The α3β3γ Subcomplex of the F1-ATPase from the Thermophilic Bacillus PS3 with the βT165S Substitution Does Not Entrap Inhibitory MgADP in a Catalytic Site during Turnover*

(Received for publication, June 24, 1996, and in revised form, August 5, 1996)

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The hydrolytic properties of the mutant αβ(βT165S)γ and wild-type αβγ subcomplexes of TF1 have been compared. Whereas the wild-type complex hydrolyzes 50 μM ATP in three kinetic phases, the mutant complex hydrolyzes 50 μM ATP with a linear rate. After incubation with a slight excess of ADP in the presence of Mg2+, the wild-type complex hydrolyzes 2 mM ATP with a long lag. In contrast, prior incubation of the mutant complex under these conditions does not affect the kinetics of ATP hydrolysis. The ATPase activity of the wild-type complex is stimulated 4-fold by 0.1% lauryl dimethylamine oxide, whereas this concentration of lauryl dimethylamine oxide inhibits the mutant complex by 25%. Compared with the wild-type complex, the activity of the mutant complex is much less sensitive to turnover-dependent inhibition by azide. This suggestion suggests that the mutant complex does not entrap substantial inhibitory MgADP in a catalytic site during turnover, which is supported by the following observations. ATP hydrolysis catalyzed by the wild-type complex is progressively inhibited by increasing concentrations of Mg2+ in the assay medium, whereas the mutant complex is insensitive to increasing concentrations of Mg2+. A Lineweaver-Burk plot constructed from rates of hydrolysis of 20–2000 μM ATP by the wild-type complex is biphasic, exhibiting apparent Km values of 30 μM and 470 μM with corresponding kcat values of 26 and 77 s⁻¹. In contrast, a Lineweaver-Burk plot for the mutant complex is linear in this range of ATP concentration, displaying a Km of 133 μM and a kcat of 360 s⁻¹.

The abbreviations used are: TF1, MF1, CF1, and YF1; the F1-ATPases contain six nucleotide binding sites. Three of these are potentially catalytic, whereas the others, for want of a defined function, are called noncatalytic nucleotide binding sites (10, 11). The 2.8 Å resolution crystal structure of MF1 shows that catalytic sites are predominantly β subunits but also contain side chains arising from α subunits. Conversely, the noncatalytic sites are mostly in α subunits, with side chains from the β subunit contributing to them (12). The nucleotide binding domains of the α and β subunits have a common topology and contain the consensus sequence GXGK(T/S), known as the Walker A motif (13) or the P-loop (14). The P-loop is found in the nucleotide binding sites of many proteins including Ras p21, elongation factor-Tu, and the RecA protein (15–18). The critical role of the P-loop in catalytic sites has been revealed by site-directed mutagenesis of the β subunits of the E. coli and YF1-ATPases (19–21). The sequence of the P-loop in the β subunit of TF1 is 18GGAGGKVGT165.

Kinetic analysis of F1-ATPases is complicated by turnover-dependent entrapment of inhibitory MgADP in a catalytic site, which occurs during ATP hydrolysis when noncatalytic sites are not saturated with ATP. Three kinetic phases are observed when MF1, TF1, and the αβγ subcomplex of TF1 hydrolyze low concentrations of ATP (8, 22, 23). An initial burst rapidly decelerates to a slow, intermediate rate, which, in turn, progressively accelerates to the final steady-state rate. Transition from the burst to the intermediate rate, which has been well characterized in MF1 (24), and CF1 (26–28), is caused by turnover-dependent entrapment of inhibitory MgADP in a catalytic site. Slow binding of ATP to noncatalytic sites relieves inhibition by promoting release of entrapped MgADP from the affected catalytic site. This is responsible for the transition.

F1F1-ATP synthases found in energy-transducing membranes couple ATP synthesis and hydrolysis to proton electrochemical gradients (1). They are composed of an integral membrane protein complex, F0, which mediates proton conduction, and a peripheral membrane protein complex, F1, which bears the catalytic sites. When separated from F0 as a soluble complex, F1 is composed of five different subunits in a stoichiometry of α3β3γεδ and functions as an ATPase (2). The αβ2γ, αβγδ, and αβγδε subcomplexes reconstituted from the isolated subunits of TF1 are active as ATPases (3–6). Both the αβγ and αβγδ subcomplexes differ from TF1 in that they are less specific for divalent cations and are insensitive to inhibition by azide (4, 5). In contrast, the catalytic characteristics of the αβγε subcomplex are very similar to those of TF1 (4, 7, 8). Moreover, it has been demonstrated that expression of a plasmid bearing the genes encoding the α, β, and γ subunits of TF1 in an unc strain of Escherichia coli leads to overproduction of the assembled αβγε complex, which can be purified in high yield (9). For these reasons, the wild-type and mutant αβγδ complexes are valuable tools for examining structure-function relationships in F1-ATPases.

