Binding of the Transition State Analog MgADP-fluoroaluminate to F₁-ATPase*

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_F. coli_ F₁-ATPase from mutant βY331W was potently inhibited by fluoroaluminate plus MgADP but not by MgADP alone. β-Trp-331 fluorescence was used to measure MgADP binding to catalytic sites. Fluoroaluminate induced a very large increase in MgADP binding affinity at catalytic site one, a smaller increase at site two, and no effect at site three. Mutation of either of the critical catalytic site residues β-Lys-155 or β-Glu-181 to Gln abolished the effects of fluoroaluminate on MgADP binding. The results indicate that the MgADP-fluoroaluminate complex is a transition state analog and independently demonstrate that residues β-Lys-155 and (particularly) β-Glu-181 are important for generation and stabilization of the catalytic transition state. Dicyclohexylcarbodiimide-inhibited enzyme, with 1% residual steady-state ATPase, showed normal transition state formation as judged by fluoroaluminate-induced MgADP binding affinity changes, consistent with a proposed mechanism by which dicyclohexylcarbodiimide prevents a conformational interaction between catalytic sites but does not affect the catalytic step per se. The fluorescence technique should prove valuable for future transition state studies of F₁-ATPase.

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F₁-ATPase is the catalytic sector of the enzyme F₆Fₒ-ATP synthase, responsible for ATP synthesis by oxidative phosphorylation in mitochondria, chloroplasts, and bacteria and also for ATP-driven proton pumping in bacteria. It consists of five different subunits in the stoichiometry α₃β₃γδε and contains three catalytic nucleotide-binding sites whose structures have been defined by x-ray crystallography at 2.8 Å resolution (1, 2). Each catalytic site lies at an interface between an α- and β-subunit of the enzyme, with most of the ligands between the nucleotide and the protein being provided by side chains from the β-subunit. Rotation of the γ-subunit within the αβ₂ hexagon has been demonstrated during MgATP hydrolysis (3), indicating that the three catalytic sites participate in catalysis in sequential fashion. Functional characterization of catalysis has been facilitated by mutagenesis of critical catalytic site residues and also by introduction of specifically engineered Trp residues into the catalytic sites. The latter technique has enabled determination of catalytic site nucleotide binding parameters in wild-type and mutant enzymes under a wide range of conditions (for recent reviews see Refs. 4 and 5).

To understand the mechanism of ATP synthesis and hydrolysis in F₁, the manner in which the catalytic transition state is attained and stabilized by catalytic site residues must be established. We have previously studied the catalytic transition state using techniques of “unisite catalysis.” Unisite catalysis is the term used to describe the single turnover of ATP hydrolysis that occurs under conditions where F₁ is present in excess over substrate MgATP (6). Complete characterization of all the rate and equilibrium constants of unisite catalysis allows one to construct Gibbs free energy diagrams for the entire catalytic pathway, including the catalytic transition state (7). Construction of difference energy diagrams for mutant versus wild-type enzymes then readily reveals situations where destabilization of the catalytic transition state has been brought about by mutagenesis (8, 9). With this technique, the roles of several critical catalytic site residues were defined in our laboratory (7–10). The x-ray structures have since confirmed their proximity to the bound substrate in the catalytic sites and revealed their actual locations in exquisite detail.

However, unisite catalysis assays require significant quantities of both enzyme and radioactive isotope and are time-consuming. An alternative approach to study the transition state that overcomes these problems would be of great value. Earlier, Vignais and colleagues (11–13) had shown that the MgADP-fluoroaluminate complex was a potent inhibitor of mitochondrial and _Escherichia coli_ F₁-ATPase. At that time, it was already recognized that the MgADP-fluoroaluminate complex bound in catalytic sites of ATP- or GTP-hydrolyzing enzymes was most likely mimicking the catalytic transition state, and the data reported by Vignais and colleagues strongly supported the view that this was also the case for F₁. Subsequent publication of x-ray crystallography structures of a variety of ATPase and GTPase enzymes with MgADP-fluoroaluminate bound in the catalytic sites (14–22) has established definitively that MgADP-fluoroaluminate complex is a catalytic transition state analog.¹

