Modulation by ADP and Mg\(^{2+}\) of the Inactivation of the F\(_1\)-ATPase from the Thermophilic Bacterium, PS3, with Dicyclohexylcarbodiimide

Massasuke Yoshida and William S. Allison

From the Department of Biochemistry, Jichi Medical School, Minamikawachi-machi, Tochigi-ken, Japan 329-04 and the Department of Chemistry, School of Medicine, University of California, San Diego, La Jolla, California 92037

The soluble F\(_1\)-ATPase from the thermophilic bacterium PS3 (TF\(_1\)) contains no endogenous adenine nucleotides and contains about 0.2 g ions of Mg\(^{2+}\)/mol which resists removal by repeated centrifugation-elution on columns of Sephadex G-50. The isolated enzyme will not bind additional Mg\(^{2+}\) added in the absence of adenine nucleotides nor is the rate of inactivation of the isolated enzyme by dicyclohexylcarbodiimide (DCCD) affected by the addition of Mg\(^{2+}\). When ADP is added to isolated TF\(_1\), a 1:1 TF\(_1\)-ADP complex is formed which is stable to repeated gel permeation on columns of Sephadex G-50 subjected to centrifugation-elution. On formation of the 1:1 TF\(_1\), ADP complex, the rate of inactivation of the enzyme by DCCD is accelerated 6-fold. The rate of inactivation of the 1:1 TF\(_1\), ADP complex by DCCD is not further stimulated in the presence of 2 mM ADP which indicates that the binding of ADP to a single site in the enzyme is sufficient to promote maximal stimulation of the inactivation. Addition of Mg\(^{2+}\) to the 1:1 TF\(_1\), ADP complex results in the binding of about 1 g ion of Mg\(^{2+}\)/mol of enzyme. The 1:1:1 TF\(_1\), ADP-Mg\(^{2+}\) complex thus formed is sluggishly inactivated by DCCD. When the Mg\(^{2+}\) is removed from the TF\(_1\), ADP-Mg\(^{2+}\) complex by treatment with trans-1,2-diaminocyclohexane-N,N',N"-tetraacetic acid, the rate of inactivation of the enzyme by DCCD is accelerated 4-fold.

Other divalent metal ions protect the 1:1 TF\(_1\), ADP complex against inactivation by DCCD. Of these, Mn\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\), and Cd\(^{2+}\), which are about as equally effective as Mg\(^{2+}\) as cofactors for the hydrolytic reaction when present at 0.2 mM, offer about equal protection of the complex against inactivation by DCCD also when present at 0.2 mM. These results indicate that the binding site for ADP in the 1:1 TF\(_1\), ADP complex is a catalytic site.

TF\(_1\), inactivated by 92% with DCCD, has the same capacity to bind ADP as the active enzyme, forming a tight 1:1 TF\(_1\), ADP complex which is stable to repeated centrifugation-elution on columns of Sephadex G-50. The 1:1 TF\(_1\), ADP complex retains its capacity to bind Mg\(^{2+}\) to form the 1:1:1 TF\(_1\), ADP-Mg\(^{2+}\) complex after it is inactivated by 88% with DCCD.

Evidence is accumulating that the F\(_1\)-ATPases contain one or more carboxylic acid side chains which are essential for the hydrolytic activity catalyzed by these enzymes (1-13). The F\(_1\)-ATPases from a variety of organisms have been inactivated by DCCD (1, 3-5, 8, 11). With the use of \(^{14}C\)DCCD it has been demonstrated that the inactivations of the F\(_1\)-ATPases, TF\(_1\), MF\(_1\), and EF\(_1\), are due to the modification of a single glutamic acid side chain in the \(\beta\) subunit of each enzyme (8-10). Surprisingly, a different glutamic acid residue, designated by an asterisk in the sequence shown below, is labeled by \(^{14}C\)DCCD in the \(\beta\) subunit of TF\(_1\), than is labeled by \(^{14}C\)DCCD in the \(\beta\) subunits of MF\(_1\) and EF\(_1\), designated by a dagger in the sequence shown below. The highly conserved amino acid sequence in the \(\beta\) subunits of these enzymes which contain both the DCCD-reactive glutamic acid residue of TF\(_1\), (E*) and the DCCD-reactive glutamic acid residue of MF\(_1\) and EF\(_1\), (E\(\dagger\)) is: -A-G-V-E*-R-T-R-E-G-N-D-E-Y-H-E\(\dagger\)-M-.

The function or functions of the DCCD-reactive glutamic acid residue of TF\(_1\) and the DCCD-reactive glutamic acid residue common to MF\(_1\) and EF\(_1\) are not known. The rate of inactivation of the F\(_1\)-ATPases by DCCD is affected by the presence of adenine nucleotides and by Mg\(^{2+}\). The rate of inactivation of TF\(_1\) by DCCD is accelerated about 7-fold in the presence of ADP while it is only slightly stimulated by ATP (8).

