Substitution of βGlu\(^{201}\) in the α\(_3\)β\(_3\)γ Subcomplex of the F\(_1\)-ATPase from the Thermophilic Bacillus PS3 Increases the Affinity of Catalytic Sites for Nucleotides*

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In the crystal structure of bovine mitochondrial F\(_{1}\)-ATPase (MF\(_{1}\)) (Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628), the side chain oxygen of βThr\(^{163}\) interacts directly with Mg\(^{2+}\) coordinated to 5'-adenylyl β\(_3\)-imidodiphosphate or ADP bound to catalytic sites of β subunits present in closed conformations. In the unliganded β subunit present in an open conformation, the hydroxyl of βThr\(^{163}\) is hydrogen-bonded to the carbonylate of βGlu\(^{199}\). Substitution of βGlu\(^{201}\) (equivalent to βGlu\(^{199}\) in MF\(_{1}\)) in the α\(_3\)β\(_3\)γ subcomplex of the F\(_1\)-ATPase from the thermophilic Bacillus PS3 with cysteine or valine increases the propensity to entrap inhibitory MgADP in a catalytic site during hydrolysis of 50 μM ATP. These substitutions lower \(K_{m}\) (the Michaelis constant for trisite ATP hydrolysis) relative to that of the wild type by 25- and 10-fold, respectively. Fluorescence quenching of \(\alpha_3(\beta_2E021C/Y341W)_{\gamma}\) and \(\alpha_2(\beta_3Y341W)_{\gamma}\) mutant subcomplexes showed that MgATP and MgADP bind to the third catalytic site of the double mutant with 8.4- and 4.4-fold higher affinity, respectively, than to the single mutant. These comparisons support the hypothesis that the hydroxyl bond observed between the side chains of βThr\(^{163}\) and βGlu\(^{199}\) in the unliganded catalytic site in the crystal structure of MF\(_{3}\) stabilizes the open conformation of the catalytic site during ATP hydrolysis.

The \(F_0F_1\)-ATP synthases found in energy-transducing membranes couple ATP synthesis and hydrolysis to proton or sodium ion electrochemical gradients. They are composed of \(F_0\), an integral membrane protein complex that mediates ion conduction, and \(F_1\), a peripheral membrane protein complex containing the catalytic sites. When separated from \(F_0\) as a soluble complex, \(F_1\) is an ATPase composed of five different subunits with \(\alpha_3\beta_3\gamma\) stoichiometry (1, 2). \(F_1\) contains six nucleotide-binding sites. Three of these participate directly in catalysis. The other three, called noncatalytic sites, do not have a well defined physiological role (2). However, it is clear that saturation of these sites with MgATP in MF\(_{1}\) \(^{1}\) prevents entrapment of inhibitory MgADP when low concentrations of ATP are hydrolyzed (3). The 2.8-Å resolution crystal structure of bovine MF\(_{1}\), determined by Abrahams \textit{et al}. (4) shows that the catalytic sites are at α/β interfaces, with most residues participating directly in catalysis located in β subunits. Noncatalytic sites are located at different α/β interfaces, with most residues contributing to the binding sites located in α subunits. In the crystal structure, the three noncatalytic sites are homogeneously liganded with MgAMP-PNP. In contrast, catalytic sites are heterogeneously liganded. One (designated β\(_{1}\)) contains MgAMP-PNP; another (designated β\(_{1}\)) contains MgADP; and the third catalytic site (designated β\(_{2}\)) is empty. The α subunits contributing to β\(_{1}\), β\(_{2}\), and β\(_{3}\) are designated α\(_{1}\), α\(_{2}\), and α\(_{3}\), respectively (4). In the crystal structure of the completely unliganded α\(_3\)β\(_3\)γ subcomplex of TF\(_{1}\) determined at 3.2-Å resolution by Shirakihara \textit{et al}. (5), the α and β subunits are arranged symmetrically. The common open conformation of β subunits in the α\(_3\)β\(_3\)γ subcomplex is essentially identical to that of β\(_{1}\) in MF\(_{1}\), and the common conformation of the α subunits is essentially identical to that of the liganded α subunits in MF\(_{1}\).

