient-driven rotation of the γ subunit to ATP synthesis at catalytic sites is somewhat disturbing. Moreover, it prevents any detailed molecular/chemical discussion of the "power strokes" expected to be involved in coupling rotation of the γ subunit to ATP synthesis at catalytic sites. A power stroke is defined here as the torque from the rotating γ subunit that upon impacting a specific $\gamma\beta$ contact region moves certain residues/regions of the β subunit through a distance x in a given amount of time, thus bringing about binding changes in the active site favorable for ATP synthesis/release. In the reaction pathway for ATP synthesis at a single catalytic site, energy derived originally from the electrochemical proton gradient is believed to promote P_i binding and ATP release (Fig. 1B). Therefore, it is predicted that at least two power strokes from the rotating γ subunit are necessary, one to act on one of the three β subunits to promote P_i binding while a second acts on another β subunit to promote ATP release.

Despite these problems, one very thoughtful, quite elegant, and very speculative "coupling" model has already been proposed (Oster and Wang, 2000). It relies heavily on information derived from (a) the three-dimensional structure of bovine heart F_1 (Abrahams *et al.*, 1994), (b) studies of the rotational properties of the γ subunit (Noji *et al.*, 1997; Omote *et al.*, 1999), (c) the recent finding that β subunits are within contact distance in the C-terminal region near isoleucine 390 (Tsunoda *et al.*, 1999), and (d) a number of assumptions, one of which relates to multisite catalytic rates. Significantly, the model predicts on thermodynamic grounds that there are two power strokes when F_1 operates in the direction of ATP hydrolysis, one related to ATP binding and the other related to phosphate release, that is, the reverse steps considered earlier for ATP synthesis. One of unique features of this model is the prediction that ATP is bound at the catalytic site on one of the F_1 β subunits via a number of hydrogen bonds ("binding zipper"), and that when "energy" from the electrochemical proton gradient is transmitted through the rotating γ subunit to this β subunit, it is done in such a way that the binding of ATP is weakened one hydrogen bond at a time until its release occurs. Thus, ATP is proposed to be gently and efficiently released from the active site to satisfy the prediction that the efficiency of converting the energy conserved in the electrochemical proton gradient into formation of ATP from ADP and P_i is close to 100% (Omote *et al.*, 1999; Yasuda *et al.*, 1998). This new view, which is believed to allow for ATP release without the dissipation of some of the conserved energy as heat, is considered by its authors to differ significantly from those views where the rotation of the γ subunit is believed to build up an elastic strain in the ATP binding subunit until a threshold is reached resulting in the instantaneous release of ATP (Reviewed in Oster and Wang, 2000). The latter process is considered very inefficient as the elastic energy stored is predicted to dissipate as heat.

As it regards future work to elucidate this second level of mechanism, that is, how γ-subunit rotation is coupled to conformational/binding changes in the three $\alpha\beta$ pairs, it is important to acquire two important pieces of information. First, the $\gamma\beta$ contact sites where the torque from the rotating γ subunit impacts on the β subunit surface need to be positively identified for each β subunit. Second, those internal residues, or secondary structural regions within the β subunit that transmit the energy derived from $\gamma\beta$ interactions to the catalytic site also need to be positively identified. Perhaps the best way to obtain this information is to acquire more X-ray structures of F_1 at high resolution and to work toward several such structures for F_0F_1 also. It seems important, however, to obtain these structures under conditions that are as physiological as possible. The structure of bovine F_1, which has been used to make most of the predictions about $\gamma\beta$ contact points, was crystallized in the presence of AMP-PNP, ADP (substoichiometric levels), $MgSO_4$, the inhibitor azide, D_2O and without phosphate (Lutter *et al.*, 1993). This apparently inhibited state of the enzyme may not be reporting the $\gamma\beta$ contacts that are most relevant to coupling during ATP synthesis, and the large conformational change that is observed in the empty β subunit (β_E) may be more a reflection of the empty state of this subunit, and/or the inhibited state of the enzyme, than the physiological state that has just released bound ATP.