The stimulatory effect of ADP on the inactivation of TF\(_1\) by DCCD is abolished by the addition of Mg\(^{2+}\). It was also observed that the addition of Mg\(^{2+}\) to native TF\(_1\) affords slight protection of the enzyme against inactivation by DCCD (8). The effects of adenine nucleotides and Mg\(^{2+}\) on the rate of inactivation by DCCD of MF\(_1\) depleted of loosely bound endogenous adenine nucleotides are very similar to those described for the inactivation of TF\(_1\) by DCCD (2, 14). In the presence of ADP or ATP the rate of inactivation of MF\(_1\) by DCCD is accelerated by about 50% while the rate of inactivation of the enzyme by DCCD is decreased by about 60% in the presence of Mg\(^{2+}\). The addition of Mg-ADP to MF\(_1\) depleted of loosely bound endogenous adenine nucleotides has very little effect on the rate of inactivation of the enzyme when compared to a control with no additions (14). The rate of inactivation of

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The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; TF\(_1\), the F\(_1\)-ATPase isolated from the plasma membranes of the thermophilic bacterium, PS3; EF\(_1\), the F\(_1\)-ATPase isolated from the plasma membranes of Escherichia coli; MF\(_1\), the F\(_1\)-ATPase isolated from bovine heart mitochondria; CF\(_1\), the F\(_1\)-ATPase isolated from chloroplast thylakoid membranes; MES, (2-(N-morpholino)ethanesulfonic acid; TEA, triethanolamine; CDTA, trans-1,2-diaminocyclohexane-N,N',N"-tetraacetic acid.
Modulation of DCCD Inactivation of TF\textsubscript{i} by ADP and Mg\textsuperscript{2+}

EF\textsubscript{i} (3) is affected differently by DCCD than is TF\textsubscript{i} and MF\textsubscript{i} in the presence of adenine nucleotides and Mg\textsuperscript{2+}. In the presence of ATP or ADP the rate of inactivation of EF\textsubscript{i} by DCCD is slowed by a factor of two. It is slowed 3- to 4-fold in the presence of Mg\textsuperscript{2+} (3). Therefore, in terms of the characteristics of inactivation by DCCD, MF\textsubscript{i} resembles TF\textsubscript{i} more than it does EF\textsubscript{i}, although MF\textsubscript{i} and EF\textsubscript{i} have a common glutamic acid residue that reacts with DCCD which is essential for the hydrolytic reaction catalyzed by the enzymes.

In order to provide clues to elucidate the function or functions of the carboxyl groups in the F\textsubscript{1}-ATPases which, when modified by DCCD abolish hydrolytic activity, an investigation was initiated to explore in more detail the characteristics of ADP and Mg\textsuperscript{2+} binding to TF\textsubscript{i}, which modulate the rate of inactivation of the enzyme by DCCD. These characteristics are presented and discussed in this report.

EXPERIMENTAL PROCEDURES

Materials—The adenine nucleotides used were of the highest purity available from Sigma. MES was purchased from Calbiochem-Behring. CDTA was purchased from Sigma. Stock solutions of DCCD (Sigma) were prepared in absolute ethanol. The divalent metal salts were of the highest purity available from Wako Chemical Company. The MgSO\textsubscript{4} was used as a special grade prepared for use in atomic absorption spectroscopy.

Preparation and Assay of TF\textsubscript{i}—TF\textsubscript{i} was prepared from plasma membranes of PSS as described in detail previously (15). The enzyme was stored as a lyophilized powder at −20°C after exhaustive dialysis of the purified enzyme against distilled water. TF\textsubscript{i} is stable for years when stored in this manner. The lyophilized enzyme was dissolved in the appropriate buffer just before each of the experiments described.

ATPase activity was determined at 25°C by a phosphate release method with an ATP-regenerating system (16) unless specified otherwise. Protein concentrations were determined with Coomassie brilliant blue as described by Bradford (17) using a standard curve prepared from lyophilized TF\textsubscript{i} for each experiment. The average of triplicate measurements for the standard curve and the unknowns was used. The centrifugation-elution method of Penefsky (18) was used to separate enzyme-bound ligands from free ligands using columns equilibrated with 50 mM TEA-SO\textsubscript{4}, pH 7.5, containing 1 μM CDTA unless specified otherwise. When the volume of the TF\textsubscript{i}-ligand solutions was 0.1 ml or less, 1.0-ml syringes were used for centrifugation-elution. For larger volumes up to 0.5 ml, centrifugation-elution was performed on 5-ml syringes.