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It has become clear that the apparent \( K_m \) observed in the millimolar range when \( F_1 \)-ATPases hydrolyze 30–5000 \( \mu \)M ATP in the absence of activating anions or activation of the enzymes by prior loading of noncatalytic sites with PP\(_i\) or ATP does not represent binding of ATP to a low affinity catalytic site but rather represents a rate acceleration caused by binding of ATP to noncatalytic sites (8, 22). Therefore, the apparent negative cooperativity in this range of ATP concentration, initially reported for rat liver MF\(_i\), by Ebel and Lardy (29) and later for bovine heart MF\(_i\) (30) and yeast \( F_1 \) (31), is an in vitro artifact and is of little, if any, physiological significance.

There have been several reports that \( K_m \) values of 1–5 \( \mu \)M and 100–200 \( \mu \)M are observed when MF\(_i\) hydrolyzes ATP (32–35). Since substoichiometric ATP binds to a single catalytic site under “unisite” conditions with a \( K_d \) of less than 1 \( \mu \)M (36), it appears that the \( K_m \) of 1–5 \( \mu \)M represents “bisite” catalysis and that the \( K_m \) of 100–200 \( \mu \)M represents “trisite” catalysis. However, Boyer (37) has suggested that the \( K_m \) of 1–5 \( \mu \)M is also an artifact associated with entrapment of inhibitory ADP in a catalytic site and contends that the \( K_m \) of 100–200 \( \mu \)M represents bimetal catalysis and that \( V_{max} \) rates are attained when two catalytic sites are saturated.

Substitution of the adjacent threonine on the C-terminal side of the lysine in the P-loop of the \( \beta \) subunit of YF\(_1\) (38) or \( E. coli \) \( F_1 \) (20) leads to enzyme with augmented ATPase activity. This substitution increases the specific activity of YF\(_1\), 3-fold, eliminates stimulation by oxyanions, and reduces sensitivity to inhibition induced by azide 25-fold (38, 39). Although these characteristics suggest that the mutant YF\(_1\) might not entrap inhibitory MgADP in a catalytic site during turnover, the kinetic analysis used by Mueller et al. (39) was not sufficiently discriminating to establish this with certainty. Rather than looking for turnover-dependent entrapment of inhibitory MgADP, they determined apparent \( K_i \) values for ADP using a phosphate release assay. The \( K_i \) values reported by Mueller et al. (39) are based on the effect of increasing, fixed ADP concentrations on the high \( K_m \) portions of biphasic Lineweaver-Burk plots. However, for reasons stated above, the apparent \( K_m \) in this concentration range does not represent ATP binding to catalytic sites but rather represents, at least for the wild-type enzyme, ATP binding to noncatalytic sites that promotes dissociation of inhibitory MgADP from a catalytic site. Therefore, the \( K_i \) values obtained represent competition of ADP with ATP for noncatalytic sites.

This investigation was initiated to determine 1) whether the \( \alpha_{\beta T165S}\gamma \) mutant subcomplex of TF\(_1\) does or does not entrap inhibitory MgADP in a catalytic site during turnover using appropriate kinetic analysis and 2) if it does not, whether the mutant subcomplex exhibits a \( K_m \) of 1–5 \( \mu \)M when it hydrolyzes low concentrations of ATP. The results obtained clearly show that the mutant complex does not entrap appreciable MgADP in a catalytic site during turnover and that it exhibits a \( K_m \) of 1–5 \( \mu \)M when it hydrolyzes low concentrations of ATP.

**EXPERIMENTAL PROCEDURES**

**Materials—**Biochemicals used in the assays and buffer components were purchased from Sigma. DuPont NEN supplied the [\(^{3}H\)ADP]. DCCD and Rhodamine 6G were purchased from Aldrich. LDAO was purchased from Calbiochem.