Although it is generally assumed that the three catalytic sites in F\(_1\)-ATPases exist in tight, loose, and open conformations, scrutiny of the crystal structure of bovine MF\(_{1}\) reveals only two distinctly different conformations. These are the open, unliganded conformation of β\(_{2}\) and the nearly identical, closed conformations in β\(_{1}\) and β\(_{3}\) liganded with MgAMP-PNP and MgADP, respectively (6). In β\(_{2}\) and β\(_{3}\), the hydroxyl oxygen of βThr\(^{163}\) is directly liganded with the Mg\(^{2+}\) ion that interacts with anionic oxygens of phosphates in bound AMP-PNP or ADP, respectively. In contrast, in β\(_{1}\), the hydroxyl of βThr\(^{163}\) is 2.9 Å from a carbonyl oxygen of βGlu\(^{199}\). These groups are 11.4 Å apart in β\(_{1}\) and β\(_{3}\), suggesting that the open conformation of β\(_{1}\) might be stabilized by the hydrogen bond between the side chains of βThr\(^{163}\) and βGlu\(^{199}\). It is interesting that the carboxyl group of βGlu\(^{199}\) in MF\(_{1}\) or the equivalent residue in \textit{Escherichia coli} F\(_{1}\) and spinach chloroplast F\(_{1}\) is specifically derivatized on inactivation of ATPase activity with dicyclohexylcarbodiimide. In contrast, when TF\(_{1}\) is inactivated with dicyclohexylcarbodiimide (7–9), βGlu\(^{199}\), the equivalent of βGlu\(^{186}\) in MF\(_{1}\), is derivatized (10).

The catalytic properties of site-directed mutants of the glutamates in TF\(_{1}\), \textit{E. coli} F\(_{1}\), and \textit{Rhodospirillum rubrum} \textit{frum} F\(_{1}\) that are equivalent to βGlu\(^{199}\) in MF\(_{1}\) also suggest a functional role for this side chain in ATP hydrolysis (11–13). Substitution of the equivalent residue with glutamine (11, 12) or cysteine (12) in TF\(_{1}\) or \textit{E. coli} F\(_{1}\) or with glutamine, lysine, or glycine in \textit{R. rubrum} F\(_{1}\) (13) significantly lowers ATP hydrolysis under saturating conditions, whereas ATPase activity is attenuated to a lesser extent when the equivalent residue is substituted with Asp (12). In the crystal structure of MF\(_{1}\), βThr\(^{163}\) is hydrogen-bonded to βGlu\(^{199}\) in the open catalytic site, whereas

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\(^{1}\) The abbreviations used are: MF, and TF, F\(_{1}\)-ATPases from bovine heart mitochondria and the thermophilic \textit{Bacillus} PS3, respectively; MgAMP-PNP, Mg-5'-adenylyl β\(_3\)-imidodiphosphate; LDAO, lauryldimethylamine oxide; CDTA, 1,2-diaminocyclohexane-N\(_2\),N\(_4\),N\(_{\alpha}\)-tetraacetic acid.
it is liganded with Mg$^{2+}$ in the two closed, ligandated catalytic sites as illustrated elsewhere (4, 6). This suggests that the hydrogen bond between the side chains observed in the empty catalytic site of MF$_1$ might function to stabilize the open conformation of the catalytic site during catalysis. If this is indeed the case, the affinity of catalytic sites for MgADP and MgATP should increase on substituting $\beta$Glu$^{201}$ in the $\alpha_2\beta_2\gamma$ subcomplex of TF$_1$ with amino acids containing side chains incapable of hydrogen bonding. To test this possibility, the steady-state kinetics of ATP hydrolysis by mutant $\alpha_2\beta_2\gamma$ subcomplexes of TF$_1$ with the $\beta$E201C substitution with and without carboxymethylation and with the $\beta$E201V substitution have been examined in detail. In addition, the affinities of the $\beta$E201C/Y341W double mutant for nucleotides have been determined by assessing quenching of the introduced tryptophan by ATP and ADP in the presence of Mg$^{2+}$.

