Characterization of the Catalytic and Noncatalytic ADP Binding Sites of the F₁-ATPase from the Thermophilic Bacterium, PS₃

(Received for publication, November 22, 1985)

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Two classes of ADP binding sites at 20 °C have been characterized in the F₁-ATPase from the thermophilic bacterium, PS₃ (TF₁). One class is comprised of three sites which saturate with [³H]ADP in less than 10 s with a $K_d$ of $10 \mu M$ which, once filled, exchange rapidly with medium ADP. The binding of ADP to these sites is dependent on Mg²⁺. [³H]ADP bound to these sites is removed by repeated gel filtrations on centrifuge columns equilibrated with ADP free medium. The other class is comprised of a single site which saturates with [³H]ADP in 3 min with a $K_d$ of 30 $\mu M$. [³H]ADP bound to this site does not exchange with medium ADP nor does it dissociate on gel filtration through centrifuge columns equilibrated with ADP free medium. Binding of [³H]ADP to this site is weaker in the presence of Mg²⁺, where $K_d$ for ADP is about 100 $\mu M$. [³H]ADP dissociated from this site when ATP plus Mg²⁺ was added to the complex while it remained bound in the presence of ATP alone or in the presence of ADP, P₃, or ADP plus P₃ with or without added Mg²⁺. Significant amounts of ADP in the 1:1 TF₁·ADP complex were converted to ATP in the presence of P₃, Mg²⁺, and 50% dimethyl sulfoxide. Enzyme-bound ATP synthesis was abolished by chemical modification of a specific glutamic acid residue by dicyclohexylcarbodiimide, but not by modification of a specific tyrosine residue with 7-chloro-4-nitrobenzofurazan. Difference circular dichroism spectra revealed that the three Mg²⁺-dependent, high affinity ADP binding sites were on the $\alpha$ subunits and that the single ADP binding site that was stable to gel filtration was on one of the three $\beta$ subunits. It has also been demonstrated that enzyme-bound ATP is formed when the TF₃·F₁ complex containing bound ADP was incubated with P₃, Mg²⁺, and 50% dimethyl sulfoxide.

The energy transducing ATP synthase complexes of bacterial plasma membranes, chloroplast thylakoid membranes, the mitochondrial inner membrane, and the chromatophore membranes of photosynthetic bacteria catalyze ATP synthesis driven by electron transport processes (1). The enzyme is composed of an integral membrane protein sector, F₀, which mediates H⁺ conduction, and a peripheral protein sector, F₁, which contains the catalytic site for ATP synthesis. F₁ is composed of five different polypeptide chains designated $\alpha$-$\epsilon$ in order of decreasing Mr. The molecular weight of F₁, from several sources is about 380,000 and the subunit stoichiometry is $\alpha_3\beta_3\gamma_8\delta_5$ (5–8). The results obtained from chemical modification studies indicate that the active sites of F₁ are located on the $\beta$ subunits alone (6, 9) or at the interface of $\alpha$ and $\beta$ subunits (10, 11).

The isolated $\alpha$ and $\beta$ subunits of bacterial F₁-ATPases can bind ATP or ADP (12–14). Excluding CF₁, for which only three adenine nucleotide binding sites have been described (15), it is generally accepted that there are six adenine nucleotide binding sites in the F₁-ATPases (16). With the exception of TF₁, which, when isolated, is free of nucleotides, the F₁-ATPases isolated from various other sources contain 1–4 mol of endogenous adenine nucleotides (15, 16). These ATPases will bind an additional 2–4 mol of adenine nucleotides/mol of enzyme when incubated with exogenous nucleotides. It has been generally accepted that the bovine mitochondrial F₁-ATPase contains three tight adenine nucleotide binding sites which are not exchangeable with medium adenine nucleotides, and that there are three loose adenine nucleotide binding sites which are exchangeable (17, 18). The former are noncatalytic sites, the function of which is uncertain, and the latter sites are catalytic. As is implied in the work of Penefsky and his colleagues, the catalytic sites, owing to cooperative interactions, show a wide range of affinities for adenine nucleotides. Cross and Nalin (18) have shown that the noncatalytic sites also exhibit a range of affinities for adenine nucleotides (18). Therefore, tightness or looseness of binding are not useful parameters for distinguishing catalytic sites from noncatalytic sites.