Purification of the wild-type \( \alpha_{\beta T165S}\gamma \) and mutant \( \alpha_{\beta T165S}\gamma \) complexes was carried out according to Matsui and Yoshida (9). The complexes were stored at 4 °C as precipitates in 70% saturated ammonium sulfate. After submitting the \( \alpha_{\beta T165S}\gamma \) and \( \alpha_{\beta T165S}\gamma \) complexes to SDS-polyacrylamide gel electrophoresis, the only protein bands that were revealed on the stained gels corresponded to the \( \alpha, \beta, \gamma \) subunits in a 3:3:1 ratio. Unless indicated otherwise, stock solutions of the complexes were prepared by removing ammonium sulfate precipitates from mother liquor by centrifugation and dissolving the pellets in either 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA. 10 \( \mu \)M of the wild-type complex or 5 \( \mu \)L of the mutant complex were assayed in medium containing 1.05 mM Mg\(^{2+}\) and 50 \( \mu \)M ATP as described in “Experimental Procedures.” Trace a, assay of 10 \( \mu \)L of the untreated wild-type complex. Trace b, assay of 10 \( \mu \)L of the wild-type complex after incubation with 1.4 \( \mu \)M excess of ADP for 30 min. Trace c, assay of 2 \( \mu \)L of the untreated mutant complex. Trace d, assay of 2 \( \mu \)L of the mutant complex after incubation with 1.4 \( \mu \)M excess of ADP for 30 min. The chart recordings were initiated within 3 s of mixing.

**Methods—**Site-directed mutagenesis was performed as described by Kunkel et al. (41). The oligonucleotide used to introduce the \( \beta T165S \) mutation was: 5′-GCT TTT GCA TGA TTA GTC AAT-3′. The oligonucleotide also contained a new site for ScaI, which allowed facile screening for the mutation. The mutation was confirmed by DNA sequencing (42) using the Sequenase 2.0 kit supplied by Amersham.

Protein concentrations were determined by the method of Bradford (43). ATPase activity was determined spectrophotometrically using an ATP regeneration system at 30 °C and pH 8.0, as specified previously (44). Unless indicated otherwise, the Mg\(^{2+}\) concentration in the assay medium was 1 mM greater than the ATP concentration.

Binding of [\(^{3}H\)ADP to the wild-type \( \alpha_{\beta T165S}\gamma \) and mutant \( \alpha_{\beta T165S}\gamma \) complexes was performed as described previously (22) with the use of 1-mL centrifuge columns of Sephadex G50 (40) under conditions specified in the legend of Fig. 4. Radioactivity was detected with a Packard 1600 TR counter using Ecosint from National Diagnostics.

**RESULTS**

**Comparison of the Effects of MgADP and Azide on the Hydrolytic Properties of the Wild-type \( \alpha_{\beta T165S}\gamma \) and the Mutant \( \alpha_{\beta T165S}\gamma \) Complexes—**Fig. 1 compares hydrolysis of 50 \( \mu \)M ATP by the wild-type and mutant complexes in the presence of an ATP-regenerating system before and after incubation of each with a slight excess of ADP in the presence of Mg\(^{2+}\) in order to load a catalytic site with MgADP (45). Trace a of Fig. 1 illustrates that three kinetic phases are exhibited during hydrolysis of 50 \( \mu \)M ATP by 10 \( \mu \)g of the wild-type complex. The
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**FIG. 2.** Comparison of the effects of azide on hydrolysis of 2 mM ATP by the wild-type α_3β_3γ and mutant α_3(βT165S)_3γ complexes with and without prior binding of MgADP to a single catalytic site. Enzyme stock solutions were prepared as described in Fig. 1. A, 4-μl samples of the wild-type complex were assayed in medium containing 3 mM Mg^{2+} and 2 mM ATP before (traces a and b) or after (traces c and d) prior incubation of 1 mg/ml stock solutions of the complex with a 1.4 M excess of ADP in the presence of 1 mM MgCl₂ for 30 min. Traces b and d were obtained with assay medium containing 1 mM NaN₃. B, samples (2 μl each) of the mutant α_3(βT165S)_3γ complex were assayed in medium containing 3 mM Mg^{2+} and 2 mM ATP before (traces a and b) or after (traces c and d) prior incubation of 1 mg/ml stock solutions of the complex with a 1.4 M excess of ADP in the presence of 1 mM MgCl₂ for 30 min. Traces b and d were obtained with assay medium containing 1 mM NaN₃.