**EXPERIMENTAL PROCEDURES**

**Materials—**Enzymes and biochemicals used in assays and buffer components were purchased from Sigma. Iodoacetic acid free of iodine was obtained from Sigma. LDAO was purchased from Calbiochem. Iodo[3H]acetate was purchased from American Radiolabeled Chemicals. Primers for mutations were purchased from Life Technologies, Inc.

**Generation of Mutant Subcomplexes—**Plasmid pKK, which carries the genes for the $\alpha$, $\beta$, and $\gamma$ subunits of TF$_1$, was used for mutagenesis and gene expression (14). Polymerase chain reaction was used to prepare mutant expression plasmids using the QuikChange site-directed mutagenesis kit (Stratagene). Wild-type pKK was used as the template for the $\beta$E201C and $\beta$E201V single mutants, and the $\beta$Y341W mutant plasmid pKK (15) was used as the template to generate the $\beta$E201CY341W double mutant. The primers 5'-GACTTTGACCTAG- GATGAAAAGATTCCG-3' and 5'-GACTTTGACCTAGCATGAAAGATT- TCCG-3' (with the changed bases underlined) and their corresponding complementary primers were used in polymerase chain reaction to generate the $\beta$E201C and $\beta$E201V mutants, respectively. The plasmids were purified with the Wizard $^\text{TM}$ Plus Miniprep DNA purification system (Promega). The mutations were confirmed by sequence analysis. The resultant pKK mutant plasmids were expressed in JM103 (unc$^-$).

**Analytical Methods—**Enzyme stock solutions were prepared by dissolving pellet ammonium sulfate precipitates obtained by centrifugation in 50 mM Tris-Cl, pH 8.0, containing 1 mM EDTA. After 1 h at room temperature, dissolved enzymes were passed through 1-ml centrifuge columns of Sephadex G-50 equilibrated with 50 mM Tris-Cl, pH 8.0, containing 0.1 mM EDTA. Protein concentrations were determined by the method of Bradford (16) using Coomassie Blue (Pierce). ATPase activity was determined spectrophotometrically using an ATP regeneration system at 30 °C and pH 8.0 (3). Unless indicated otherwise, the Mg$^{2+}$ concentration in the assay medium was 1 mM in excess of the ATP concentration. The NADH used in the assays contained negligible ADP. Radioactivity was determined by liquid scintillation counting in Ecoscint (National Diagnostics, Inc.).

**Carboxymethylation of the $\beta$E201C Mutant Subcomplex—**Iodoacetate (100 mM) adjusted to pH 7.0 with NaOH was added to a final concentration of 2 mM to a 2 mg/ml solution of the $\beta$E201C mutant subcomplex in 50 mM Tris-Cl, pH 8.0, containing 0.1 mM EDTA. The reaction mixture was incubated for 30 min at room temperature, at which time excess iodoacetate was removed by passing it through a centrifuge column of Sephadex G-50 equilibrated with 50 mM Tris-Cl.

**Preliminary experiments showed that treatment of the $\beta$E201C subcomplex with 2 mM iodo[3H]acetate leads to parallel activation of ATPase activity and incorporation of covalently bound [3H]acetate. The first 2 mol of [3H]acetate were incorporated within 7 h, whereas incorporation of a third mol was not complete after another 17 h when 2.7 mol of reagent were incorporated per mol of enzyme. After carboxymethylation for 24 h, the specific activity increased from 1.1 to 2.9 pmol of ATP hydrolyzed min$^{-1}$ mg$^{-1}$. Although the wild-type $\alpha_2\beta_2\gamma$ subcomplex contains cGys$^{396}$, only 0.2 mol of [3H]acetate were incorporated per mol of enzyme when it was treated with iodo[3H]acetate under the same conditions without altering ATPase activity. Therefore, modification of cGys$^{396}$ is responsible for the increase in ATPase activity observed with the mutant subcomplex.**