Analysis of Enzyme-bound Adenine Nucleotides—Enzyme-bound adenine nucleotides were determined by anion exchange high performance liquid chromatography. Bound nucleotides were released from TF\textsubscript{i} by adding 2 μl of 60% perchloric acid to 50 μl of the enzyme-adenine nucleotide complex. After adding perchloric acid to the mixture, it was incubated at 0°C for 30 min at which time denatured enzyme was removed by centrifugation. Then 2 μl of 9.2 M KOH was added to the supernatants. The pH of the supernatants was adjusted to neutrality as indicated by pH test paper by the addition of small volumes of 9.2 M KOH or 85% H\textsubscript{3}PO\textsubscript{4}. The precipitated KClO\textsubscript{4} in the supernatant was applied to a Toya Soda IEX-540 column (4 × 300 mm) which was equilibrated with 400 mM sodium phosphate, pH 6.0. The column was eluted isocratically with the same buffer at a flow rate of 0.8 ml/min using a Waters model 204 liquid chromatograph. Nucleotides were monitored at 254 nm. Peak areas were determined by automatic integration. Using this method adenine nucleotides released from the enzyme were rapidly analyzed in the concentration range of 10 to 500 pmol.

Analysis of Enzyme-bound Mg\textsuperscript{2+}—The Mg\textsuperscript{2+} content of the enzyme was determined by atomic absorption spectroscopy using a Varian model 150 flame atomic absorption analyzer. The buffer in which the enzyme was dissolved, 50 mM TEA-SO\textsubscript{4}, pH 7.3, containing 1 μM CDTA, contained up to 0.8 μM contaminating Mg\textsuperscript{2+}. Therefore, due to this background it was necessary to use enzyme concentrations greater than 10 mg/ml (26 PM) to ensure accurate analysis of Mg\textsuperscript{2+} contributed by the enzyme. Standardization was carried out by the method of additions. For a series of solutions with different enzyme concentrations, each was mixed with Mg\textsuperscript{2+} solutions of increasing concentration and then subjected to analysis. The Mg\textsuperscript{2+}:TF\textsubscript{i} ratios were determined from the average of the values obtained for each proteic concentration.

RESULTS

Sensitization of TF\textsubscript{i} to DCCD Inactivation by Pretreatment with ADP or ATP—Table I shows that treatment of TF\textsubscript{i} with ADP or ATP followed by centrifugation-elution on Sephadex G-50 (18) leaves the enzyme in a form more sensitive to inactivation by DCCD. ADP is more effective in this capacity than is ATP. Sensitization of TF\textsubscript{i} to DCCD inactivation is not promoted by AMP or P\textsubscript{i}. When TF\textsubscript{i}, sensitized to DCCD inactivation in the manner described above, is subsequently treated with Mg\textsuperscript{2+}, inactivation by DCCD becomes sluggish as shown in Table I. The TF\textsubscript{i}·ADP complex responds rapidly to the addition of Mg\textsuperscript{2+} as is illustrated by the lower curve in Fig. 1. The addition of DCCD to the TF\textsubscript{i}·ADP complex prepared by centrifugation-elution led to a rapid rate of inactivation. When Mg\textsuperscript{2+} was added 25 min after the inactivation was initiated, the rapid inactivation ceased abruptly. The upper curve in Fig. 1 shows that the addition of ADP to a reaction mixture containing DCCD and TF\textsubscript{i} leads to an abrupt increase in the rate of inactivation of the enzyme.

Binding of ADP and Mg\textsuperscript{2+} to TF\textsubscript{i}, Correlated with Sensitivity to Inactivation by DCCD—The amount of ADP and Mg\textsuperscript{2+}

| Additions before centrifugation-elution | Rate of inactivation by DCCD |
|----------------------------------------|-----------------------------|
| None*                                  | 2 mM ADP\textsuperscript{**} 2 mM Mg\textsuperscript{2+} |
|                                        | 10\textsuperscript{6} 1/min |
| None                                   | 3                            23 |
| 2 mM AMP                               | 23                           |
| 2 mM ADP                               | 22                           |
| 2 mM ATP                               | 11                           |
| 2 mM P\textsubscript{i}                | 3                            |

*Additions after centrifugation-elution.