The final rate of ATP hydrolysis in trace a is 5.4 μmol of ATP hydrolyzed/mg/min. In contrast, trace c shows that 50 μmol ATP is hydrolyzed by 2 μg of the mutant complex with a slightly accelerating rate before a final steady-state rate of 30 μmol of ATP hydrolyzed/mg/min is attained. After incubation with a 1.4 M excess of ATP plus Mg^{2+}, the wild-type complex hydrolyzes 50 μmol ATP with an extended lag, which is illustrated by trace b. After prior incubation with ATP plus Mg^{2+}, the mutant complex hydrolyzes 50 μmol ATP with a clearly perceptible lag (trace d), albeit one that is much less pronounced than that observed for the wild-type complex under the same conditions (trace b).

Fig. 2 compares hydrolysis of 2 mM ATP by 5.5 μg of the wild-type α_3β_3γ complex or 1.5 μg of the mutant complex before and after incubation of each with a 1.4 M excess of ADP in the presence of 1 mM Mg^{2+}. The effects of azide on hydrolysis of 2 mM ATP by the wild-type and mutant complexes are also compared. Traces a and c of Fig. 2A compare hydrolysis of 2 mM ATP by the wild-type complex before and after incubation with ATP plus Mg^{2+}. As observed for intact TF₁, as shown in previous studies (8), the final rate of ATP hydrolysis in trace a is 18 μmol/min. The lag becomes more pronounced after incubating the wild-type complex with a 1.4 M excess of ATP plus Mg^{2+}, as illustrated by trace b. However, the final rate of ATP hydrolysis in trace c is also 18 μmol/min. Trace b of Fig. 2A illustrates inhibition that develops during turnover when the wild-type complex hydrolyzes 2 mM ATP when 1.0 mM NaN₃ is present in the assay medium. Maximal inhibition developed within 3 min after initiating the assay. The final specific activity exhibited in the trace is about 0.45 μmol of ATP hydrolyzed/min. After incubating the wild-type complex with a 1.4 M excess of ATP plus Mg^{2+} and 1.0 mM NaN₃ prior to assay, the rate of ATP hydrolysis is slowed to 0.15 mol/mg/min, which is illustrated by trace d of Fig. 2A.

Fig. 2B shows that the ATPase activity of the mutant α_3(βT165S)_3γ complex responds differently than that of the wild-type complex to prior incubation with ATP plus Mg^{2+} and also shows that the mutant complex is much less sensitive to turnover-dependent inhibition by azide. Comparison of traces a and c of Fig. 2B shows that prior incubation of the mutant complex with ATP plus Mg^{2+} has very little effect on the kinetics of hydrolysis of 2 mM ATP. Nearly identical lags are exhibited before a final rate of 85 μmol of ATP hydrolyzed/min is attained with or without prior incubation of the mutant complex with ATP plus Mg^{2+}. Trace b shows that only slight turnover-dependent inhibition develops in the early stage of hydrolysis of 2 mM ATP by the mutant complex in the presence of 1.0 mM NaN₃. However, it is clear that inhibition slowly develops in the latter part of the trace. Comparison of traces d of Fig. 2, A and B, shows that the mutant complex is inhibited to a much lesser extent than the wild-type complex when it is incubated with a 1.4 M excess of ATP plus Mg^{2+} in the presence of 1.0 mM NaN₃ prior to assay. The slowly accelerating rate in the time interval illustrated in trace d of Fig. 2B corresponds to about 6 μmol of ATP hydrolyzed/min. This is 40-fold greater than that catalyzed by the wild-type complex under the same conditions (trace d of Fig. 2A).

**Comparison of the Effects of LDAO and Rhodamine 6G on the ATPase Activities of the Wild-type α_3β_3γ and Mutant α_3(βT165S)_3γ Complexes**—It has been shown in previous studies that the ATPase activities of TF₁ and the wild-type α_3β_3γ complex are stimulated about 4-fold by LDAO (7, 8). Therefore, the observation that hydrolysis of 2 mM ATP catalyzed by the mutant α_3(βT165S)_3γ complex is inhibited by about 25% in the presence of 0.06% LDAO was unexpected. This observation prompted a comparison of the dependence of the ATPase activities of the wild-type and mutant complexes on increasing concentrations of LDAO. Fig. 3A shows that the steady-state rate of hydrolysis of 2 mM ATP catalyzed by the wild-type complex (open circles) is progressively augmented by increasing concentrations of the detergent until maximal stimulation of about 4-fold is observed at 0.15% LDAO, in agreement with previously reported results (8). In contrast, LDAO inhibits...
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Figure 4. Comparison of the rates of release of Mg$^{3+}$ADP from the wild-type $\alpha_\beta\beta_2\gamma$ and mutant $\alpha_\beta(\beta T165S)_3\gamma$ induced by 40 $\mu$M ATP. Solutions of 1 mg/ml (2.8 $\mu$M) wild-type (●, ○) or mutant (▲, △) complexes were incubated with 2 mM Mg$^{2+}$, and the concentrations of LDAO were varied as specified.