Mechanism of ATP Synthesis and Release at the Active Sites

The third and final stage of mechanism noted in Fig. 1A that each individual ATP synthase molecule must deal with is the payoff stage, namely the formation of ATP from ADP and P_i in the presence of Mg^{++} and the release of the newly formed ATP. Much work by many investigators during the last two decades of the past century eventually arrived at a reaction pathway for ATP synthesis at a single catalytic site very similar or identical to that depicted in Fig. 1B. The roots of these efforts lie in the classic paper by Grubmeyer *et al.* (1982). In Fig. 1B, ADP is shown to bind to F_1 in the presence of Mg^{++} to form an F_1-(MgADP) intermediate where the MgADP is bound at the catalytic site located at an α/β interface. In a subsequent step, P_i, also in the presence of Mg^{++}, binds

to F_1 at the same catalytic site but at a different location within the site. Significantly, the binding of P_i to F_1 is generally believed to be an energy requiring step, although this view may not be shared by all investigators. The resultant F_1-(ADP/Mg^{++}/P_i) intermediate then goes through a transition state where the β phosphate oxygen of ADP bearing a negative charge is placed in line with the phosphorus atom of P_i, at which time the former makes a nucleophilic attack on the latter resulting in both the leaving of water and the formation of an F_1-(MgATP) complex. Finally, in the last step of the reaction, MgATP is released from the enzyme in a step that also requires energy. Significantly, the formation of MgATP on the enzyme is believed to require very little energy as it proceeds with an equilibrium constant near 0.5 (Grubmeyer *et al.,* 1982). Rather, it is believed that release of MgATP bound to the catalytic site may be the major energy requiring step in the reaction.

Prior to the elucidation of three-dimensional structures of F_1, a number of residues important for facilitating catalysis and therefore the reaction pathway shown in Fig. 1B had been identified by a variety of approaches (Reviewed in Futai *et al.* 2000; Weber and Senior, 1997). For the most part, the long overdue F_1 structures (Abrahams *et al.*, 1994; Bianchet *et al.*, 1998; Shirakihara *et al.*, 1997) confirmed that most of these previously identified residues are sufficiently near MgAMP-PNP (on the β_{TP} subunit of the bovine heart F_1 structure), or near ADP + P_i (on the $\beta_{ADP,Pi}$ subunits of the rat liver F_1 structure) to play a role in binding or catalysis. Among the most important of these residues in the mammalian F_1 numbering system are β-lysine 162, β-arginine 189, and α-arginine 373, which interact with the nonbridging oxygens of the γ-phosphoryl group of ATP; threonine 163, which helps position the γ-phosphoryl group while interacting with Mg^{++}; glutamic acid 188, which is believed to serve as a catalytic base; and tyrosine 345, which forms part of a hydrophobic pocket for binding the purine. Two of these amino acid residues, β-lysine 162 and β-threonine 163, lie at the end of a loop called the "P-loop," which is found in many nucleotide binding proteins and has the general consensus GX_4GKT/S. In the β-subunit of the F_1 unit of ATP synthases this P-loop sequence is GGAGVGKT. Mutational analysis in *E. coli* of P-loop residues corresponding to β-lysine 162 and β-threonine 163 together with the residue corresponding to β-arginine 189 multiply to an activity loss of 10^{14}–$10^{15.6}$ in k_{cat} attesting to the importance of these residues (Reviewed in Mildvan, 1997). Significantly, in the bovine heart F_1 structure there is just enough room between the γ-phosphoryl group of ATP and β-glutamic acid 188 to accommodate a water molecule, supporting the view, together with mutational analysis, that this residue is in fact a catalytic base. Therefore, the simplified view of the mechanism for the ATP hydrolysis reaction catalyzed by F_1 is that the β-glutamic acid 188 facilitates catalysis by removing a proton from water, the resulting hydroxyl ion of which makes an attack on the γ phosphorus atom of ATP bound to the P-loop, Mg^{++}, and other amino acids. This event facilitates the cleavage of the terminal bond of ATP and the formation of ADP and P_i. Conversely, during ATP synthesis the same catalytic base, β-glutamic acid 188, is considered to participate in the "extraction" of a water molecule from ADP and P_i, also bound at or near the P-loop, thus facilitating the formation of ATP.

From the information summarized here, it seems clear that the reaction pathway for ATP synthesis catalyzed by ATP synthases summarized in Fig. 1B is facilitated by a number of amino acid residues near or within the P-loop region of catalytic sites on F_1 β subunits. It also seems clear that the structure of bovine heart F_1 (Abrahams *et al.*, 1994) is an excellent indicator of residues important for substrate/product binding and catalysis at active sites, perhaps much better than as an indicator of those residues important for coupling γ subunit rotation to catalysis. The rat liver structure (Bianchet *et al.*, 1998) also appears to be an excellent indicator of those residues believed to be catalytically important.