![Fig. 1. The effect of added ADP or Mg\textsuperscript{2+} on the rate of inactivation of TF\textsubscript{i} by DCCD.](http://www.jbc.org)
bound to TF, under various conditions of pretreatment followed by centrifugation-elution is presented in Table II which also shows the rate of inactivation of the enzyme by 0.15 mM DCCD in the presence and absence of 2 mM ADP. The isolated enzyme used in this study did not contain bound ADP nor did it contain bound ATP. The amount of Mg$^{2+}$ detected in the isolated enzyme by atomic absorption spectroscopy was not stoichiometric as shown in the control of Table II. Pretreatment of the enzyme with CDTA or Mg$^{2+}$ did not change the Mg$^{2+}$ content of TF, nor did it affect its inactivation by DCCD as shown by Experiments 1 and 2 of Table II. Incubation of TF, with ADP in the absence of Mg$^{2+}$ leads to the binding of 1.5 mol of ADP/mol of TF, after a single centrifugation-elution as shown in Experiment 3a of Table II. This value decreased to 1.1 mol of ADP/mol of TF, when the TF, - ADP complex was subjected to a second centrifugation-elution step as shown by Experiment 3b. Both complexes are equally sensitive to inactivation by DCCD in the presence and absence of 2 mM ADP. This shows that the binding of 1 mol of ADP per mol of TF, is sufficient to cause maximum stimulation of inactivation by DCCD. The TF, - ADP complex obtained after the second centrifugation-elution step is stable. It has been stored for 1 week at 4°C with no change in the amount of bound ADP when examined by centrifugation-elution and with no change in its sensitivity to inactivation by DCCD. However, a single ammonium sulfate precipitation removed 20% of the ADP bound to the TF, - ADP complex which may explain the absence of endogenous ADP in the preparation of TF, used in this study and the very low content of endogenous adenine nucleotides usually found in TF, preparations (19).

Fig. 2 shows the effect of increasing Mg$^{2+}$ concentrations on the rate of inactivation of the TF, - ADP complex by DCCD. Saturation occurs at \( \leq 20 \mu M \) Mg$^{2+}$ showing that the TF, - ADP complex has a high affinity for Mg$^{2+}$ which does not exist in the apoenzyme. When 2 mM ADP is present in the inactivation medium, higher concentrations of Mg$^{2+}$ are required to saturate the complex for inhibition of DCCD inactivation. Medium ADP acts like medium EDTA by chelating Mg$^{2+}$, thus reducing the concentration of free Mg$^{2+}$ available to bind the ADP moiety of the TF, - ADP complex. Mg$^{2+}$ binds in amounts stoichiometric with bound ADP when it is added to the TF, - ADP complex as shown by Experiment 3c of Table II. When TF, was pretreated with ADP and Mg$^{2+}$ simultaneously and then subjected to a single centrifugation-elution step, 3 mol of both ADP and Mg$^{2+}$ were bound per mol of TF,.

### Table II

**Correlation of ADP and Mg$^{2+}$ binding to TF, with its rate of inactivation by DCCD**

| Experiment | Pretreatment | Mol ADP/mol TF, | Mol$\dagger$ Mg$^{2+}$/mol TF, | 10$^3$ k$_{max}$ min$^{-1}$ (no additions) | 10$^3$ k$_{max}$ min$^{-1}$ with 2 mM ADP |
|------------|--------------|----------------|-------------------------------|---------------------------------|-----------------------------------|
| Control 1  | None         | 0              | 0.2                           | 5                               | 28                                |
| 2          | 10 mM CDTA   | 0              | 0.3                           | 5                               | 29                                |
|            | a. Dialysis  | 0              | 0.3                           | 5                               | 29                                |
| 3          | 5 mM Mg$^{2+}$ | 0              | 0.3                           | 5                               | 29                                |
|            | a. CE after 1 h | 1.5             | 0.3                           | 30                              | 28                                |
|            | b. 2nd CE after 12 h | 1.1             | 0.2                           | 29                              | 28                                |
|            | c. Mg$^{2+}$ brought to 5 mM | 1.0             | 1.1                           | 6                               | 6                                 |
|            | in effluent b, then CE after 1 h | 1.0             | 1.1                           | 6                               | 6                                 |
| 4          | 2 mM ADP plus 5 mM Mg$^{2+}$ | 3.0             | 3.2                           | 8                               | 8                                 |
|            | a. CE after 1 h | 3.0             | 3.2                           | 8                               | 8                                 |
|            | b. 2 additional CE's at 12-h intervals | 1.1             | 0.9                           | 9                               | 9                                 |
|            | CDTA brought to 20 mM in effluent b, then CE after 12 h | 0.9             | 0.1                           | 30                              | 36                                |