**RESULTS**

**Comparison of Hydrolysis of 50 $\mu$M ATP by the Wild-type and Mutant Subcomplexes—**It has been shown that the wild-type subcomplex hydrolyzes 50 $\mu$M ATP in three kinetic phases (18). A burst rapidly decelerates to an intermediate, slow phase that slowly accelerates to a final rate that approaches the initial rate. Transition from the burst phase to the intermediate phase is caused by turnover-dependent entrapment of MgADP in a catalytic site, and transition from the intermediate phase to the final rate reflects slow binding of ATP to noncatalytic sites that promotes dissociation of the inhibitory MgADP from the affected catalytic site. Earlier studies have shown that only a single, rapid kinetic phase is observed when the wild-type subcomplex hydrolyzes 50 $\mu$M ATP in the presence of low concentrations of LDAO, indicating little or no entrapment of inhibitory MgADP during turnover. Entrapment of inhibitory MgADP is augmented when the wild-type subcomplex is assayed in the presence of azide (15). To assess the propensity of mutant $\alpha_2\beta_2\gamma$ subcomplexes to entrap inhibitory MgADP during turnover, the characteristics of hydrolysis of 50 $\mu$M ATP by the wild-type and mutant subcomplexes in the presence or absence of 0.06% LDAO or 1 mM NaN$_3$ were compared. The results of this comparison are illustrated in Fig. 1. The numbers associated with the traces in Fig. 1 are the approximate specific activities of the subcomplexes at the final 30-s interval recorded. Trace a in panel A-I illustrates the three kinetic phases exhibited when the wild-type $\alpha_2\beta_2\gamma$ subcomplex hydrolyzed 50 $\mu$M ATP. In contrast, in the presence of LDAO, the rate of ATP hydrolysis by the wild-type subcomplex was nearly linear (trace c). LDAO stimulated the ATPase activity of the wild-type subcomplex ~3-fold. Trace b shows that hydrolysis of 50 $\mu$M ATP in the presence of 1 mM NaN$_3$ proceeded with an initial burst that rapidly decelerated to a severely inhibited rate. It was previously shown that the $\alpha_2\beta_2\gamma$ subcomplex is much less sensitive than the wild type to turnover-dependent inhibition induced by azide during hydrolysis of 2 mM ATP (15). Comparison of traces b in panels A-I and A-II shows that hydrolysis of 50 $\mu$M ATP by the $\alpha_2\beta_2\gamma$ subcomplex was less sensitive than that by the wild type to turnover-dependent inhibition induced by 1 mM NaN$_3$. Comparison of traces c in these panels shows that LDAO had a much smaller stimulatory effect on the $\alpha_2\beta_2\gamma$ subcomplex than on the wild type.

**Fig. 1 (panel B-I) illustrates that the $\beta$E201C mutant subcomplex hydrolyzed 50 $\mu$M ATP in three kinetic phases both in the presence and absence of LDAO. Whereas a prolonged intermediate phase was observed in the absence of LDAO (trace a), a relatively brief intermediate phase was observed in the presence of the detergent (trace c). The characteristics of hydrolysis of 50 $\mu$M ATP by the $\beta$E201V mutant were similar to those of the $\beta$E201C mutant, except that the rates of hydrolysis were much slower under each condition. Panel B-II illustrates the hydrolysis of 50 $\mu$M ATP by the carboxymethylated $\beta$E201C mutant. Carboxymethylation of the mutant subcomplex stimulated the rate of hydrolysis of 50 $\mu$M ATP ~3.5-fold in the absence of LDAO (trace a). The rate of hydrolysis of 50 $\mu$M ATP by the carboxymethylated enzyme was stimulated ~6-fold and...
was nearly linear in the presence of LDAO (trace c). Comparison of traces b in panels A-I, B-I, and B-II shows that the βE201C and carboxymethylated βE201C mutant subcomplexes were much more sensitive than the wild type to inhibition by azide and that carboxymethylation of the βE201C subcomplex slightly relieved inhibition by azide. Comparison of panels A-II and B-III illustrates that the α3(βY341W)3γ double mutant was a much more sluggish enzyme in the presence and absence of LDAO and was much more sensitive than the α3(βE201C)γ subcomplex to turnover-dependent inhibition by azide.