Figure 3. Effect of LDAO and rhodamine 6G on the ATPase activity of wild-type $\alpha_\beta\gamma$ or mutant $\alpha_\beta(\beta T165S)_3\gamma$. Enzyme stock solutions were prepared as described in Fig. 1. Samples, 1 $\mu$l each of the wild-type complex (●) or mutant complex (○), were assayed in medium containing 2 mM ATP plus 3 mM Mg$^{2+}$ and the concentrations of LDAO (●) or rhodamine 6G (○) specified.

Comparison of the Sensitivity of the ATPase Activity of Wild-Type $\alpha_\beta\gamma$ and Mutant $\alpha_\beta(\beta T165S)_3\gamma$ Complexes to Inactivation by DCCD—Inactivation of TF1 by DCCD derivatizes Glu$^{190}$ (48) which is equivalent to Glu$^{188}$ of MF1. In the deduced crystal structure of MF1, the carboxylic acid side chain of Glu$^{188}$ is positioned appropriately to act as the general base that activates the attacking water molecule during ATP hydrolysis (12). The rate of inactivation of TF1 by DCCD is accelerated 7-fold when ADP binds to a single catalytic site (49), whereas the addition of Mg$^{2+}$ to form the 1:1:1 TF1-ADP-Mg complex or the addition of Mg$^{2+}$ directly to TF1 decreases the rate of inactivation by DCCD. These opposing effects suggest that reactivity of DCCD with the carboxylate side chain Glu$^{190}$ is sensitive to conformational changes induced by binding of ligands to a catalytic site. Therefore, it was of interest to compare the inactivation of the wild-type and mutant complexes by DCCD in the presence of ADP, ADP plus Mg$^{2+}$, or Mg$^{2+}$ alone. Table II compares the pseudo-first-order rate con-
Desalted stock solutions contained 1 mg/ml of the mutant or wild-type complexes in 50 mM Tris-HCl, pH 8.0. Samples (6.0 μl of the wild-type or 1.0 μl of the mutant complex) were assayed in 50 mM Hepes-KOH, pH 8.0, which contained 50 μM ATP and the concentrations of MgCl₂ indicated or in the same buffer, which contained 2 mM ATP and the concentrations of MgCl₂ indicated using the ATP regenerating system described previously (44).

TABLE I

| [MgCl₂] | Hydrolysis of 50 μM ATP | Hydrolysis of 2 mM ATP |
|---------|------------------------|------------------------|
| mut     | Wild-type | Mutant | Wild-type | Mutant |
| 1       | 4.5       | 27     | 18         | 86     |
| 2       | 3.3       | 27     | 13         | 85     |
| 4       | 9         | 85     |
| 5       | 7         | 85     |
| 10      | 5         | 82     |

TABLE II

| Complex   | Additions | \(k_{\text{inact}}\) | \(k_{\text{inact}}\) Percentage of \(a_ββγ\) with no additions |
|-----------|-----------|-----------------|--------------------------------------------------|
| Wild-type | None      | \(1.6 \times 10^{-2}\) | 100                                             |
| Wild-type | ADP       | \(3.9 \times 10^{-2}\) | 244                                             |
| Wild-type | ADP + Mg²⁺| \(5.6 \times 10^{-3}\) | 35                                              |
| Wild-type | Mg²⁺      | \(6.0 \times 10^{-3}\) | 38                                              |
| Mutant    | None      | \(7.7 \times 10^{-3}\) | 100                                             |
| Mutant    | ADP       | \(7.8 \times 10^{-3}\) | 100                                             |
| Mutant    | ADP + Mg²⁺| \(6.3 \times 10^{-3}\) | 82                                              |
| Mutant    | Mg²⁺      | \(6.7 \times 10^{-3}\) | 87                                              |

Fig. 5. Comparison of Lineweaver-Burk plots obtained for the the wild-type \(αβγ\) and mutant \(α(βT165S)γ\) complexes. Assays were conducted as described (57) with a 1 mM excess of Mg²⁺ over the ATP concentration. Initial rates were determined between 15 and 45 s after adding the wild-type or mutant complex to the assay mixture. A, double reciprocal plots of initial rates of hydrolysis of 20–2000 μM ATP by the wild-type (●) and mutant (■) complexes. B, a double reciprocal plot of the initial rate of hydrolysis of 4–2000 μM ATP catalyzed by the mutant complex.