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The adenine nucleotide and Mg$^{2+}$ content, protein concentration, and rate of inactivation by DCCD in the presence and absence of ADP were carried out as described under "Experimental Procedures" after pretreating the enzyme with ADP and/or Mg$^{2+}$ as described above. The centrifugation-elutions (CE) were performed on columns of Sephadex G-50 equilibrated with 50 mM TEA-NO$_3$, pH 7.3, containing 1 \( \mu M \) CDTA. Control, TF (2 mg) was dissolved in 0.10 ml of 50 mM TEA-NO$_3$, pH 7.3, containing 1 \( \mu M \) CDTA. Experiment 1, TF (5 mg) was dissolved in 0.13 ml of 20 mM TEA-NO$_3$, pH 7.3, containing 10 \( \mu M \) CDTA and was then dialyzed against the same buffer for 12 h at 25°C at which time it was dialyzed against deionized water. The dialyzed enzyme was lyophilized and then dissolved in 0.10 ml of 50 mM TEA-NO$_3$, pH 7.3, containing 1 \( \mu M \) CDTA to produce effluent 1a from which samples were removed for the analyses listed. Experiment 2, TF (2 mg) was dissolved in 0.10 ml of TEA-NO$_3$, pH 7.3, containing 1 \( \mu M \) CDTA and 5 mM MgSO$_4$. After 1 h at 25°C this solution was subjected to centrifugation-elution to produce effluent 2a from which samples were removed for analyses. Experiment 3, TF (2 mg) was dissolved in 0.60 ml of 50 mM TEA-NO$_3$, pH 7.3, containing 1 \( \mu M \) CDTA and 5 mM MgSO$_4$. After 1 h at 25°C this solution was subjected to centrifugation-elution to produce effluent 3a from which samples were removed for analyses. The remainder of effluent 3a was incubated for 12 h at which time it was subjected to centrifugation-elution to produce effluent 3b from which samples were removed for the analyses listed. To the remainder of effluent 3b (0.12 ml) was added 2.4 \( \mu l \) of 0.18 M MgSO$_4$. The resulting solution was subjected to centrifugation-elution after 1 h to produce effluent 3c from which samples were removed for analyses. Experiment 4, TF (13 mg) was dissolved in 0.60 ml of 50 mM TEA-NO$_3$, pH 7.3, containing 1 \( \mu M \) CDTA, 5 mM MgSO$_4$, and 2 mM ADP. After 1 h at 25°C the solution was subjected to centrifugation-elution to produce effluent 4a from which samples were removed for analyses. The remainder of effluent 4a was incubated for 12 h at which time it was subjected to centrifugation-elution, and the effluent was incubated an additional 12 h at which time it was subjected to centrifugation-elution to produce effluent 4b from which samples were removed for analyses. To the remainder of effluent 4b (0.19 ml) was added 48 \( \mu l \) of 0.10 M CDTA. The resulting solution was incubated at 25°C for an additional 12 h at which time it was subjected to the third centrifugation-elution to produce effluent 4c from which samples were removed for analyses.
as shown by Experiment 4a. However, when the TF₁-ADP-Mg²⁺ complex obtained in this manner was subjected to two more centrifugation-elution steps, ADP and Mg²⁺ were released simultaneously. After the intermediate centrifugation-elution step 1.4 mol of ADP and 1.5 mol of Mg²⁺ were bound per mol of TF₁.

The Mg²⁺ in the TF₁-ADP-Mg²⁺ complex can be removed by the addition of CDTA as shown in Experiment 4c of Table II. Readdition of Mg²⁺ to the TF₁-ADP complex so obtained by resolution with CDTA followed by centrifugation-elution led to the reformation of the TF₁-ADP-Mg²⁺ complex which again reacted sluggishly with DCCD. The Mg²⁺ moiety of the TF₁-ADP-Mg²⁺ complex does not appear to be firmly bound. When the ternary complex is diluted after isolation in buffers to inactivation by DCCD increases slowly, indicating that the Mg²⁺ component dissociates slowly.

![Image of Fig. 2](http://www.jbc.org/)

**FIG. 2.** Rate of inactivation of the TF₁-ADP complex by DCCD as a function of [Mg²⁺]. The TF₁ was loaded with ADP by treating 7.2 µg of the enzyme in 300 µl of 50 mM TEA-SO₄, pH 7.3, with 2 mM ADP for 1 h at 20 °C at which time it was subjected to gel filtration on Sephadex G-50 which was equilibrated and eluted with 50 mM TEA-SO₄, pH 7.3. The inactivation mixtures, 300 µl each in 50 mM TEA-SO₄, pH 7.3, contained 120 µg of TF₁, 0.15 mM DCCD, and the MgSO₄ concentrations indicated. Other additions were: none (Ο); 2 mM ADP (●); and 2 mM EDTA (△).