Comparison of the Steady-state Kinetic Parameters of the Wild-type and Mutant α3β3γ Subcomplexes—Entrapment of inhibitory MgADP in a catalytic site when F1-ATPases hydrolyze ATP introduces an increase in slope in Lineweaver-Burk plots at high ATP concentration. This increased slope reflects ATP binding to noncatalytic sites rather than catalytic sites. Binding of ATP to noncatalytic sites promotes dissociation of inhibitory MgADP from the affected catalytic site, thus effectively increasing the concentration of active enzyme (3). The steady-state kinetic parameters obtained for the hydrolysis of 2–2000 μM ATP by the wild-type and mutant enzyme subcomplexes are summarized in Table I. The $K_{noncat}$ values in Table I were determined from extrapolation of the linear segment of the Lineweaver-Burk plots with highest slope. In cases where plots with only two distinct slopes were obtained, $K_{noncat}$ was estimated from the intercept on the negative abscissa of the segment of lower slope. In cases where plots with three distinct slopes were observed, the segment with intermediate slope was extrapolated to estimate $K_{noncat}$. To obtain reliable $K_{noncat}$ and $k_{cat}$ values for the α3(βE201C)3γ subcomplex, ATP hydrolysis was examined over the range of 2–5000 μM ATP rather than that of 2–2000 μM ATP examined with the other subcomplexes.

The Lineweaver-Burk plot for the wild-type subcomplex revealed a $K_{noncat}$ value for bisite ATP hydrolysis of 1.7 μM and a $K_{cat}$ value of 43 μM for trisite ATP hydrolysis. The $K_{noncat}$ value for the wild-type enzyme is 180 μM. The $K_{noncat}$ values varied from 170 to 420 μM for the mutant subcomplexes. The very low rates of ATP hydrolysis observed when the α3(βE201C)3γ and α3(βE201V)3γ subcomplexes hydrolyzed low concentrations of ATP precluded estimation of $K_{noncat}$ values. However, following carboxymethylation with iodoacetate, the ATPase activity of the α3(βE201C)3γ subcomplex increased significantly, thus allowing estimation of a $K_{noncat}$ value of ~1 μM.

The $K_{noncat}$ values obtained for the α3(βE201C)3γ, α3(βE201V)3γ, and α3(βE201C/Y341W)3γ mutants were 25-, 10-, and 13-fold lower than that of the wild type, respectively.
MgATP binds to the three catalytic sites with widely different affinities in the presence of LDAO with the wild-type and \( \alpha_\beta \beta_\gamma \) subcomplex. This corresponds to the high affinity catalytic site characterized by maximal rates of ATP hydrolysis of the linear segment of the Lineweaver-Burk plots with high-est slope. They represent maximal rates of ATP hydrolysis when catalytic and noncatalytic sites are saturated with ATP.

Comparison of MgATP and MgADP Binding to the \( \alpha_\beta \beta_\gamma \) and \( \alpha_\beta \beta_\gamma + \text{LDAO} \) Subcomplexes in the Presence and Absence of LDAO—By monitoring quenching of fluorescence of the introduced tryptophan, the titration curve for the \( \alpha_\beta \beta_\gamma \) subcomplex was obtained by subtracting the number of occupied sites determined from the fluorescence measurements from the concentration of ATP present at each point in the titration.