The isolated TF₁-ATPase does not contain endogenous adenine nucleotides. When ADP is added to the isolated enzyme in the presence of Mg²⁺, 4 mol of ADP are bound per mol of enzyme as detected by a nitrocellulose filter binding assay (19). It has also been shown that a stable 1:1 TF₁·ADP complex is formed when ADP is added to the isolated enzyme followed by multiple gel filtrations (20). The formation of the 1:1 TF₁·ADP complex is accompanied by a 7-fold acceleration in the rate of inactivation of the enzyme by DCCD. Addition of Mg²⁺ to the 1:1 TF₁·ADP complex, followed by gel filtration, led to the formation of a stable 1:1:1 TF₁·ADP·Mg²⁺ complex.

1 The abbreviations used are: CF₁, the F₁-ATPase from spinach thylakoid membranes; TF₁, the F₁-ATPase from the thermophilic bacterium, PS₃; MFl, the Fl-ATPase from bovine heart mitochondria; MES, 2-(N-morpholino)ethanesulfonic acid; DCCD, dicyclohexylcarbodiimide; CDTA, trans-1,2-diaminocyclohexane-N,N',N''-tetraacetic acid; EEDQ, 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; NmCl, 7-chloro-4-nitrobenzofuranaz; Nbf-, 4-nitrobenzofuranaz; AMP-PNP, adenyly-5'-ylimidodiphosphate; MgSO₄, dimethyl sulfoxide; HPLC, high performance liquid chromatography.
complex which was slowly inactivated by DCCD. We report here a more thorough characterization of the TF₁-ADP complexes formed in the presence and absence of Mg²⁺ that includes identification of the subunits with which the different complexes are associated and identification of the ADP binding site that is involved in enzyme-bound ATP synthesis in the presence of 50% dimethyl sulfoxide (21).

EXPERIMENTAL PROCEDURES

Materials—[2,8-³H]ADP was obtained from New England Nuclear. ADP and ATP were purchased from Kyowa Hakko. Dimethyl sulfoxide and other chemical reagents were the products of Wako Chemicals, Tokyo. Commercial ADP was purified on Dowex 1 X-4 resin to remove contaminating ATP as follows. Dowex 1 X-4 in a 3-ml column was washed with 20 ml of 1 M HCl and then washed with water until pH of the effluent was that of the water used as wash. After the column was equilibrated with 60 mM HCl, 1.7 ml of 0.3 M ADP in 60 mM HCl, pH 1.5, was applied on the column. ADP did not bind and was eluted from the column with 60 mM HCl. The combined, collected fractions containing ADP were neutralized with NaOH. After determination of the ADP concentration, the solution was divided into fractions containing ADP were neutralized with NaOH. After determination of the ADP concentration, the solution was divided into three equal parts which were then denatured protein was removed by centrifugation. The supernatants were neutralized with 5 ml of 5 M KClO₃ and were placed in an ice bath for 30 min. The precipitated KClO₃ was then removed by centrifugation. The supernatant was injected on to a HPLC column that was equilibrated with 0.4 M sodium phosphate, pH 6.0, at 0.8 ml/min at room temperature. Adenine nucleotides were detected by absorbance at 254 nm. Concentrations were determined by automatic integration of the eluting peaks which were compared with those of standard solutions of known ADP and ATP which were subjected to the same analysis.

Other Analytical Methods—ATPase activity was assayed at 20 °C by monitoring inorganic phosphate release as described earlier (22). In experiments illustrated in Fig. 5, ATPase activity was monitored at 20 °C by a coupled enzyme assay. Reaction mixtures contained 50 mM Tris-SO₄, pH 8.0, 5 mM MgSO₄, 10 mM KCl, 2 mM phosphoenolpyruvate, 0.32 mM NADH, 10 µg/ml pyruvate kinase, and 10 µg/ml lactate dehydrogenase. Reactions were initiated by the addition of TF₁ or the TF₁-ADP complexes to 1 ml of the assay mixture and the rate of increase of absorbance at 340 nm was monitored at 0.8 ml/min at room temperature. Adenine nucleotides were determined by the method of Bradford (25). When perchloric acid precipitation preceded protein assays (as was used for the data presented in Table III) to remove residual triethanolamine which interferes with the Lowry protein assay, the precipitate was resuspended in 200 µl of 5% perchloric acid and centrifuged. The precipitate was then dissolved in 100 µl of 1% sodium dodecyl sulfate solution and protein was determined by the Lowry method (26). The molecular weight of TF₁ was taken to be 380,000 (7).

