Does the γ Subunit Move to an Abortive Position for ATP Hydrolysis when the $F_1 \cdot ADP \cdot Mg$ Complex Isomerizes to the Inactive $F_1^* \cdot ADP \cdot Mg$ Complex?

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F_1-ATPases transiently entrap inhibitory MgADP in a catalytic site during turnover when noncatalytic sites are not saturated with ATP. An initial burst of ATP hydrolysis rapidly decelerates to a slow intermediate rate that gradually accelerates to a final steady-state rate. Transition from the intermediate to the final rate is caused by slow binding of ATP to noncatalytic sites which promotes dissociation of inhibitory MgADP from the affected catalytic site. Evidence from several laboratories suggests that the γ subunit rotates with respect to α/β subunit pairs of F_1-ATPases during ATP hydrolysis. The $\alpha_3\beta_3$ and $\alpha_3\beta_3\delta$ subcomplexes of the TF_1-ATPase do not entrap inhibitory MgADP in a catalytic site during turnover, suggesting involvement of the γ subunit in the entrapment process. From these observations, it is proposed that the γ subunit moves into an abortive position for ATP hydrolysis when inhibitory MgADP is entrapped in a catalytic site during ATP hydrolysis.

INTRODUCTION

The F_0F_1-ATP synthases of energy-transducing membranes couple transmembrane proton electrochemical gradients to ATP synthesis. The F_0 sector is an integral membrane protein complex that mediates proton translocation. F_1, which is attached to F_0 by a narrow stalk, is a peripheral membrane protein complex which contains the catalytic sites. When removed from the membrane as a soluble complex containing five gene products in the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$, F_1 is an ATPase. In the crystal structure of F_1 from bovine heart mitochondria $(MF_1)^3$ determined by Abrahams *et al.* (1994), the elongated α and β subunits are arranged alternately to form a roughly spherical $\alpha_3\beta_3$ hexamer in which an antiparallel coiled-coil contributed by the amino- and carboxyl-termini of the γ subunit is present as a core.

Kinetic analyses of the F_1-ATPases have demonstrated that they hydrolyze ATP with positive cooperativity of catalysis and negative cooperativity of substrate binding. The MF_1 and *E. coli* F_1-ATPases bind substoichiometric ATP to a single catalytic site with K_d's of 10^{-12} M and 10^{-9} M, respectively. However, ATP bound to the single catalytic site is hydrolyzed extremely slowly with a k_{cat} of about 10^{-3}

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[3] Abbreviations: MF_1, TF_1, and CF_1, the F_1-ATPases from bovine heart mitochondria, the plasma membrane of the thermophilic Bacillus PS3, and spinach chloroplasts, respectively; $ndMF_1$, MF_1 depleted of endogenous nucleotides; and LDAO, lauryl dimethylamine oxide.

s^{-1} (Penefsky and Cross, 1991; Senior, 1990). Eadie–Hofstee plots constructed from the initial rates of hydrolysis of 0.2–4000 μM ATP by MF_1, TF_1, and the $\alpha_3\beta(T_{165}S)_3\gamma$ subcomplex of TF_1 are biphasic (Gresser *et al.*, 1982; Wong *et al.*, 1984; Allison *et al.*, 1995). In the case of the $\alpha_3\beta(T_{165}S)_3\gamma$ subcomplex, K_{m1} is about 1 μM and has an associated $k_{cat\ 1}$ of about 14 s^{-1} (Allison *et al.*, 1995). For MF_1, K_{m1} is also about 1 μM and $k_{cat\ 1}$ is about 20 s^{-1} (Gresser *et al.*, 1982; Wong *et al.*, 1984). K_{m2} is about 130 μM for each of these enzymes. In the case of MF_1, $k_{cat\ 2}$ is about 750 s^{-1} whereas $k_{cat\ 2}$ of the $\alpha_3\beta$ $(T_{165}S)_3\gamma$ subcomplex of TF_1 is about 350 s^{-1}. From the kinetic analyses, it can be concluded that a rate acceleration of $10–20 \times 10^3$ accompanies the transition from unisite to bisite catalysis and a rate acceleration of about 30–40 accompanies the transition from bisite to trisite catalysis. Therefore, the F_1-ATPases do not attain maximal velocity until three catalytic sites are saturated with ATP. Weber *et al.* (1993) have reached the same conclusion by correlating binding of MgATP to the $\beta Y_{331}W$ mutant of *E. coli* F_1 with ATPase activity. They determined binding of MgATP to the mutant enzyme by monitoring quenching of intrinsic tryptophan fluorescence. According to the crystal structure of MF_1 (Abrahams *et al.*, 1994), the incorporated tryptophan residue interacts with the adenine moiety of adenine nucleotides bound to catalytic sites.