When the βY331W mutation is engineered into catalytic sites of _E. coli_ F₁, the fluorescence of residue β-Trp-331 provides a sensitive and specific probe of catalytic site nucleotide binding (23). The fluorescence signal can readily be monitored at low enzyme concentration, and because the aromatic ring of residue β-331 stacks against the adenine ring of bound nucleotide (24), the fluorescence signal is totally quenched upon binding of adenine nucleotide in the catalytic sites. We therefore hypothesized that in βY331W mutant enzyme (which, it should be stated, shows normal catalytic behavior in ATP synthesis and hydrolysis), the MgADP-fluoroaluminate complex should be readily detected as a very high affinity species by fluorescence titration. In this paper, we use this approach to measure the effects of fluoroaluminate on MgADP binding to MgADP-fluoroaluminate complex is generated by incubating together enzyme, MgADP, AlCl₃, and NaF. The fluoroaluminate species bound in the x-ray structures in different enzymes (14–22) was either AlF₄⁻ or AlO₂⁻. The fluoroaluminate species that occurs at the catalytic site of F₁ is not yet known from x-ray data, and we will refer to it here simply as fluoroaluminate.

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catalytic sites in normal, mutant, and DCCD\(^2\)-inhibited enzyme.

**EXPERIMENTAL PROCEDURES**

*Preparation of Enzyme*—Wild-type F\(_1\) was from strain SWM1 (25), \(\beta Y331W\) mutant F\(_1\) was from strain SWM4 (23), pK155Q/\(\beta Y331W\) mutant F\(_1\) was from strain pSWM31 (24), and \(\beta E181Q/\(\beta Y331W\) mutant F\(_1\) was from strain pSWM32 (24). Purification of F\(_1\) was as in Ref. 26. Before use the enzymes were passed twice through 1-mL centrifuge columns of Sephadex G-50 in 50 mM Tris/SO\(_4\), pH 8.0, as described (27), which was shown to effectively remove catalytic site-bound nucleotide ("native" enzyme). Nucleotide-depleted F\(_1\) (depleted of both catalytic and noncatalytic site-bound nucleotide) was prepared as described by Senior et al. (28). Before use, it was passed once through a centrifuge column equilibrated with 50 mM Tris/SO\(_4\), pH 8.0 (as above), and then EDTA was added to a final concentration of 2 mM. Then, after 1 h at room temperature, the enzyme was washed through a centrifuge column equilibrated in 50 mM Tris/SO\(_4\), pH 8.0.

*Inhibition of F\(_1\)-ATPase by MgADP-fluoroaluminate*—F\(_1\) (0.24 mg/mL) was preincubated at room temperature in 50 mM Tris/SO\(_4\), pH 8.0, with or without 2.5 mM MgADP, AlCl\(_3\), and NaF. Aliquots (100 \(\mu\)L) were removed, and ATPase activity was assayed in a total volume of 1 mL containing 50 mM Tris/SO\(_4\), pH 8.5, 10 mM NaATP, and 4 mM MgCl\(_2\) at room temperature for 2–5 min, at which time the reaction was stopped by the addition of 1 mL of 10% sodium dodecyl sulfate. F\(_1\) release was linear with time and was measured as described in Ref. 29.

*Inhibition of F\(_1\)-ATPase by DCCD*—This was carried out as described by Weber et al. (27) using 500 \(\mu\)M DCCD. The enzyme was assayed as above.

**RESULTS**

*Inhibition of ATP Hydrolysis Activity of \(\beta Y331W\) F\(_1\) by MgADP-fluoroaluminate Complex but Not by MgADP Alone*