RESULTS

TF₁ Has Two Classes of Binding Sites for ADP—Binding of [³H]ADP to TF₁ has been determined with a membrane filtration method and by a gel-filtration method using centrifuge columns (19, 20). When the TF₁-[³H]ADP complex, bound to nitrocellulose filters, was washed with ADP-free buffer, [³H]ADP did not dissociate. With this method, the binding of [³H]ADP to TF₁, in the presence of Mg²⁺ was biphasic with respect to time as shown in Fig. 1A, and biphasic with respect to [³H]ADP concentration as shown in Fig. 1B. The migration of [³H]ADP with TF₁ in the rapid phase. An additional mol of [³H]ADP was present during the slow phase. The fast phase of binding was nearly complete in J s, the shortest measurement permitted by this method. To
ADP Binding Sites of the TF1-ATPase

**FIG. 1.** The binding of [3H]ADP to TF1 in the presence of Mg2+. A, a solution of 3.9 μM TF1 and 250 μM [3H]ADP in 50 mM triethanolamine sulfate, pH 7.3, containing 1 mM MgSO4 was incubated at 20 °C. At the times indicated, 7.5-μl samples were diluted with either 200 μl of 50 mM triethanolamine sulfate, pH 7.3, containing 1 mM MgSO4 (total binding) or with 200 μl of the same buffer also containing 1 mM ADP (nonexchangeable binding). The diluted solutions were subjected to filtration on Millipore filters immediately (total binding (0)) or after 25 min at 20 °C (nonexchangeable binding (●)). The membrane binding assay is described under “Experimental Procedures.” B, solutions containing 4.4 μM TF1 and the indicated concentrations of [3H]ADP in 50 mM triethanolamine sulfate, pH 7.3, containing 1 mM MgSO4 were incubated for 3 h at 20 °C. Then 7.5-μl samples were diluted with 200 μl of buffer in the presence (○) or absence (□) of 1 mM ADP as described under A. The diluted samples were subjected to the membrane filter assay as described above.

determine if bound [3H]ADP exchanged with added, nonradioactive ADP, samples were removed from the reaction mixtures at the times indicated in Fig. 1A, supplemented with nonradioactive ADP to a final concentration of 1 mM, incubated for 25 min, and then subjected to the membrane filtration procedure. 90% of the [3H]ADP bound in the fast phase exchanged with medium ADP, while the [3H]ADP bound in the slow phase was unaffected by medium ADP.

The second order rate constant for the binding of [3H]ADP in the fast phase was calculated to be greater than 3 × 10^8 M⁻¹ s⁻¹ and that for the slow binding phase was found to be 2 M⁻¹ s⁻¹. After subtracting a residual fast binding component (0.3 [3H]ADP/TF1) from the experimental values obtained for nonexchangeable binding, the same value of 2 M⁻¹ s⁻¹ was obtained for the binding of nonexchangeable ADP in the lower curve of Fig. 1A. These results show that in the presence of Mg2⁺ three sites bind [3H]ADP rapidly and that the [3H] ADP bound to these sites freely exchanges with medium ADP, while a single site binds [3H]ADP slowly and that the [3H] ADP bound to this site exchanges very slowly with medium ADP.

The binding of [3H]ADP to TF1, as a function of [3H]ADP concentration is illustrated in Fig. 1B. Scatchard plots of these data revealed the presence of two binding sites with differing affinities. Three mol of [3H]ADP bound to TF1, with a Kd of about 10 μM and 1 mol of [3H]ADP bound with a Kd of 100 μM. When excess, nonradioactive ADP was added 3 h subsequent to the addition of [3H]ADP, the amount of [3H] ADP bound at high affinity sites decreased by 90%, while the amount of [3H]ADP bound at the low affinity site was unchanged. The Kd for the nonexchangeable, low affinity [3H] ADP binding site was calculated to be 100 μM. From these results it is also clear that in the presence of Mg2⁺ there are at least two classes of ADP binding sites in TF1, one of which is comprised of three sites which bind and exchange ADP rapidly, and the other of which is made up of a single site which binds and exchanges ADP slowly.