The binding change mechanism developed by Boyer (1993) to explain the unusual cooperative behavior of F_1-ATPases is the working hypothesis for most current investigations designed to establish the molecular mechanism of the F_1-ATPase and the F_0F_1-ATP synthase. A basic tenet of the binding change mechanism is sequential participation of catalytic sites which is supported by substantial experimental evidence (Boyer, 1993). To accommodate the $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit stoichiometry of MF_1 with sequential firing of catalytic sites, Gresser *et al.* (1982) proposed that during ATP hydrolysis, the $\alpha_3\beta_3$ hexamer rotates with respect to a core made up of the three minor subunits. The asymmetric arrangement of the α and β subunits around the coiled-coil comprised of the N- and C-termini of the γ subunit in the deduced crystal structure of MF_1 (Abrahams *et al.*, 1994) is consistent with this argument. The elegant experiment recently reported by Duncan *et al.* (1995), in which it was demonstrated that a specific site on the γ subunit switches from one β subunit to another, apparently in a sequential pathway during ATP hydrolysis catalyzed by the *E. coli* F_1-ATPase, provides strong evidence in favor of

rotational catalysis. Differences in cross-linking of β subunits to the γ or ε subunits of *E. coli* F_1, which depend on the state of ligation of the catalytic sites reported by Aggeler *et al.* (1995), also support rotational catalysis. Therefore, any mechanism proposed for ATP synthesis or hydrolysis should take into account sequential participation of catalytic sites and positional interchange of the γ subunit with respect to the α/β subunit pairs which comprise each of the three catalytic sites.

Comparison of the crystal structure of MF_1 (Abrahams *et al.*, 1994) with that of the $\alpha_3\beta_3$ subcomplex of TF_1 (Y. Shirakihara, personal communication) suggests that closing of catalytic sites from an open conformation accompanies binding of MgATP to catalytic sites. In the crystal structure of MF_1, the noncatalytic nucleotide binding sites in α subunits are homogeneously liganded with Mg·AMP-PNP, whereas the catalytic sites are heterogeneously liganded. One catalytic site, designated β_{TP}, contains Mg·AMP-PNP, another, designated β_{DP}, contains Mg·ADP, and the third, designated β_E, is empty (Abrahams *et al.*, 1994). In MF_1, the three liganded noncatalytic sites and the two liganded catalytic sites are in closed, nearly superimposable conformations, whereas β_E is in an open conformation. In contrast, in the crystal structure of the $\alpha_3\beta_3$ subcomplex of TF_1, which is free of bound nucleotides, the three β subunits are in open conformations and the α subunits are in closed conformations (Y. Shirakihara, personal communication). The open conformation of the β subunits in the crystal structure of the $\alpha_3\beta_3$ subcomplex of TF_1 is nearly superimposable with that of β_E in the crystal structure of MF_1, whereas the closed conformation of the unliganded α subunits in the $\alpha_3\beta_3$ subcomplex of TF_1 is nearly superimposable with the closed conformations of the liganded α and liganded β subunits of MF_1.

ISOMERIZATION OF THE F_1·ADP·Mg COMPLEX TO THE F_1^*·ADP·Mg COMPLEX ACCOMPANIES ENTRAPMENT OF INHIBITORY MgADP IN A CATALYTIC SITE OF F_1

MF_1 depleted of nucleotides (ndMF_1), and TF_1 and the $\alpha_3\beta_3\gamma$ subcomplex of TF_1, which do not contain endogenous nucleotides, hydrolyze low concentrations of ATP in three kinetic phases. A burst rapidly decelerates to an intermediate phase, which, in turn, accelerates to a final rate approaching the initial rate

$$F_1.ADP.Mg \overset{\text{slow}}{\underset{}{\rightleftharpoons}} F_1{*}.ADP.Mg \overset{N_3^-}{\underset{}{\rightleftharpoons}} F_1{*}.ADP.Mg.N_3^-$$

Scheme I.