**TABLE III**

The divalent metal ion specificity of the ATPase reaction and for protection against inactivation by DCCD

Divalent Metal Ions That Protect TF₁ against Inactivation by DCCD—The relative effectiveness of various divalent metal ions which protect the TF₁-ADP complex against inactivation by DCCD when present at a concentration of 0.2 mM is shown in Table III. Also shown in Table III is the relative effectiveness of the same metal ions as cofactors in the ATPase reaction. It is clear that those divalent metal ions (Mn²⁺, Zn²⁺, Co²⁺, and Mg²⁺) which are the most active catalytically are also the most effective in protecting the TF₁-ADP complex against inactivation by DCCD. This suggests that the divalent metal ions protect TF₁ against inactivation by DCCD when they are bound to the catalytic site of the enzyme. We have no explanation for the observation that Cd²⁺, Ni²⁺, and Fe²⁺ appear to be more active as coenzymes when assayed with TF₁, than do when they are assayed with the TF₁-ADP complex. The ATPase activity is very low in the presence of 0.2 mM Ca²⁺ when compared to its activity in the presence of 0.2 mM Mg²⁺. This appears to contradict results previously reported which showed that 5 mM Ca²⁺ is as effective as 5 mM Mg²⁺ as cofactor for the hydrolytic reaction catalyzed by TF₁ (15). However, at 5 mM both divalent metal ions saturate the enzyme and, therefore, the differences observed between Ca²⁺ and Mg²⁺ at low nonsaturating concentrations were not exhibited.

The Binding of ADP to TF₁ Inactivated with DCCD—When TF₁ was inactivated by 92% with DCCD the enzyme retained its capacity to bind ADP at pH 7.3 as shown by comparison of Experiment 3 of Table II with Experiment 2 of Table IV. The DCCD-inactivated enzyme, like native TF₁, binds 1 mol of ADP tightly which is not removed by repeated centrifugation-elutions. When Mg²⁺ and ADP are added together to TF₁, ADP is retained as shown by Experiment 4a. However, when the TF₁-ADP-Mg²⁺ complex obtained in this manner was subjected to two more centrifugation-elution steps, ADP and Mg²⁺ were released simultaneously. After the intermediate centrifugation-elution step 1.4 mol of ADP and 1.5 mol of Mg²⁺ were bound per mol of TF₁.

![Image of Table IV](http://www.jbc.org/)

**TABLE IV**

Binding of ADP to TF₁ inactivated with DCCD

The DCCD-inactivated enzyme was prepared as follows. To 15 µg of TF₁ in 1.0 ml of 50 mM Na-MES, pH 5.5, containing 1 µM CDTA was added 20 µl of 10 mM DCCD. 150 min after initiating the inactivation a second 20 µl dose of DCCD was added. After 10 h 92% inactivation was attained at which time the reaction mixture was subjected to centrifugation-elution (CE) on Sephadex G-50 equilibrated with 50 mM TEA-SO₄, pH 7.3, containing 1 mM CDTA. Experiment 1, samples of the DCCD-inactivated enzyme were removed for the determination of ADP and protein. Experiment 2, to 0.4 ml of the DCCD-inactivated enzyme was added 16 µl of 100 mM ADP. This mixture was incubated for 1 h at 25 °C at which time it was subjected to centrifugation-elution to produce effluent 2a. Samples of effluent 2a were removed for analyses and the remainder was incubated an additional 12 h at 25 °C at which time it was subjected to centrifugation-elution to produce effluent 2b. Samples of effluent 2b were removed for analyses. Experiment 3, to 0.2 ml of DCCD-inactivated TF₁ was added 8 µl of 100 mM ADP and 10 µl of 100 mM MgSO₄. This mixture was incubated for 1 h at 25 °C at which time it was subjected to centrifugation to produce effluent 2a. Samples of effluent 2a were removed for analyses and the remainder was incubated an additional 12 h at 25 °C at which time it was subjected to centrifugation-elution. The resulting effluent was incubated an additional 4 h at 25 °C at which time it was subjected to another centrifugation-elution to produce effluent 2b. Samples of effluent 2b were then removed for analyses.
TABLE V
Binding of Mg$$^{2+}$$ to the TF$$\_1$$-ADP complex after inactivation by DCCD

| Treatment of the TF$$\_1$$-ADP complex | mol ADP/mol TF$$\_1$$ | g ions Mg$$^{2+}$$/mol TF$$\_1$$ |
|---------------------------------------|----------------------|-------------------------------|
| A. CE before DCCD inactivation        | 0.1                  | 0.2                           |
| B. CE after DCCD inactivation         | 0.9                  | 0.1                           |
| C. CE after addition of Mg$$^{2+}$$ to effluent B to 2 mM | 0.8                  | 0.9                           |

or TF$$\_1$$ inactivated with DCCD, 3 mol of ADP remained bound per mol of enzyme after a single centrifugation step as shown by Experiment 4a of Table II and Experiment 3a of Table IV. After repeated centrifugation-elutions the ADP content of both the native and DCCD-inactivated TF$$\_1$$ decreased to about 1 mol per mol of enzyme when the initial loading with ADP took place in the presence of Mg$$^{2+}$$ as shown in Experiment 3 of Table IV.