Conditions for potent inhibition of mitochondrial and wild-type *E. coli* F\(_1\)-ATPase by MgADP in combination with AlCl\(_3\) and NaF, which together form fluoroaluminate complexes, have been documented by Vignais and colleagues (11–13). Here it was necessary to confirm that the \(\beta Y331W\) mutant enzyme was also subject to the same inhibition. Table I shows the data. It was found that inclusion of AlCl\(_3\) and NaF had no effect on the fluorescence spectrum of the \(\beta Y331W\) enzyme. Initial experiments were done on native enzyme, i.e. enzyme that had been passed sequentially through two 1-mL Sephadex G-50 columns as described under "Experimental Procedures." This treatment removes catalytic site-bound nucleotide but not endogenous noncatalytic site-bound nucleotide (27). For MgADP binding in the absence of fluoroaluminate (Fig. 1, circles), NaADP was added to enzyme in 50 mM Tris/SO\(_4\), pH 8.0, in the presence of 2.5 mM MgSO\(_4\), and the data were similar to what we have previously published (23, 24, 31). We previously determined that a model assuming two types of binding site, with one site of higher and two sites of lower affinity, gives a satisfactory fit to these data (23, 24, 31). When the same model was used here, calculated values of binding parameters were as follows: \(K_{d1} = 0.08 \mu\)M, \(n_1 = 1.21\); \(K_{d2} = 14 \mu\)M, \(n_2 = 1.62\). For MgADP binding in the presence of fluoroaluminate (Fig. 1, triangles), NaADP was added to enzyme in 50 mM Tris/SO\(_4\), pH 8.0, in the presence of 2.5 mM MgSO\(_4\), 0.5 mM AlCl\(_3\), and 5 mM NaF. The effect of fluoroaluminate was very obvious, with a large increase in binding of MgADP occurring at low MgADP concentrations. It was found that a theoretical model assuming three binding sites of different affinities gave a better fit to these data than a model with two types of site. Calculated values for binding parameters were as follows: \(K_{d1} = 0.06 \mu\)M, \(K_{d2} = 0.66 \mu\)M, \(K_{d3} = 40 \mu\)M. For comparison, if the data for MgADP binding in the absence of fluoroaluminate (Fig. 1, circles) were analyzed using the same three-site model, the calculated values for binding parameters were as follows: \(K_{d1} = 0.05 \mu\)M, \(K_{d2} = 2.54 \mu\)M, \(K_{d3} = 47 \mu\)M. (The three-site model was equally satisfactory as the two-site model for these data.) Overall, the Fig. 1 binding curves indicated that fluoroaluminate induced a very large increase in affinity for MgADP at site one, a significant effect at site two, and little or no effect at site three.

However, it should be noted that in the presence of fluoroaluminate (Fig. 1, triangles), at low concentrations of added NaADP, the stoichiometry of bound catalytic site MgADP considerably exceeded the actual amount of ADP added. The F\(_1\)

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\(\text{ADP/fluoroaluminate Binding to F}_1\)-ATPase 7053

**Table I**

*Inhibition of ATPase activity of \(\beta Y331W\) F\(_1\) by MgADP-fluoroaluminate complex*

| Additions | ATPase activity |
|-----------|----------------|
| None      | 100            |
| 2.5 mM MgADP | 92             |
| 1 mM AlCl\(_3\) | 97            |
| 5 mM NaF  | 97             |
| 1 mM AlCl\(_3\), 5 mM NaF | 97          |
| 2.5 mM MgADP, 1 mM AlCl\(_3\) | 95          |
| 2.5 mM MgADP, 5 mM NaF | 96          |
| 2.5 mM MgADP, 1 mM AlCl\(_3\), 5 mM NaF | 0          |

*Preparation of Enzyme*—Wild-type F\(_1\) was from strain SWM1 (25), \(\beta Y331W\) mutant F\(_1\) was from strain SWM4 (23), pK155Q/\(\beta Y331W\) mutant F\(_1\) was from strain pSWM31 (24), and \(\beta E181Q/\(\beta Y331W\) mutant F\(_1\) was from strain pSWM32 (24). Purification of F\(_1\) was as in Ref. 26. Before use the enzymes were passed twice through 1-mL centrifuge columns of Sephadex G-50 in 50 mM Tris/SO\(_4\), pH 8.0, as described (27), which was shown to effectively remove catalytic site-bound nucleotide ("native" enzyme). Nucleotide-depleted F\(_1\) (depleted of both catalytic and noncatalytic site-bound nucleotide) was prepared as described by Senior et al. (28). Before use, it was passed once through a centrifuge column equilibrated with 50 mM Tris/SO\(_4\), pH 8.0 (as above), and then EDTA was added to a final concentration of 2 mM. Then, after 1 h at room temperature, the enzyme was washed through a centrifuge column equilibrated in 50 mM Tris/SO\(_4\), pH 8.0.