Discussion

The major conclusion emerging from this study is that the mutant \(α(βT165S)γ\) complex, unlike the wild-type \(αβγ\) complex, does not entrap appreciable inhibitory MgADP in a catalytic site when it hydrolyzes ATP in the presence of a regener-
ating system. Differences in the hydrolytic properties of the mutant and wild-type complexes that support this contention are the following. 1) The wild-type complex hydrolyzes 50 μM ATP in three phases; an initial burst rapidly decelerates to a slower, intermediate phase, which, in turn, accelerates to the final steady-state rate. In contrast, the mutant complex hydrolyzes 50 μM ATP with a slightly accelerating rate. The final steady-state rate of the mutant complex is 5-fold greater than that observed for the wild-type complex. 2) The rate of hydrolysis of 50 μM or 2 mM ATP by the mutant complex is not affected by increasing the concentration of Mg\(^{2+}\) in the assay medium. In contrast, the rate of hydrolysis of 50 μM or 2 mM ATP catalyzed by wild-type complex is markedly suppressed by increasing the concentration of Mg\(^{2+}\) in the assay medium. 3) The mutant complex is much less sensitive than the wild-type complex to turnover-dependent inhibition when it hydrolyzes ATP in the presence of azide. 4) A Lineweaver-Burk plot of the initial rates of hydrolysis of 20–4000 μM ATP catalyzed by the mutant complex is linear rather than biphasic as observed for the wild-type enzyme. It has been shown with MF1, that biphasic Lineweaver-Burk plots for hydrolysis of ATP in this concentration range are caused by slow binding of ATP to noncatalytic sites, which promotes dissociation of inhibitory MgADP from a catalytic site (22).

To explain transient entrapment of inhibitory MgADP in a single catalytic site during ATP hydrolysis by MF1, it has been hypothesized that an active (24) or a readily activable (50) F\(_{1}\)ATPase-Mg complex is in equilibrium with an inactive F\(_{1}\)\(^{\gamma}\)-ADP-Mg complex. It has been proposed that turnover-dependent inhibition by azide is caused by stabilization of the F\(_{1}\)\(^{\gamma}\)-ADP-Mg complex (51, 52). The postulated equilibria are illustrated in Scheme I.

\[
F_{1}\text{ADP.Mg} \rightleftharpoons F_{1}^{\gamma}\text{ADP.Mg} \rightleftharpoons N_{i} \rightleftharpoons F_{1}^{\gamma}\text{ADP.Mg.N}_{i}
\]

**Scheme I**

The results presented here are entirely consistent with the proposed equilibria. The kinetic characterization described suggests that, in the case of the α\(_{i}\)(β\(_{i}\)δ\(_{i}\))γ complex, the equilibrium between the F\(_{1}\)ADP-Mg and F\(_{1}\)\(^{\gamma}\)-ADP-Mg complexes is predominantly in favor of the F\(_{1}\)\(^{\gamma}\)-ADP-Mg complex. This argument is consistent with the observation that preloading a catalytic site of the mutant complex with MgADP only slightly affects the initial rate of hydrolysis of 50 μM ATP and has essentially no effect on the hydrolysis of 2 mM ATP. It is also consistent with the observation that the mutant complex is much less sensitive to turnover-dependent inhibition when assayed in the presence of azide but becomes substantially inhibited when incubated with MgADP in the presence of azide prior to assay. Presumably, this occurs with slow formation of the more stable F\(_{1}\)\(^{\gamma}\)-ADP-Mg\(_{i}\) complex at a catalytic site.