Of the various steps in the reaction pathway summarized in Fig. 1B, the two that are currently receiving the most attention are the transition state step and the step involving the release of bound ATP. These two very important steps in the reaction pathway are considered separately as there appear to be some significant differences among the views of various investigators.

Transition State

Detailed knowledge of the transition state in an enzyme catalyzed reaction is important for several reasons. First, it is at the transition state that the reacting species are optimally aligned for interaction. Second, it is in this state that the bond-making and bond-breaking steps occur. Third, because of the former two reasons, it is predicted that something has changed in the enzyme to bring it from its substrate bound "ground" state(s) into the transition state, that is, a conformational change is predicted to occur. The transition state is also important for the purpose of designing potent inhibitors of catalysis, known as transition state analogs. Such analogs can have therapeutic action in a number of diseases. For example, a transition state analog of an ATP synthase molecule, if selectively delivered to the mitochondria of a cancer or parasitic cell, may be able to destroy the cell.

For enzymatic reactions involving phosphate bond cleavage or formation, it is frequently desirable to obtain knowledge about the transition state for any one or all of the reasons just stated. To do this, one needs to have a chemical compound that looks structurally like what one would predict the transition state to look like. A favorite compound of choice with an excellent track record for enzymes involving phosphate is vanadate (Cremo *et al.*, 1989, 1990; Grammer *et al.*, 1990; Gresser and Tracy, 1990; Holloway and Melnick, 1986; Linquist *et al.*, 1973; Smith and Rayment, 1996; Westheimer, 1987; Wlodawer *et al.*, 1983). To understand this, it is instructive to consider the geometry of phosphate within an ATP molecule before its interaction with water, and during its interaction with water in the transition state of an ATPase catalyzed reaction. Within ATP, the terminal phosphate (γ-P) has a tetrahedral structure but in the transition state, when the γ-P has aligned with and interacted with water, the geometry of the resultant adduct is predicted to be trigonal bipyramidal in an SN_2 like reaction. Vanadate can assume a trigonal bipyramidal structure and is able to substitute for the terminal phosphate (γ-P) of ATP in the transition state and inhibit the ATPase reaction. Another desireable feature of vanadate is that it is photoreactive (Cremo *et al.*, 1989). Therefore, when one uses vanadate to trap an ATP dependent reaction in the transition state it is possible to introduce ultraviolet light, which via a series of complex chemical events, results in the cleavage of the peptide at that position in the transition state where it contacts the γ-P of ATP (Cremo *et al.*, 1989; Grammer *et al.*, 1990). Therefore, using this strategy one can identify the amino acid within an ATPase that is nearest to the γ-P of ATP in the transition state.

An excellent example of the use of vanadate to gain insight into an ATPase reaction is in the case of myosin, which exhibits a very similar reverse reaction pathway (Bagshaw and Trentham, 1973) to that outlined in Fig. 1B for ATP synthases. Biochemical studies show that vanadate in the presence of MgADP inhibits the ATPase reaction catalyzed by myosin and, upon the introduction of ultraviolet light, peptide bond cleavage takes place at the third position within the P-loop GE**S**GAGKT (Cremo *et al.*, 1989, 1990), indicating that this serine is very near the γ-P of ATP in the transition state. This was confirmed by solving, via X-ray crystallography at 1.9 Å, the structure of the myosin head trapped as a MgADP-vanadate complex (Smith and Rayment, 1996). Compelling evidence that the most probable transition state was formed was the finding in the structure of the predicted trigonal bipyramidal geometry of vanadate.

Using the studies conducted on the myosin headpiece as a guide, we applied the vanadate trapping approach to the F_1 headpiece of the ATP synthase of rat liver (Ko *et al.*, 1997). Here, we found that, as in the case of myosin, MgADP + vanadate markedly inhibits the ATPase activity of F_1, and that ultraviolet light induces cleavage at the third position of the P loop at the catalytic site. In F_1, the β subunit P loop sequence is GG**A**GVGKT with the third position corresponding to an alanine (residue 158) that is conserved in the β subunits of all known ATP synthases. These results, taken together with those carried out on myosin (Cremo *et al.*, 1989, 1990; Smith and Rayment, 1996), strongly imply that, in the presence of MgADP and vanadate, F_1 is trapped in an SN_2 reaction-like transition state with the predicted trigonal bipyramidal geometry, and that in this state the γ-P of ATP is near alanine 158 of the P-loop. The latter is an intriguing finding as in the F_1 β subunit structures of bovine heart (β_{TP}) and rat liver ($\beta_{ADP,Pi}$) (Abrahams *et al.*, 1994; Bianchet *et al.*, 2000), which are closely representative, respectively, of product and substrate "ground state" forms (Fig. 1B), alanine 158 in the P-loop is at nonbonding distances from the γ-P of ATP (4.78 Å) and from P_i /ADP (>7 Å). Therefore, during the reaction pathway shown in Fig. 1B, the P-loop is predicted to undergo significant movement as the reaction proceeds from substrate or product bound ground states to the transition state. We have suggested that this movement of alanine 158 away from its ground state position to a position near the γ-P of ATP may provide a transient hydrophobic environment for facilitating the removal of water from ADP and P_i during ATP synthesis.