*Inhibition of F\(_1\)-ATPase by MgADP-fluoroaluminate*—F\(_1\) (0.24 mg/mL) was preincubated at room temperature in 50 mM Tris/SO\(_4\), pH 8.0, with or without 2.5 mM MgADP, AlCl\(_3\), and NaF. Aliquots (100 \(\mu\)L) were removed, and ATPase activity was assayed in a total volume of 1 mL containing 50 mM Tris/SO\(_4\), pH 8.5, 10 mM NaATP, and 4 mM MgCl\(_2\) at room temperature for 2–5 min, at which time the reaction was stopped by the addition of 1 mL of 10% sodium dodecyl sulfate. F\(_1\) release was linear with time and was measured as described in Ref. 29.

*Inhibition of F\(_1\)-ATPase by DCCD*—This was carried out as described by Weber et al. (27) using 500 \(\mu\)M DCCD. The enzyme was assayed as above.

**Fluorescence Measurements**—Fluorescence measurements were made at room temperature in 50 mM Tris/SO\(_4\), pH 8.0. A SPEX Fluorolog 2 or Amino-Bowman 2 spectrofluorometer was used. The excitation wavelength was 285 nm, and fluorescence emission at 360 nm was used as the signal (23, 27). For MgADP titration in absence of fluoroaluminate, the buffer contained 2.5 mM MgSO\(_4\), and NaADP was added. For MgADP titration in presence of fluoroaluminate, the buffer contained 2.5 mM MgSO\(_4\), 0.5 mM AlCl\(_3\), and 5 mM NaF, and NaADP was added. For ATP depletion (absence of Mg\(^{2+}\)), the buffer contained 0.5 mM EDTA, and NaADP was added. Enzyme was preincubated 60 min at room temperature before fluorescence signals were measured to allow full inhibition by fluoroaluminate to be attained. Background signals (buffer) were subtracted, and inner filter and volume effects were corrected by performing parallel titrations with wild-type F\(_1\). Nucleotide binding parameters were analyzed by fitting theoretical curves to the measured data points assuming theoretical models with one, two, or three types of binding sites as described in detail previously (23, 24). MgADP concentrations were calculated using the stability constant of 78 \(\mu\)M from Ref. 30.

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The abbreviations used are: DCCD, dicyclohexylcarbodiimide; AMPPNP, adenosine 5'-(\(\beta\)-\(\gamma\)-imino)triphosphate.
concentration in Fig. 1 was 100 nM, and total MgADP concentration is plotted on the x axis. (It is assumed that total MgADP is equivalent to ADP added, since MgSO₄ concentration was 2.5 mM). A possible explanation for the anomaly could be that endogenous ATP or ADP is released from noncatalytic sites and becomes sequestered into catalytic sites by fluoroaluminate, because the MgADP-fluoroaluminate is bound at catalytic site one with extremely high affinity.

This possibility was investigated as follows. Native enzyme was preincubated in the presence of 2.5 mM MgSO₄, 0.5 mM AlCl₃, and 5 mM NaF, and then ATPase activity was assayed and found to be inhibited by 60%. In a parallel fluorescence experiment, the addition of 2.5 mM MgSO₄, 0.5 mM AlCl₃, and 5 mM NaF to enzyme in 50 mM Tris/SO₄, pH 8.0, induced a 10% quench of fluorescence. This fluorescence quench is equivalent to a catalytic site occupancy of 0.6 mol/mol, which is in excellent agreement with the degree of inhibition seen. In contrast, when nucleotide-depleted enzyme (depleted of catalytic and noncatalytic site nucleotide) was tested similarly, there was no inhibition of ATPase and only a small quench of fluorescence. Therefore, we concluded that in native enzyme, significant transfer of nucleotide from noncatalytic to catalytic sites did occur in presence of fluoroaluminate.

MgADP binding experiments were therefore repeated using nucleotide-depleted enzyme. For the data in the absence of fluoroaluminate (Fig. 2, circles), a model with two types of binding site gave a satisfactory fit to the data as seen previously (23, 24, 31), and the following values were calculated: $K_{d1} = 0.13 \mu M, n_1 = 1.29; K_{d2} = 6.8 \mu M, n_2 = 1.37$. These values are similar to our previously published values for nucleotide-depleted F₁ and are similar to the values for native enzyme (see above). A model with three sites of differing affinities gave an equally satisfactory fit with calculated values of $K_{d1} = 0.07 \mu M, K_{d2} = 1.1 \mu M, K_{d3} = 14 \mu M$.