The Binding of Mg$$^{2+}$$ to the TF$$\_1$$-ADP Complex Inactivated with DCCD—When the 1:1 TF$$\_1$$-ADP complex was inactivated by 88% with DCCD, the bound ADP in the complex resisted removal by centrifugation-elution as shown in Table V. After inactivation with DCCD the 1:1 TF$$\_1$$-ADP complex retained its capacity to bind Mg$$^{2+}$$ as shown by comparison of Experiment 3c of Table II with Experiment C of Table V. Therefore, the DCCD-reactive glutamic acid residue in TF$$\_1$$ which, when modified leads to inactivation of the enzyme, does not appear to function in binding the Mg$$^{2+}$$ moiety of Mg$$^{2+}$$-adenine nucleotide complexes as was suggested by us previously (8, 10).

DISCUSSION

From the results presented it is clear that Mg$$^{2+}$$ does not bind tightly to TF$$\_1$$ in the absence of bound adenine nucleotides nor does the addition of Mg$$^{2+}$$ to TF$$\_1$$ free of adenine nucleotides protect the enzyme against inactivation by DCCD. Although the TF$$\_1$$ used in the experiments reported here was completely free of endogenous adenine nucleotides, some preparations of the enzyme have been found to contain small amounts of endogenous ADP. The presence of a small amount of ADP in the TF$$\_1$$ preparation examined by us previously (8) might have been responsible for the slight protective effect that was observed when DCCD inactivation was carried out in the presence of Mg$$^{2+}$$ but in the absence of exogenous adenine nucleotides. The observation that TF$$\_1$$ contains very little endogenous Mg$$^{2+}$$ differs considerably from the results of Senior and his colleagues who have reported that isolated MF$$\_1$$ contains 1 g ion of tightly bound Mg$$^{2+}$$ per mol which remains bound even after the tightly bound adenine nucleotides were removed from the enzyme (20, 21). Isolated EF$$\_1$$ has been reported to contain about 2 g ions of Mg$$^{2+}$$ per mol (21). To explain the fact that MF$$\_1$$ depolymerizes when the tightly bound Mg$$^{2+}$$ is removed from it, Senior has postulated that the tightly bound Mg$$^{2+}$$ is an integral component of the active enzyme complex which might function to stabilize the quaternary structure of the complex (20). From the results presented here, it is clear that TF$$\_1$$ does not require endogenous Mg$$^{2+}$$ for stabilization of its quaternary structure.

Scheme 1 summarizes the sensitivity of different TF$$\_1$$ complexes to DCCD inactivation which have been isolated after incubating the enzyme with ADP or ADP plus Mg$$^{2+}$$ and then subjecting it to centrifugation-elution in buffers not containing the ligands. When TF$$\_1$$ binds only ADP the rate of inactivation of the enzyme is stimulated as designated by an asterisk in Scheme 1. This increased sensitivity of TF$$\_1$$ to inactivation by DCCD is probably due to the binding of ADP to a single catalytic site for the following reasons. 1) The 1:1 TF$$\_1$$-ATP complex has been isolated and shows maximal stimulation of DCCD inactivation as indicated in Scheme 1. 2) Only those divalent metal ions which are active catalytically protect the 1:1 TF$$\_1$$-ADP complex against inactivation by DCCD. 3) It has been shown that the ADP in the 1:1 TF$$\_1$$-ADP complex can be converted to ATP when the complex is incubated with Mg$$^{2+}$$ and Pi in 50% dimethyl sulfoxide under slightly acidic conditions (22).

The observations that the occupancy of a single catalytic site of TF$$\_1$$ by ADP is sufficient to promote full acceleration of inactivation of the enzyme by DCCD raises interesting questions concerning the stoichiometry of modification of the enzyme by the reagent. It has been shown that the complete inactivation of TF$$\_1$$ with [14C]DCCD in the presence of 1 mM ADP is accompanied by the modification of 1.6-1.8 copies of the β subunit to form a single N-γ-glutamyl derivative of dicyclohexyl[14C]urea with each β subunit modified (8). This suggests that selective modification of two of the three β subunits of the enzyme with DCCD is sufficient to abolish all ATPase activity. However, as has been discussed in detail elsewhere (10), it is not certain that the O-acylisourea formed on the initial reaction of the essential carboxyl group with DCCD rearranges quantitatively to form the N-acrylurea which is isolated. Thus firm experimental evidence is not available to distinguish between "two-thirds of the sites reactivity" and "all of the sites reactivity" when TF$$\_1$$ is inactivated by DCCD. Cross and Nalin have suggested on the basis of diminished binding of [3H]adenyl 5'-yl imidodiphosphate to MF$$\_1$$ inactivated with DCCD that inactivation of the mitochondrial enzyme proceeds with "two-thirds of the sites reactivity" (23). However, since DCCD modifies different glutamic acid side chains when it inactivates TF$$\_1$$ and MF$$\_1$$ (8), the observations of Cross and Nalin with MF$$\_1$$ may not bear on the stoichiometry of carboxyl group modification which occurs when TF$$\_1$$ is inactivated by DCCD.