The \( k_{\text{cat}} \) values tabulated were determined from extrapolation of the linear segment of the Lineweaver-Burk plots with highest slope. They represent maximal rates of ATP hydrolysis when catalytic and noncatalytic sites are saturated with MgATP.

\[
\begin{array}{cccccc}
\text{Subcomplex} & K_{\text{cat}} & K_{\text{nucat}} & K_{\text{macat}} & k_{\text{cat}} \\
\alpha_\beta \beta_\gamma & 1.7 & 43 & 180 & 120 \\
\alpha_\beta \beta_\gamma + \text{LDAO} & 5 & 100 & 200 & 2 \\
\alpha_\beta \beta_\gamma (\beta \text{E201C})_3 \beta_\gamma & 71 & 100 & 180 & 16 \\
\alpha_\beta \beta_\gamma + \text{LDAO} & 5 & 100 & 200 & 2 \\
\text{carboxymethylated } \alpha_\beta \beta_\gamma (\beta \text{E201C})_3 \beta_\gamma & 71 & 100 & 180 & 16 \\
\alpha_\beta \beta_\gamma (\beta \text{Y341W})_3 \beta_\gamma & 71 & 100 & 180 & 16 \\
\alpha_\beta \beta_\gamma (\beta \text{Y341W})_3 \beta_\gamma + \text{LDAO} & 5 & 100 & 200 & 2 \\
\alpha_\beta \beta_\gamma (\beta \text{E201C}/\beta \text{Y341W})_3 \beta_\gamma & 71 & 100 & 180 & 16 \\
\alpha_\beta \beta_\gamma (\beta \text{E201C}/\beta \text{Y341W})_3 \beta_\gamma + \text{LDAO} & 5 & 100 & 200 & 2 \\
\end{array}
\]

\( a \) Cases in which a change in slope on Lineweaver-Burk plots was not obtained from extrapolations.

\( b \) Cases in which the optimal ATP/Mg\(^{2+}\) ratio for the wild type in the presence of 11 mM Mg\(^{2+}\) was 11.74 mM Mg\(^{2+}\), but in the absence of LDAO, the ATPase activity of the wild-type subcomplex was inhibited by free Mg\(^{2+}\), which presumably provokes entrapment of inhibitory MgADP in a catalytic subcomplex. Complex behavior was exhibited during titration of the second catalytic site with MgATP in the absence of LDAO. Under these conditions, a theoretical binding curve with a single \( K_d \) that fit the experimental data in this region of the titration curve could not be generated by computer using the equation described by Weber et al. (17). However, when catalytic sites of the \( \alpha_\beta (\beta \text{Y341W})_3 \beta_\gamma \) subcomplex were titrated with MgATP in the presence of LDAO, a theoretical binding curve was generated from which \( K_m \) and \( K_d \) values of 0.9 and 47 \( \mu \text{M} \), respectively, were estimated. Fig. 2 illustrates that two catalytic sites of the \( \alpha_\beta (\beta \text{E201C}/\beta \text{Y341W})_3 \beta_\gamma \) double mutant were nearly saturated at the lowest concentration of ATP added to it in the presence of Mg\(^{2+}\), but in the absence of LDAO. When titrated

The \( K_m \) values tabulated were determined from extrapolation of the linear segment of the Lineweaver-Burk plots with highest slope. They represent maximal rates of ATP hydrolysis when catalytic and noncatalytic sites are saturated with MgATP.
The TF\textsubscript{1} βE201C Mutation Increases Affinity for Nucleotides

3

![Graph of occupied catalytic sites versus free MgADP concentration](Image)

**FIG. 3.** Titration of the catalytic sites of the βY341W and βE201C/βY341W mutant subcomplexes with MgADP in the presence and absence of 0.06% LDAO. ○, βY341W without LDAO; □, βY341W with 0.06% LDAO; ■, βE201C/βY341W without LDAO; ▲, βE201C/βY341W with 0.06% LDAO. The concentration of free MgADP was obtained as described for free MgATP in the legend to Fig. 2.

| Conditions | α\textsubscript{βY341W}\textsubscript{βγ} | α\textsubscript{βE201C/βY341W}\textsubscript{βγ} |
|------------|-------------------|-------------------|
| K\textsubscript{d}, MgATP | 0.9 µM | 0.2 µM |
| K\textsubscript{d}, MgATP + LDAO | 21 µM | 6.9 µM |
| K\textsubscript{d}, MgADP | 14 µM | 3.2 µM |
| K\textsubscript{d}, MgADP + LDAO | 43 µM | 7.4 µM |

* In the absence of LDAO, K\textsubscript{d} values could not be estimated from the complex patterns of fluorescence quenching obtained when the α\textsubscript{βY341W}\textsubscript{βγ} and α\textsubscript{βE201C/βY341W}\textsubscript{βγ} mutant subcomplexes were titrated with MgATP or MgADP.