In the presence of fluoroaluminate (Fig. 2, triangles), again a very large increase in binding stoichiometry at low MgADP concentrations was seen. These experiments utilized 26 nM F₁ concentration, which, under our experimental conditions, is at the low range permissible for a satisfactory fluorescence signal. In Fig. 2, we have plotted bound catalytic site MgADP versus calculated free, nonbound MgADP concentration (x axis). Even at the lowest concentration of free MgADP (1.7 nM), the first catalytic site was already filled when fluoroaluminate was present. A fit of the data to a model assuming two types of binding site was unsatisfactory, but a model assuming three sites of different affinities gave a satisfactory fit, and the calculated binding parameters were as follows: $K_{d1} \ll 1 \text{ nM}, K_{d2} = 0.06 \mu M, K_{d3} = 5.6 \mu M$. At concentrations of MgADP below the lowest point shown in Fig. 2, calculated free MgADP concentration was undependable, because it represented a tiny fraction of the total MgADP present (bound plus free). The value for $K_{d3}$ should be taken only as indicating an extremely high affinity for MgADP-fluoroaluminate at catalytic site one and not as an accurate estimate of binding affinity. A value far below 1 nM would not be surprising, Fig. 2 showed that $K_{d2}$ was also decreased in the presence of fluoroaluminate as compared with its absence.

Summarizing the data of Figs. 1 and 2, fluoroaluminate had a very large effect on MgADP binding at catalytic site one, a moderate effect at site two, and no significant effect at site three. The very tight MgADP-fluoroaluminate complex formed at catalytic site one may be regarded with confidence as a transition state analog, and it may well be that a complex with partial transition state-like properties forms also at catalytic site two. As noted under “Discussion,” there is prior evidence for binding of 2 mol of MgADP-fluoroaluminate/mol of F₁.

**Titration of βY331W F₁ with ADP in the Presence of AlCl₃ and NaF**

Fig. 3 shows binding of ADP, in the absence of Mg²⁺, to native βY331W mutant enzyme. It is evident that fluoroaluminate did not influence ADP binding in the absence of Mg²⁺. The data were analyzed assuming a model with one class of binding site, as used previously for ADP binding (23, 24, 31), and gave values of $K_d = 28 \mu M$ and $n = 2.8$ in the absence or presence of Mg²⁺.

**Fig. 1.** Titration of native βY331W F₁ with MgADP in the presence or absence of fluoroaluminate. ▲, presence of fluoroaluminate; ○, absence of fluoroaluminate. Protein concentration was 100 nM. The lines are fits to a theoretical model assuming two types of binding site (○) or three binding sites of different affinities (▲) (see “Results”). Total (bound plus unbound) MgADP concentration is plotted on the x axis. For further details, see “Experimental Procedures.”

**Fig. 2.** Titration of nucleotide-depleted βY331W F₁ with MgADP in the presence or absence of fluoroaluminate. ▲, presence of fluoroaluminate, enzyme concentration = 26 nM; ○, absence of fluoroaluminate, enzyme concentration = 50 nM. The lines are fits to theoretical models as described in the legend to Fig. 1. Free, unbound MgADP concentration is plotted on the x axis. For further details, see “Experimental Procedures.”
fluoroaluminate. Therefore, a transition state analog did not form under these conditions. This is consistent with x-ray structures of enzymes complexed with MgADP-fluoroaluminate, which show that Mg$^{2+}$ plays an important role in forming the complex itself and in liganding the complex to the enzyme catalytic site. Inhibition of F$_1$-ATPase by fluoroaluminate requires the presence of Mg$^{2+}$ (13), and the enzyme is inactive in the absence of Mg$^{2+}$.

Taken together, the results of Table I and Figs. 1–3 demonstrate that the fluorescence of residue β-Trp-331 provides a sensitive probe of high affinity binding of the MgADP-fluoroaluminate complex and of formation of a catalytic transition state-like complex, at the first, highest affinity catalytic site of F$_1$-ATPase.

**Effects of Mutations of Critical Catalytic Site Residues on Binding Affinity for the MgADP-fluoroaluminate Complex**

*Residue β-Lys-155—β-Lys-155* is the Lys residue of the Walker A consensus sequence. In the x-ray structure, it is seen to lie close to the phosphate moieties of the catalytic site-bound nucleotide (1), and as will be discussed later, it has previously been implicated as a critical functional residue. Here our goal was to determine its role in stabilizing the catalytic transition state using binding of MgADP-fluoroaluminate as the assay.