(Jault and Allison, 1993, 1994; Paik *et al.*, 1994; Jault *et al.*, 1995). Transition from the burst to the intermediate phase is caused by turnover-dependent entrapment of MgADP in a catalytic site. Slow binding of ATP to noncatalytic sites which promotes dissociation of inhibitory MgADP from the affected catalytic site is responsible for the transition of the intermediate rate to the final rate.

Inhibitory MgADP can also be loaded onto a catalytic site of F_1-ATPases by incubating them with a slight excess of ADP in the presence of Mg^{2+} prior to assay (Vasilyeva *et al.*, 1980; Drobinskaya *et al.*, 1985). After preloading a catalytic site of MF_1 and TF_1 with MgADP, long lags are introduced before final steady-state rates of ATP hydrolysis are attained. The activation observed during these assays is accelerated as the ATP concentration in the medium is increased in the 50–2000 μM range. This indicates that binding of ATP to noncatalytic sites promotes release of the inhibitory MgADP from the affected catalytic site (Jault *et al.*, 1995, 1996). Milgrom and Boyer (1990) and Chemyak and Cross (1992) have shown that prior treatment of $ndMF_1$ or MF_1, respectively, with stoichiometric 2-N_3-ADP plus Mg^{2+} followed by irradiation labels a catalytic site. Therefore, it is clear that the inhibitory MgADP is bound to a catalytic site.

Since turnover-dependent entrapment of MgADP in a catalytic site occurs during ATP hydrolysis in the presence of a regenerating system containing excess pyruvate kinase and phospho*enol*pyruvate, the ADP in the inhibitory $F_1 \cdot ADP \cdot Mg$ complex is derived from ATP hydrolysis at the affected catalytic site. In contrast, the observation that the extent of turnover-dependent entrapment of inhibitory MgADP in a catalytic site of MF_1, CF_1, or the $\alpha_3\beta_3\gamma$ subcomplex of TF_1 increases markedly with increasing concentration of Mg^{2+} in the assay medium, suggests that the Mg^{2+} in the inhibitory complex is derived from the medium (Guerrero *et al.*, 1990; Murataliev, 1992; Jault *et al.*, 1996).

To explain transient entrapment of inhibitory MgADP in a single catalytic site during ATP hydrolysis catalyzed by MF_1, it has been postulated that an active (Vasilyeva *et al.*, 1982) or a readily activatable (Milgrom and Murataliev, 1989) $F_1 \cdot Mg \cdot ADP$ complex is in equilibrium with an inactive $F_1{*} \cdot Mg \cdot ADP$ complex.

It has also been postulated that turnover-dependent inhibition of the F_1-ATPases by azide is caused by stabilization of the $F_1{*} \cdot ADP \cdot Mg$ complex (Vasileyeva *et al.*, 1982; Hyndman *et al.*, 1994). The equilibria between the three postulated complexes are illustrated in Scheme I.

EVIDENCE SUGGESTING THAT THE γ SUBUNIT IS LINKED TO THE $F_1 \cdot ADP \cdot Mg$ TO $F_1{*} \cdot ADP \cdot Mg$ ISOMERIZATION