α\textsubscript{βY341W}\textsubscript{βγ} mutant was 72% of that observed at the optimal Mg\textsuperscript{2+} concentration of 2 mM. In the presence of LDAO, the ATPase activity of the α\textsubscript{βY341W}\textsubscript{βγ} subcomplex was slightly stimulated when the Mg\textsuperscript{2+} concentration was greater than the ATP concentration, and no inhibition was observed at 11 mM Mg\textsuperscript{2+}.

**DISCUSSION**

It was previously reported that substitution of βGlu\textsuperscript{201} in TF\textsubscript{1} (12) or its equivalent in E. coli F\textsubscript{1} (11) lowers ATPase activity compared with the wild type. However, this is the first demonstration that substitution of this residue with amino acids containing side chains that are incapable of hydrogen bonding increases the affinity of catalytic sites for nucleotides in the presence of Mg\textsuperscript{2+}. When compared with the wild type, substitution of βGlu\textsuperscript{201} in the α\textsubscript{β}\textsubscript{βγ} subcomplex of TF\textsubscript{1} with valine or cysteine decreases k\textsubscript{cat} and K\textsubscript{m3}, the Michaelis constant for trisite ATP hydrolysis. Consistent with the kinetic analyses, direct binding measurements obtained from nucleotide-induced quenching of fluorescence of introduced tryptophan in the α\textsubscript{βY341W}\textsubscript{βγ} and α\textsubscript{βE201C/βY341W}\textsubscript{βγ} subcomplexes demonstrated that the βE201C substitution increases the affinities of the second and third catalytic sites for MgATP and MgADP. The K\textsubscript{m3} values determined for binding of MgATP to the α\textsubscript{βE201C/βY341W}\textsubscript{βγ} mutant subcomplexes are 25-, 10-, and 13-fold lower than that of the wild type, respectively, whereas the K\textsubscript{m3} value determined for binding of MgATP to the α\textsubscript{βE201C/βY341W}\textsubscript{βγ} double mutant is only 8.4-fold lower than that for binding of MgATP to the α\textsubscript{βY341W}\textsubscript{βγ} subcomplex. This discrepancy may reflect that the K\textsubscript{d} values determined from fluorescence quenching measurements are true binding constants, whereas K\textsubscript{m3} values are complex rate constants rather than true binding constants.

The overall findings of this study support the hypothesis under scrutiny that proposes that the hydrogen bond between the hydroxyl of βThr\textsuperscript{163} and the carboxylate of βGlu\textsuperscript{199} present in βGlu, but not in βE or βN, in the crystal structure of MF\textsubscript{1} (4) stabilizes the open conformation of the catalytic site during catalysis. The observation that carboxymethylation of α\textsubscript{βE201C}\textsubscript{βγ} increases k\textsubscript{cat} and K\textsubscript{m3} provides additional support for this hypothesis. Also consistent with this hypothesis are earlier reports demonstrating that the βT165S substitution in TF\textsubscript{1} increases K\textsubscript{m3} and k\textsubscript{cat} (23, 24). Replacement of threonine by serine in this position might decrease the affinity of MgATP bound to closed catalytic sites by interacting less strongly with Mg\textsuperscript{2+} and/or by interacting more strongly with βGlu\textsuperscript{199} in the open conformation of the catalytic site.