Milgrom and Muratallow (50) and Bulgyn and Vinogradov (53) have reported that the MF1-ADP-Mg complex slowly isomerizes to the MF1\(^{\gamma}\)-ADP-Mg complex. Although the molecular basis for the isomerization remains obscure, results presented here suggest that changes in the conformation of the affected catalytic site might be involved. Unlike the wild-type complex and TF1, the rate of inactivation of the α\(_{i}\)(β\(_{i}\)δ\(_{i}\))γ complex by DCCD is not accelerated by ADP, and compared with the wild-type complex and TF1, it is only slightly decelerated by Mg\(^{2+}\) in the presence or absence of ADP. These observations suggest that the active site of the mutant complex does not undergo the same ligand-induced conformational changes that occur in the wild-type complex. In the crystal structure of MF1, the equivalent of Thr\(_{\mu 165}\) of TF1 is liganded to the Mg\(^{2+}\) ions complexed with ADP or AMP-PNP at the catalytic sites designated β\(_{TP}\) and β\(_{TP}\), respectively (12). A hypothetical explanation for the turnover-dependent entrapment of MgADP in a catalytic site of wild-type F\(_{1}\)-ATPase is the following. In the absence of saturation of noncatalytic sites with ATP, MgP, dissociates first at low frequency during ATP hydrolysis leaving ADP bound to a catalytic site. Binding of Mg\(^{2+}\) from the medium to the catalytic site containing bound ADP forms the F\(_{1}\)\(^{\gamma}\)-ADP-Mg\(_{i}\) complex.

Although the mutant complex does not entrap substantial inhibitory MgADP in a catalytic site during turnover, a slightly accelerating rate is exhibited when it hydrolyzes 50 μM ATP, and a distinct lag is observed when it hydrolyzes 2 mM ATP. To explain this apparent dilemma, we suggest that slow binding of ATP to noncatalytic sites during turnover causes a slight activation, which is independent of promotion of dissociation of inhibitory MgADP from a catalytic site. If this is indeed the case, the accelerations attributed to the binding of ATP to noncatalytic sites of the wild-type complex would reflect dissociation of inhibitory MgADP from a catalytic site plus the acceleration observed for the mutant complex.

The linear Lineweaver-Burk plot exhibited for the initial rate of hydrolysis of 20–2000 μM ATP by the mutant complex is consistent with the finding that it does not entrap appreciable inhibitory MgADP in a catalytic site during turnover. It was shown earlier that the apparent negative cooperativity displayed on Lineweaver-Burk plots for hydrolysis of ATP in this concentration range by MF1 is caused by slow binding of ATP to noncatalytic sites, which promotes dissociation of inhibitory MgADP from the affected catalytic site (22). The negative cooperativity exhibited on the Lineweaver-Burk plot constructed from the initial rates of hydrolysis of 4–2000 μM ATP catalyzed by the mutant complex is consistent with previously reported results (54). It was shown that aneadie Hofstee plot constructed from the initial rates of hydrolysis of 0.1–2000 μM ATP is biphasic, revealing K\(_{m}\) values of 1.4 μM and 110 μM with associated k\(_{cat}\) values of 14 and 340 s\(^{-1}\), respectively. The K\(_{m}\) values derived from the Lineweaver-Burk plot shown in Fig. 5B are about 5 μM and 110 μM with respective k\(_{cat}\) values of about 38 and 400 s\(^{-1}\). The K\(_{m}\) of 1–5 μM observed for the mutant complex is clearly not an artifact associated with entrapment of inhibitory MgADP in a catalytic site as proposed by Boyer (37). Therefore, we interpret the K\(_{m}\) of 1–5 μM associated with an associated k\(_{cat}\) of about 20 s\(^{-1}\) to represent catalysis when only two catalytic sites are saturated with ATP and the K\(_{m}\) of about 120 μM with an associated k\(_{cat}\) of about 350 s\(^{-1}\) to represent catalysis when three catalytic sites are saturated with substrate. Using a different approach, Weber et al. (55) also conclude that the β3331W mutant of the E. coli F\(_{1}\)-ATPase attains maximal velocity when three catalytic sites are saturated with ATP. It should be pointed out that in their correlation of quenching of tryptophan fluorescence with ATPase activity as ATP concentration was increased, Weber et al. (55) failed to detect significant “bisite catalysis.” However, more recently, using the same method to correlate catalytic site occupancy with TNP-ATP hydrolysis, Weber and Senior (56) reported that the V\(_{max}\) for TNP-ATP hydrolysis under bisite conditions is about 40% of the V\(_{max}\) observed under trisite conditions.

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