Unlike the $\alpha_3\beta_3\gamma$ subcomplex and F_1-ATPases in general, the $\alpha_3\beta_3$ and $\alpha_3\beta_3\delta$ subcomplexes of TF_1 are not inhibited by azide (Miwa and Yoshida, 1989; Yokoyama *et al.*, 1989). In contrast to the $\alpha_3\beta_3\gamma$ subcomplex and TF_1, the $\alpha_3\beta_3$ subcomplex is not activated by LDAO. It has been shown that LDAO activates TF_1 and the $\alpha_3\beta_3\gamma$ subcomplex by accelerating dissociation of inhibitory MgADP from a catalytic site which is promoted by the binding of ATP to noncatalytic sites (Paik *et al.*, 1994; Jault *et al.*, 1995, 1996). To explain these observations, we propose that the slow isomerization of the $F_1 \cdot ADP \cdot Mg$ complex to the inactive $F_1{*} \cdot ADP \cdot Mg$ complex reflects movement of the γ subunit to an abortive position for ATP hydrolysis. Binding of ATP to noncatalytic sites slowly reverses the isomerization by promoting movement of the γ subunit from the abortive position into a position for productive ATP hydrolysis. In the case of TF_1 and the $\alpha_3\beta_3\gamma$ subcomplex, LDAO appears to facilitate repositioning of the γ subunit into a conformation for productive ATP hydrolysis. LDAO, by itself, does not promote dissociation of inhibitory MgADP loaded onto a single catalytic site (Jault *et al.*, 1995, 1996). Therefore, it appears to accelerate repositioning of the γ subunit which is induced by saturating noncatalytic sites with ATP. In other words, it facilitates cooperativity between noncatalytic sites liganded with ATP and the catalytic site liganded with inhibitory MgADP.

A MODEL FOR A PRODUCTIVE TURNOVER OF ATP HYDROLYSIS WHEN NONCATALYTIC SITES ARE NOT SATURATED WITH ATP

Figure 1 represents a hypothetical pathway for a single, productive turnover of ATP hydrolysis when
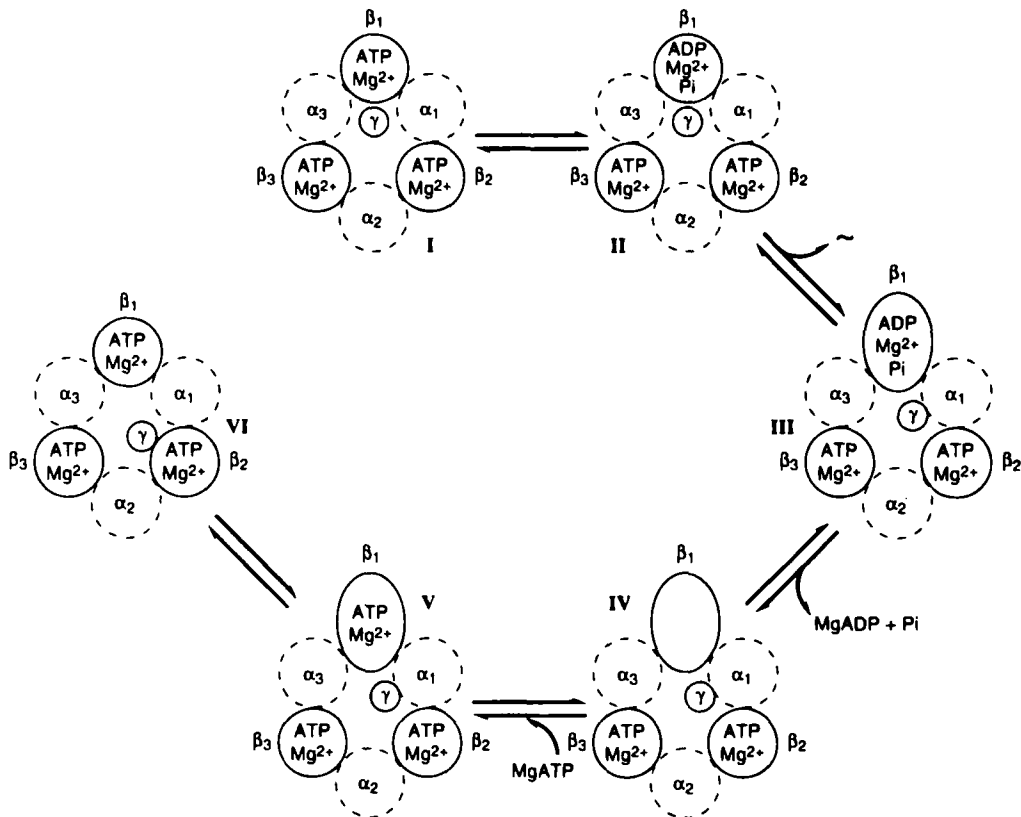
Fig. 1. A model for a productive turnover of the F_1-ATPase when catalytic sites are saturated with ATP and noncatalytic sites are not saturated with ATP. Details of the model are described in the text.