In experiments with R. rubrum ATP synthase, Nathanson and Gromet-Elhanan (13) found that β-less chromatophores reconstituted with recombinant β subunits in which the equivalent of βGlu\textsuperscript{201} in TF\textsubscript{1} was substituted with glutamine, gly-
The finding that β-less *R. rubrum* chromatophores reconstituted with mutant β subunits containing substitutions at the equivalent of βGlu201 in TF₁ have substantial ATP synthase activity (13) is consistent with the models. During ATP synthesis, the energy-requiring step is closing the catalytic site of βₖ when loosely bound MgADP and Pᵢ are converted to tightly bound MgATP (31–33). The results presented here suggest that substitution of βGlu201 stabilizes the closed conformation of the catalytic site, thus favoring the energy-dependent closing of βₖ depicted in Fig. 5B. However, by stabilizing the closed conformation of β₇, substitution of βGlu201 with residues containing side chains incapable of hydrogen bonding would hinder dissociation of MgATP, thus accounting for the comparatively small decrease in rates of ATP synthesis observed with *R. rubrum* chromatophores containing the mutant β subunits (13). During ATP hydrolysis by the αβγ subcomplex of TF₁, substitution of βGlu201 would also favor closing when βₖ binds MgATP. However, in this case, by stabilizing the closed conformation of β₇, the substitutions will significantly attenuate formation of the transition state for hydrolysis, thus significantly reducing the overall rate.

All mutants containing βGlu201 substitutions examined in this study have an increased propensity over the wild type to entrap inhibitory MgADP in a catalytic site during ATP hydrolysis. Kinetic analyses showed that the α₁(βY341W)₃γ subcomplex has less tendency than the wild type to entrap inhibitory MgADP in a catalytic site during turnover. However, complex behavior was observed at low nucleotide concentrations when the tryptophan fluorescence of the α₁(βY341W)₃γ and α₁(βE201C/Y341W)₃γ subcomplexes was titrated with MgATP or MgADP. In the absence of LDAO, it was not possible to estimate *Kₘ* values from the titration data. However, when the mutant subcomplexes were titrated with MgATP or MgADP in the presence of LDAO, clearly distinguishable *Kₘ* values could be estimated. This is consistent with observations indicating that LDAO promotes dissociation of inhibitory...
MgADP during ATP hydrolysis. These are as follows. 1) In the presence of LDAO, the wild-type αβ2γ subcomplex hydrolyzes 50 μM ATP linearly rather than in three kinetic phases exhibited in the absence of LDAO illustrated in Fig. 1. 2) The wild-type αβ2γ subcomplex preloaded with MgADP in a single catalytic site hydrolyzes 2 mM ATP with a long lag when assayed in the absence of LDAO, whereas the lag disappears when the preloaded enzyme is assayed in the presence of LDAO (18). 3) [3H]ADP preloaded onto a single catalytic site of the wild-type αβ2γ subcomplex dissociates very slowly when the enzyme is diluted into assay medium containing 40 μM ATP, whereas when the assay medium contains LDAO, the preloaded [3H]ADP dissociates rapidly (18).

The complex binding observed during titrations with MgATP and MgADP in the absence of LDAO is consistent with an equilibrium between F1MgADP and F1*MgADP that has been proposed to explain transient inhibition of F1-ATPases by MgADP during ATP hydrolysis (34). F1MgADP represents MgADP bound to F1 in an inactive conformation.

Titrations of catalytic sites of the αβ3Y341Wγ subcomplex with MgATP or MgADP previously reported from this laboratory (15). In the titrations shown here, the number of sites occupied is plotted against free MgATP or MgADP concentration rather than total MgATP or MgADP concentration as previously reported in Ref. 15. Conversion of the data on the abscissas of the titration curves in Ref. 15 from total MgATP or MgADP to free MgATP or MgADP results in titration curves that closely resemble those illustrated in Figs. 2 and 3 of this study. In the previous study (15), titration curves were presented that suggested that binding of MgATP to the αβ3Y341Wγ subcomplex was only slightly affected by the presence of 0.06% LDAO, whereas Fig. 2 clearly shows that LDAO shifts the titration curve for the subcomplex to lower affinity when it is titrated with MgATP. The curve shift illustrated in Fig. 2 is supported by the demonstration that LDAO overcomes entrapment of inhibitory MgADP during hydrolysis of 50 μM ATP by the αβ3Y341Wγ subcomplex (Fig. 1) and that LDAO overcomes inhibition induced by free Mg2+ in the assay medium (Fig. 4).

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