Fig. 4A shows titration of the native βK155Q/βY331W enzyme with MgADP in the presence and absence of fluoroaluminate. The two curves are essentially identical, showing there was no enhancement of MgADP binding by fluoroaluminate, in marked contrast to the results seen with the parent βY331W mutant F$_1$ in Fig. 1. This result demonstrates that formation of the catalytic transition state does not occur in the βK155Q mutant, giving direct confirmation of the role of β-Lys-155 in stabilizing the transition state.

*Residue β-Glu-181—This residue was earlier implicated as a critical catalytic residue by mutagenesis studies (see “Discussion”). In the x-ray structure (1), residue β-Glu-181 is seen to be located at some distance (4.1 Å) from the γ-phosphate, but it is close to a water molecule that appears hydrogen-bonded to the carboxyl oxygens and could be the substrate water for hydrolysis. Our goal was to analyze the role of β-Glu-181 in stabilizing the transition state using the MgADP-fluoroaluminate binding assay.

Fig. 4B shows titration of the native βE181Q/βY331W enzyme with MgADP in presence and absence of fluoroaluminate. There was no enhancement of MgADP binding by fluoroaluminate, demonstrating that formation of the catalytic transition state does not occur in the βE181Q mutant.

**Effects of DCCD Inhibition on Binding Affinity for the MgADP-fluoroaluminate Complex**

DCCD reacts with *E. coli* F$_1$, at residue β-Glu-192, the carboxyl oxygens of which are 16.6 Å away from the γ-phosphate of catalytic site-bound MgAMPPNP in the x-ray structure (1).
too far removed for this residue to be involved directly in transition state stabilization. Nevertheless, DCCD inhibits steady-state ATPase potently. In a recent paper (32), we proposed a mechanism for this inhibition (see “Discussion”), in which DCCD does not inhibit the catalytic step per se. It was therefore of interest to determine the effect of DCCD-reaction on formation of the transition state. As we showed previously, the DCCD-inhibited βY331W enzyme is readily amenable to assay of nucleotide binding by fluorescence assay (27).

Native βY331W mutant F₁ was reacted with DCCD as described under “Experimental Procedures.” MgATPase activity was inhibited by 99%. MgADP binding to DCCD-inhibited enzyme was then determined in the presence and absence of fluoroaluminate, as shown in Fig. 5. In the absence of fluoroaluminate, the data were similar to those obtained for the uninhibited enzyme as in Fig. 1. A model with two classes of binding site gave a satisfactory fit to the data, and the following values were calculated: $K_{d1} = 0.02 \mu M, n_1 = 0.6; K_{d2} = 4.2 \mu M, n_2 = 1.98$. A model with three sites of different affinities gave an equally satisfactory fit, and the calculated values were as follows: $K_{d1} = 0.07 \mu M, K_{d2} = 3.7 \mu M, K_{d3} = 11.3 \mu M$. Therefore, reaction with DCCD had little effect on MgADP binding affinity.

However, in the presence of fluoroaluminate (Fig. 5, triangles), it was apparent that MgADP binding affinity was very greatly increased at low MgADP concentrations, just as for uninhibited enzyme in Fig. 1. We again used a model assuming three sites of different affinities to calculate binding parameters from these data. The calculated values were as follows: $K_{d1} < 1 \mu M; K_{d2} = 0.15 \mu M; K_{d3} = 29 \mu M$. The value for $K_{d1}$ should again not be taken as an accurate assessment of binding affinity for MgADP-fluoroaluminate at catalytic site one, since this site was already filled at the lowest concentration of MgADP tested. The main point to be made is that the results with DCCD-inhibited enzyme in Fig. 5 were essentially the same as those for uninhibited enzyme in Fig. 1. We conclude that in the DCCD-inhibited enzyme a very large increase in binding affinity for MgADP was induced by fluoroaluminate at catalytic site one and therefore that DCCD reaction had little, if any, detrimental effect on formation of the transition state.

### DISCUSSION

MgADP-fluoroaluminate complex is known to mimic the catalytic transition state in numerous ATPase and GTPase enzymes (14–22). Here we used binding of MgADP-fluoroaluminate to determine formation of the transition state in F₁-ATPase. Binding affinity for MgADP in the presence of fluoroaluminate at each of the three catalytic sites in F₁ was determined using the fluorescence signal of residue β-Trp-331, engineered specifically into the catalytic sites in the βY331W mutant (23, 24).