three catalytic sites are saturated and noncatalytic sites are not saturated with ATP. Under these conditions, inhibitory MgADP is transiently entrapped in a catalytic site during turnover (Jault and Allison, 1993, 1994). Consistent with the x-ray crystal structure of the $\alpha_3\beta_3$ subcomplex of TF_1 (Y. Shirakihara, personal communication), the empty α subunits (stippled circles) are shown in closed conformations. State I represents an instant when three catalytic sites are saturated with substrate. Consistent with the crystal structure of MF_1 (Abrahams et al., 1994), the three liganded catalytic sites are shown in closed conformations. In the model, β_1 represents the first catalytic site filled and corresponds to the high-affinity catalytic site ($k_d < 10^{-9}$ M) described by Penefsky and Cross (1991), β_2 represents the second catalytic site filled characterized by a K_m of about 1 μM observed on Eadie–Hofstee plots under conditions for bisite catalysis, and β_3 represents the third catalytic site

filled characterized by a K_m of about 130 μM observed on Eadie–Hofstee plots under conditions for trisite catalysis (Allison et al., 1995; Jault et al., 1996).

During the productive turnover illustrated in Fig. 1, the State II to State III transition is the energy-yielding step in which the hydrolytic products formed in the State I to State II transition drive the simultaneous opening of β_1 and movement of the γ subunit from β_1 to α_1. MgADP and Pi then leave the open catalytic site of β_1 in the State III to State IV transition. In the State IV to State V transition, MgATP binds to the empty, open catalytic site of β_1 which, in turn, closes to form State VI. The closing of β_1 in the State V to State VI transition is accompanied by movement of the γ subunit from α_1 to β_2. State VI is equivalent to State I and is poised for another round of ATP hydrolysis in which β_2 contains the hydrolytic site.

A MODEL FOR TURNOVER-DEPENDENT ISOMERIZATION OF F$_1$ INTO THE INACTIVE F$_1$*·ADP·Mg COMPLEX WHEN NONCATALYTIC SITES ARE NOT SATURATED WITH ATP

The model depicted in Fig. 2 represents a series of steps leading to the formation of the inactive F$_1$*·ADP·Mg complex (State VIa) during ATP hydrolysis when noncatalytic sites are not saturated with ATP. The abortive steps begin with the State III to State IVa transition. It is assumed that in this transition, MgPi dissociates at low frequency from the open catalytic site formed on movement of the γ subunit from β$_1$ to α$_1$ in the State II to State III transition. Binding of Mg^{2+} from the medium to ADP bound to the catalytic site of β$_1$ present in an open conformation depicted in State IVa leads to State Va which is equivalent to the F$_1$·ADP·Mg complex proposed by Vasilyeva et al. (1982) and Milgrom and Murataliev (1989). The isom-

erization of the F$_1$·ADP·Mg complex to the F$_1$*·ADP·Mg complex is proposed to occur with closing of the catalytic site of β$_1$ which is accompanied by counterclockwise movement of the γ subunit from α$_1$ to β$_1$ in the State Va to State VIa transition. The stabilization of the F$_1$*·ADP·Mg complex by binding azide is illustrated by the State VIa to State VIIa transition. It is probable that β$_2$ and/or β$_3$ empty in the abortive pathway leading to State VIa. For the sake of simplicity, these possibilities are not included in the model.