![Scheme 1. Summary of the sensitivity of the various complexes of TF$$\_1$$ and ADP and Mg$$^{2+}$$ to inactivation by DCCD.](http://www.jbc.org/)

The moles of ADP and Mg$$^{2+}$$ bound to TF$$\_1$$ have been reduced to the nearest integer for convenience. CE, centrifugation-elution.
Modulation of DCCD Inactivation of TF₁ by ADP and Mg²⁺

Feldman and Sigman have shown that isolated CF₁ will synthesize enzyme-bound ATP under slightly acidic conditions (24). Isolated MF₁ (25) and isolated TF₁ (22) will also synthesize enzyme-bound ATP under certain conditions. Moreover, the studies with the latter two enzymes have provided evidence which suggests that the DCCD-reactive glutamic acid residue of TF₁ has a different functional role than the DCCD-reactive glutamic acid residue to MF₁. Sakamoto and Tomonura have shown that MF₁ will synthesize up to 0.6 mol of bound [³²P]ATP per mol of the MF₁ in the presence of 30% dimethyl sulfoxide, ADP, ³²P, and Mg²⁺ at slightly acidic pH (25). They also showed that MF₁ inactivated with DCCD retained full capacity to synthesize enzyme-bound ATP under the same conditions. The differential effect of DCCD on the hydrolytic reaction opposed to the artificial synthesis catalyzed by MF₁ is similar to the well documented observation that isolated MF₁ modified with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole will not catalyze ATP hydrolysis, but will catalyze ATP synthesis when added to depleted submitochondrial particles (26, 27). Enzyme-bound ATP is also formed when F₁ and Mg²⁺ are added to the TF₁-ADP complex in 50% dimethyl sulfoxide under slightly acidic conditions (22). In contrast to what is observed with MF₁, enzyme-bound ATP is no longer formed under these conditions when the TF₁-ADP complex is inactivated with DCCD.²

Therefore, the DCCD-reactive glutamic acid side chain of TF₁ appears to be essential for both ATP synthesis and ATP hydrolysis while the DCCD-reactive glutamic acid side chain of MF₁ is essential for ATP hydrolysis but apparently not for ATP synthesis. These results suggest that the DCCD-reactive glutamic acid residue of TF₁ has a direct role in the reversible reaction catalyzed by the enzyme while the DCCD-reactive glutamic acid side chain of MF₁ does not.

If the DCCD-reactive glutamic acid residue of TF₁ does indeed have a direct functional role in catalysis then why are the corresponding glutamic acid residues in the β subunits of MF₁ and EF₁ unreactive with DCCD? Very little information is available which bears directly on this dilemma. However, several reports have appeared which indicate that individual F₁-ATPases contain more than one essential carboxyl group which can be distinguished by their reactivities with different electrophilic reagents. For example, Pougeois and his colleagues have presented evidence that N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline and DCCD modify different carboxyl groups when MF₁ is inactivated by these reagents (2, 3, 12). Vallesjos and his colleagues (6, 7, 13) have provided evidence that Woodward’s reagent K inactivates CF₁ (6), TF₁ (7), and the F₁-ATPase from Rhodospirillum rubrum chromatophores (13) by modification of a different carboxyl group in each of these enzymes than is modified during inactivation with DCCD. It is possible that the essential carboxyl groups in these ATPases which react with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline or Woodward’s reagent K might correspond to the DCCD-reactive glutamic acid residue of TF₁. It is important to note that the participation of a carboxyl group does not necessarily mean that the hydrolytic reaction catalyzed by the mitochondrial F₁-ATPase has been obtained from kinetic analyses (28). Godinot and Penin have demonstrated the participation of a carboxyl group in the hydrolytic reaction catalyzed by MF₁ from pig heart by careful examination of the pH rate profiles of the enzyme as a function of buffer ion species, organic solvents, and temperature (28). From this examination they concluded that Vₘₐₓ and Vₘₐₓ/Kₘ of ATP hydrolysis depend on the presence of the conjugate base of a carboxyl group with a pKₐ of 5.4–5.9.

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