In a follow-up study (Ko *et al.*, 1999), we have shown further that formation of the transition state just described for the ATP synthase reaction (Fig. 1B) can occur in the presence of only Mg^{++} and V_i, that is, without the need for added ADP. V_i in the trapped transition state under these conditions corresponds to the "P" that subsequently becomes the γ-P of the reaction product MgATP, where Mg^{++} is coordinated to both the β and γ Ps of ATP. Therefore, these findings suggest that when the transition state is formed in the ATP synthase reaction there is a shift or "sliding" of the Mg^{++} coordination. Taken together with the results of the studies described earlier, we visualize that in the transition state of the ATP synthase catalyzed reaction, Mg^{++} preferentially coordinates with the P that will become the γ-P of ATP, perhaps to facilitate its interaction with the catalytic base (glutamic acid 188), while that part of the P-loop containing alanine 158 swings into the catalytic site increasing its hydrophobicity, thus promoting efficient removal of formed water (Fig. 2). Significantly, these movements that occur during transition state formation in the ATP synthase reaction are likely to be promoted and/or facilitated in part by the rotating γ subunit

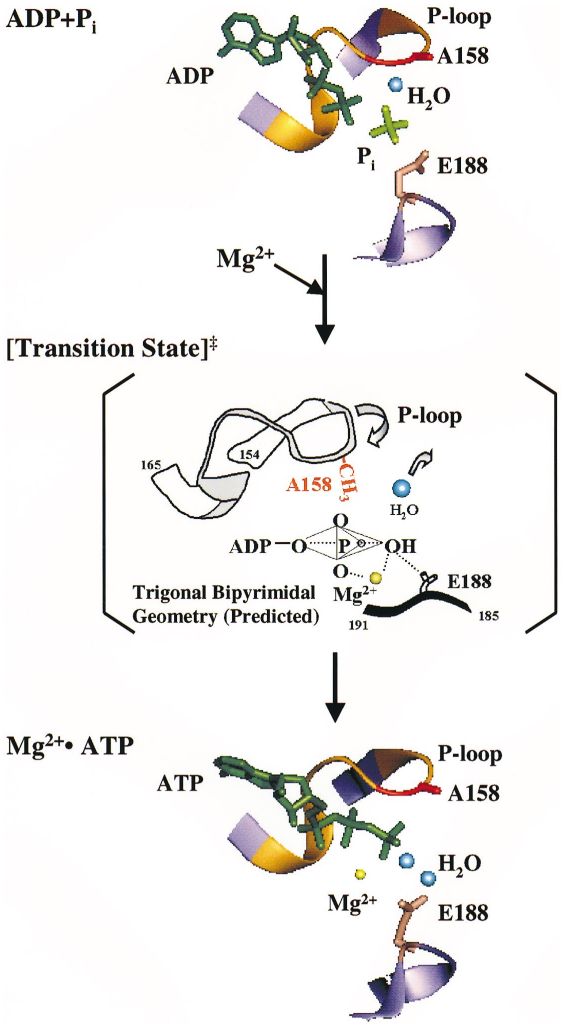

Fig. 2. Authors' view of transition state formation in the ATP synthase catalyzed reaction. The illustration presented is based on (1) the X-ray structure of rat liver F_1 obtained in the absence of Mg^{++} (top) in which two of the β subunits contain ADP and P_i (Bianchet *et al.*, 1998); (2) studies with vanadate which, in the presence of MgADP or Mg^{++} alone, induce a remodeling of the active site in the proposed transition state such that alanine 158 within the P-loop moves within contact distance of what will become the γ-P of ATP; and (3) the X-ray derived structure of the β_{TP} subunit of bovine heart F_1, which contains MgAMP-PNP, a MgATP analog (Abrahams *et al.*, 1994). The order in which Mg^{++} enters the reaction is not known. (The figure has been reproduced from Ko *et al.*, 1999, with permission.)

as we suggested earlier (Ko *et al.*, 1997). Therefore, it seems likely that there is a relationship between coupling and transition state formation in ATP synthase catalyzed reactions. Finally, what has been stated here about the properties of the transition state in the reaction pathway catalyzed by rat liver F_1, may also apply to plant F_1 from chloroplasts, where evidence for transition state formation has been found also in the presence of vanadate (Hochman *et al.*, 1993).