This model is consistent with the observation reported by Minkov et al. (1980) that inhibition of the oligomycin-sensitive ATPase of submitochondrial particles by exposure to ADP in the presence of Mg^{2+} is not accompanied by loss of capacity for oxidative phosphorylation. Assuming that the γ subunit rotates in a clockwise direction during ATP hydrolysis as illustrated in Fig. 1, it would then rotate in a counterclockwise direction during ATP synthesis. Therefore, it is
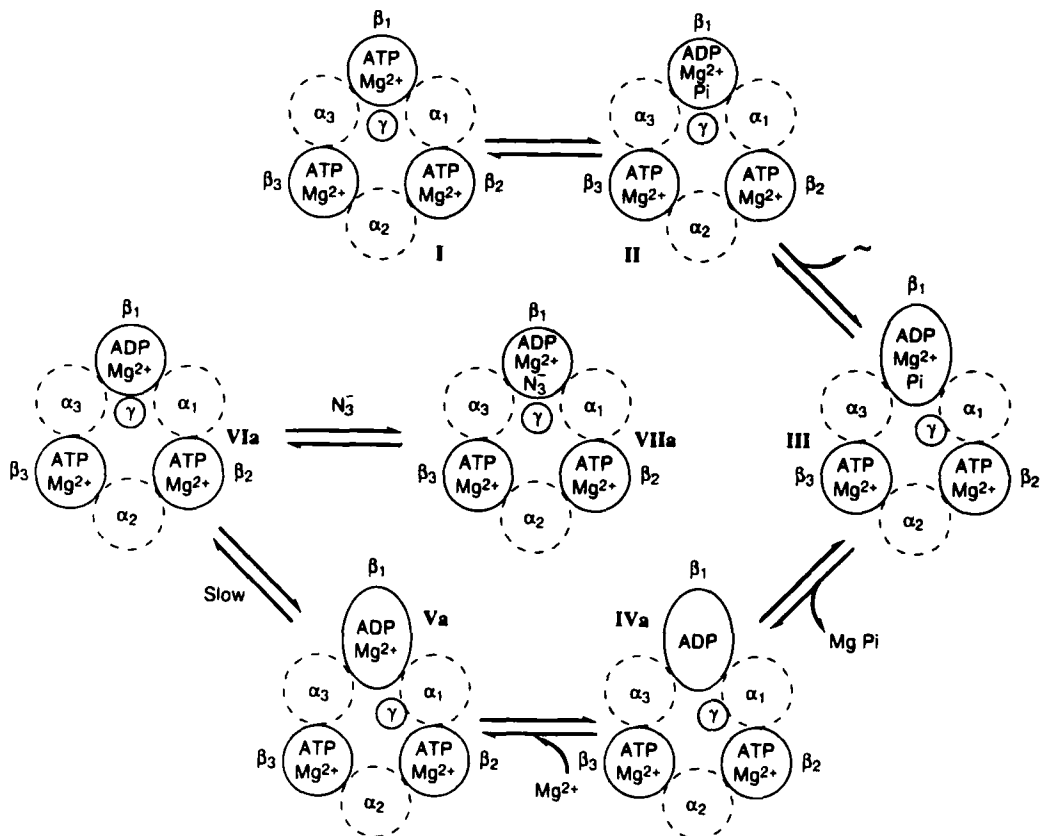

Fig. 2. A model for an abortive turnover of the F$_1$-ATPase when catalytic sites are saturated with ATP and noncatalytic sites are not saturated with ATP. Details of the model are described in the text.

logical to assume that the catalytic site on β_1 depicted in State VIa of Fig. 2 is poised for a round of ATP synthesis once Pi is bound to it. This argument is supported by the demonstration of Feldman and Sigman (1982) that latent CF_1, which contains inhibitory MgADP entrapped in a catalytic site, synthesizes enzyme-bound ATP when it binds $H_2PO_4^-$.

More recently, Syroeshkin *et al.* (1995) reported that ATP-consuming reactions catalyzed by submitochondrial particles are strongly inhibited by azide, whereas catalysis of ATP synthesis is not inhibited. These observations may also be consistent with the model shown in Fig. 2 if the catalytic site on β_1 containing the bound $ADP \cdot Mg \cdot N_3^-$ complex depicted in State VIIa is also poised for a round of ATP synthesis. Although Kasahara and Penefsky (1978) reported that MF_1 contains a high-affinity binding site for Pi that is sensitive to azide, they also reported that it contains a low-affinity binding site for Pi which is insensitive to azide. The relationship of the high- and low-affinity binding sites on soluble MF_1 for Pi to the binding site for Pi in the F_0F_1-ATP synthase that participates in oxidative phosphorylation has not been established. Therefore, the model presented in Fig. 2 may also provide an explanation for the anomalous observation reported by Syroeshkin *et al.* (1995) that azide strongly inhibits ATP-consuming reactions, whereas it does not affect oxidative phosphorylation catalyzed by submitochondrial particles.

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