The results leave no doubt that MgADP-fluoroaluminate is a transition state analog in F₁-ATPase. Extensive biochemical data had previously strongly indicated that this was the case (11–13). We found that MgADP binding affinity at catalytic site one was very greatly enhanced by fluoroaluminate and that Mg$^{2+}$ was required for this effect to occur. Furthermore, MgADP binding was not enhanced at all by fluoroaluminate in mutant enzymes (βK155Q and βE181Q) where side-chains of critical catalytic site residues, located close to bound nucleotide substrate, are modified. It should be noted that neither of these residues plays any role in liganding the Mg$^{2+}$ of the magnesium-nucleotide substrate (31). This new approach for determination of the transition state is rapid, uses a low concentration of enzyme in small volumes, and avoids the use of radioactivity, features that make it attractive for future studies of the transition state in normal, mutant, or inhibited enzyme.

As well as causing a very large increase in binding affinity for MgADP at the first catalytic site, fluoroaluminate had a smaller effect on MgADP binding at site two, indicating that an MgADP-fluoroaluminate complex might also be bound to site two. In previous work, it was demonstrated that the stoichiometry of binding of MgADP-fluoroaluminate complex to mitochondrial F₁ was 2 mol/mol (12, 13), based on measurement of bound ADP, aluminum, and fluoride. Later work (33) using kinetic methods with αβγ subcomplex of thermophilic bacillus PS3 F₁ has also supported a total binding stoichiometry of 2 mol of MgADP-fluoroaluminate/mol; however, this paper emphasized that full inhibition of ATPase was already achieved at a binding stoichiometry of 1 mol/mol. Taken overall, we believe the data indicate that a true transition state complex forms at catalytic site one, and a second MgADP-fluoroaluminate complex, possibly with partial transition state-like structure can also form at catalytic site two but not at site three.

As we have pointed out in a recent review (5), it is an assumption common to all current proposals for the catalytic mechanism of F₁-ATPase and ATP synthase that, under steady-state conditions at $V_{max}$, the actual chemical bond cleavage and synthesis reaction of ATP hydrolysis or synthesis occurs in only one of the three catalytic sites at any one time. This work supports this assumption, if one accepts the above arguments that only one catalytic site forms a true transition state complex. Presumably, formation of the catalytic transition state conformation in any one catalytic site precludes its simultaneous formation in either of the other two sites. In this behavior, F₁-ATPase is similar to the ABC transporter P-glycoprotein, where experiments using the transition state analog vanadate have demonstrated that, although both nucleotide-binding sites of P-glycoprotein are capable of catalysis, the transition state conformation can only be attained by one site at any one time (34).

In Fig. 4A, we show that fluoroaluminate did not enhance

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1 We did not examine the effect of mutation of any of the known Mg$^{2+}$-liganding residues of F₁ (β-Thr-156, β-Glu-185, or β-Asp-242; Ref. 31) in this work, because, based on x-ray crystallography studies (14–22), it may be anticipated that all will prevent binding of the MgADP-fluoroaluminate complex.
MgADP binding in the βK155Q/γY331W mutant, indicating that the catalytic transition state did not form. Residue β-Lys-155 is established as critical for catalysis. Mutagenesis of this residue impairs steady-state ATP hydrolysis rate by 3 or more orders of magnitude (9, 35). Studies of the Glu and Gln mutant enzymes revealed that β-Lys-155 is functionally important for binding of MgATP, through the γ-phosphate, particularly at catalytic sites one and two (9, 10, 24). However, it plays no functional role in MgADP binding (24). Unisite experiments (9, 10) indicated that it contributes to stabilization of the catalytic transition state, in agreement with the present data. We can therefore describe the role of this residue in catalysis as follows. It provides binding energy in MgATP hydrolysis by binding the MgATP and then stabilizes the transition state but not the product MgADP. In the synthesis direction, movement of this residue away from the γ-phosphate of tightly bound MgATP, during the rotation-induced “binding change,” is critical to allow release of MgATP.