In concluding this subject, it should be noted that several investigators have obtained data on complexes of F_1 inhibited by MgADP-fluoroaluminate, one of which includes an X-ray derived structure at 2.5 Å involving bovine heart F_1 (Braig *et al.*, 2000). The authors of this work suggest that the bovine heart F_1-MgADP-fluoroaluminate complex formed is representative of a possible transition state. However, in the structure there is no evidence for the trigonal bypyrimidal geometry expected for an ATPase catalyzed reaction (see preceding discussion) and found in the X-ray derived structure of the MgADP-V_i treated myosin ATPase head at 1.9 Å (Smith and Rayment, 1996) and other enzymes (Lindvist *et al.*, 1994; Wlodawer *et al.*, 1983). In myosin, aluminum fluoride does form a complex with the corresponding nucleotide diphosphate but the coordination of the aluminum is square bipyramidal (Fisher *et al.*, 1995), not trigonal bypyrimidal as expected for the most probable transition state. Interestingly, a second team of investigators working with a MgADP-fluoroaluminate complex of *E. coli* F_1 also refer to this complex as representative of a transition state (Reviewed in Senior *et al.*, 2000), while a third team working with MgADP-fluoroaluminate complexes of the bovine heart F_1 and the thermophilic *Bacillus* F_1 believe these complexes to be more representative of the ground state than the transition state (Allison *et al.*, 2000). Despite the vigorous debates that are likely to ensue over this matter in the future, a wealth of useful information about the ATP synthase reaction mechanism is likely to result when the dust has settled.

Release of Bound ATP

The last step in the reaction pathway for an ATP synthase catalyzed reaction at a single catalytic site on F_1 is the release of bound ATP (Fig. 1B). Considerable interest has developed about this step as it is believed from studies of both isolated and membrane bound F_1 that ATP is synthesized from ADP and P_i at a single catalytic site with an equilibrium constant near 0.5, and that the resulting ATP is bound with a very high affinity (Cross *et al.*, 1982; Grubmeyer *et al.*, 1982; Souid and Penefsky, 1995). Consequently, it is predicted that a considerable energy input will be necessary to release the tightly bound ATP. In addition, it is generally assumed that this event will be accompanied by a large conformational change. A major unresolved issue here is whether or not this final step does in fact proceed under physiological conditions with

a large conformational change. As indicated earlier in this review, the X-ray derived structure of bovine heart F_1 has been considered to be consistent with the predictions of the binding change mechanism for ATP synthesis as one of the three β subunit remains empty and exhibits a conformation markedly different from the other two that have bound nucleotide (Abrahams *et al.*, 1994). Although other interpretations are possible, the empty β subunit is commonly assumed to be the result of a rather dramatic conformational change accompanying the release of tightly bound ATP.

The elucidation of a second X-ray derived structure of F_1 (rat liver; Bianchet *et al.*, 1998), crystallized in the presence of ATP, ADP, and P_i, and showing all β subunits occupied with nucleotide (or nucleotide + P_i) and in similar conformations, has added a new dimension to the issue of whether empty β subunits that have undergone large conformational changes exist under physiological conditions. In addition, recent biochemical data obtained on *E. coli* F_1 has underscored the importance of full occupancy of the three catalytic sites for maximal activity Lobau *et al.*, 1997. Therefore, the possibility remains open that during oxidative phosphorylation *in vivo*, an empty β subunit is the result of a very short-lived event, and when this event occurs it does so without the need for a dramatic conformational change as currently assumed. Nevertheless, it would seem that it is perhaps most prudent at the present time to assume, as we have done (Bianchet *et al.*, 1998, 2000), that both X-ray derived structures of F_1, that is, the rat liver and the bovine heart, may represent physiologically relevant configurations on the main pathway for ATP synthesis. However, future efforts should be made to capture additional snapshots, via X-ray crystallography, of both enzymes under more physiological conditions. This is an essential endeavor as the bovine enzyme was crystallized under conditions that can be interpreted as inhibitory and the rat liver enzyme was crystallized in the absence of Mg^{++}, a cation required for ATP synthesis.

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