In Fig. 4B, we show that the βE181Q/γY331W mutant F1 also showed no formation of the catalytic transition state complex. Mutagenesis of residue β-Glu-181 reduces the steady-state ATP hydrolysis rate by several orders of magnitude, depending on the mutation (9, 36, 37). In the Gln mutant, which reduces hydrolysis by more than 3 orders of magnitude, it was found that binding affinities of MgATP, MgADP, ATP, and ADP are all essentially normal (24, 31), demonstrating that the effect on catalysis was not in any way due to perturbation of binding of magnesium-nucleotide substrate and that residue β-Glu-181 is not involved in Mg\(^2\+\) liganding. In agreement with the current work, previous unisite experiments had indicated that the catalytic transition state was destabilized in this mutant (9). A similar conclusion can be drawn from the (less complete) set of unisite parameters presented for the Ala mutant (36).

One can conclude, therefore, that residue β-Glu-181 is uniquely involved in generation of the catalytic transition state and that it does not accelerate catalysis by providing nucleotide substrate binding energy. In the x-ray structure, β-Glu-181 is located 4.1 Å from the γ-phosphate of MgAMPPNP (1) and appears to be hydrogen-bonded to a water molecule through its carboxyl oxygens. Its role in generating and stabilizing the transition state is probably linked to its role in immobilizing and polarizing the substrate water, therefore.

The experiments with DCCD provide additional information regarding the mechanism of inhibition by this covalent inhibitor. Multisite MgATP hydrolysis is inhibited potently by DCCD, but unisite catalysis is inhibited only partly (38). At 99% inhibition of multisite catalysis, as achieved in this work, the stoichiometry of DCCD reaction (with residue β-Glu-192) is 2 mol/mol of F\(_1\) (38). Tryptophan fluorescence assays of MgATP binding to the DCCD-inhibited mutant enzymes βY331W and βF148W have recently been described and have led to a description of the mechanism of inhibition by DCCD (32). Briefly, it was concluded that DCCD acts by blocking a conformational signal transmission event between catalytic sites that occurs upon binding of MgATP to catalytic site three and that leads subsequently, in the catalytic MgATPase mechanism of Weber and Senior (5), to rapid hydrolysis of MgATP already bound at catalytic site one. Our explanation of DCCD-inhibition proposes that the actual catalytic step is not affected by DCCD reaction. The present experiments strongly support our proposed mechanism for DCCD inhibition by demonstrating that binding of MgADP-fluoroaluminate occurs normally in DCCD-reacted enzyme. The ability of the enzyme to attain the catalytic transition state is not affected by DCCD, although overall only 1% of activity remains. As noted above, DCCD reacts at a distance (16.6 Å) from the catalytic site and could not interfere directly with transition state formation or stabilization.

Abrahams et al. (1) in their analysis of the x-ray structure of F\(_1\) suggested that reaction with DCCD could inhibit by impeding conversion of the catalytic sites between conformational states, similar to our proposal, but also they suggested that DCCD might inhibit by impeding nucleotide access and binding to the catalytic sites, due to introduction of the bulky cyclohexyl moiety into a conical tunnel, which was proposed to allow nucleotide ingress and egress. While the results of this study would be in agreement with the latter suggestion (and it is pertinent that DCCD does partly inhibit unisite catalysis (38)), we feel that our previous data (32) favor the inhibitory mechanism discussed here and in Ref. 32. The fact that thermodynamic binding constants (K\(_d\)) are not affected by DCCD modification (Ref. 27 and this paper) requires that any change in association rate constants has to be accompanied by an exactly parallel change in dissociation rate constants. In the uninhibited enzyme, the distribution of nucleotides on the catalytic sites under V\(_{max}\) steady-state conditions (1 MgATP and 2 MgADP) establishes product MgADP release as the rate-limiting step of the overall hydrolysis reaction (5, 32, 39). If the main effect of DCCD reaction were reduction of substrate binding and product release rates by the same factor, the product release step would still be rate-limiting, and the MgATP/MgADP distribution should remain the same. However, it is changed to 1.6:1.4 in the inhibited enzyme (32), indicating that DCCD acts immediately subsequent to the MgATP binding step. It further may be noted that mutagenesis of the DCCD target residue β-Glu-192 to Gln significantly inhibits multisite ATP hydrolysis (by 93%; Ref. 9), and this occurs without introduction of significant new bulk in this region of the protein.

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