The binding of [3H]ADP to TF1, in the absence of Mg2⁺ was also examined and is illustrated in Fig. 2. In the absence of Mg2⁺, most of the [3H]ADP bound slowly and, once it was

**FIG. 2.** The binding of [3H]ADP to TF1 in the absence of Mg2⁺. The constituents of the solutions were: A, 3.2 μM TF1 and 250 μM [3H]ADP in 50 mM triethanolamine sulfate, pH 7.3, containing 10 μM CDTA; and B, 4.4 μM TF1, and the indicated concentrations of [3H]ADP in 50 mM triethanolamine sulfate, pH 7.3, containing 10 μM CDTA. The other experimental conditions were the same as those described in Fig. 1 except that 10 μM CDTA was present in the buffer instead of 1 mM MgSO4. The symbols are: ○, total binding; and ●, nonexchangeable binding.
was in fact obtained by repeated gel filtrations of the same mixture that was used to determine nonexchangeable binding when the isolated Dichroism—Characteristic changes in circular dichroism occurred with a second order rate constant of 4.4 M\(^{-1}\) s\(^{-1}\). The same rate constant was obtained when the rate of the rapid binding component was subtracted from the rate of total binding of [\(^3\)H]ADP shown in the upper curve. The number of mol of [\(^3\)H]ADP estimated to bind per mol of TF\(_1\) was 0.85 for the slow component of the upper curve of Fig. 2A, and 0.7 for the nonexchangeable binding shown in the lower curve of Fig. 2A. A Kd value of 30 \(\mu\)M was estimated for both the total binding (upper curve) and nonexchangeable binding (lower curve) of Fig. 2B. It is noteworthy that the affinity of [\(^3\)H]ADP to the nonexchangeable binding site is greater in the absence of Mg\(^{2+}\). In the absence of Mg\(^{2+}\), the total number of mol of [\(^3\)H]ADP that were estimated to be bound per mol of TF\(_1\) (upper curve, Fig. 2B) was 1.07 and the number of nonexchangeable mol of [\(^3\)H]ADP bound per mol of TF\(_1\) was 1.02 (lower curve, Fig. 2B). Thus, in the absence of Mg\(^{2+}\), only a single, slow-to-saturate, nonexchangeable site binds ADP. Since [\(^3\)H]ADP which bound to TF\(_1\) in the presence of nonradioactive ADP and Mg\(^{2+}\), the same slow-to-saturate and slow-to-exchange site is occupied by ADP in the presence and absence of Mg\(^{2+}\).

We have previously shown that when mixtures of TF\(_1\) and ADP were incubated for 2 h in the presence of Mg\(^{2+}\) and then were subjected to repeated gel filtration on centrifuge columns equilibrated in the absence of ADP, a 1:1 TF\(_1\)-ADP complex or a 1:1:1 TF\(_1\)-ADP-Mg\(^{2+}\) complex were isolated. These complexes, especially the 1:1 TF\(_1\)-ADP complex were stable to many further manipulations (20). The rates of formation of these complexes, which are stable to gel filtration on centrifuge columns, are illustrated in Fig. 3A. The binding of ADP to form the complexes was slow, exhibiting a second order rate constant of 2 and 4 s\(^{-1}\) M\(^{-1}\) in the presence or absence of Mg\(^{2+}\), respectively. From the data of Fig. 3B, Kd values of about 100 and 30 \(\mu\)M were estimated for complexes formed in the presence and absence of Mg\(^{2+}\), respectively. Under these conditions Mg\(^{2+}\) depresses ADP binding.

Table I summarizes the parameters obtained from assessment of ADP binding to TF\(_1\) by the membrane filter method and by gel filtration on centrifuge columns. For ADP binding sites were apparent in these analyses. Three of them bound ADP with relatively high affinity, exchanged rapidly with medium ADP, and depended on the presence of Mg\(^{2+}\). ADP binding to the fourth site was of relatively low affinity, was nonexchangeable, and was slightly inhibited by Mg\(^{2+}\). It is clear that the single, nonexchangeable ADP binding site characterized by the membrane filter assay is the same as the binding site which is stable to multiple gel filtrations on centrifuge columns. The 1:1:1 TF\(_1\)-[\(^3\)H]ADP-Mg\(^{2+}\) complex was in fact obtained by repeated gel filtrations of the same mixture that was used to determine nonexchangeable binding of [\(^3\)H]ADP.

**Subunit Localization of the ADP Binding Sites by Circular Dichroism—**Characteristic changes in circular dichroism occurred when the isolated \(\alpha\) or \(\beta\) subunits bind adenine nucleotides as described previously (12). Based on changes in circular dichroism, it has been possible to identify the subunits which contain the three high affinity, exchangeable ADP binding sites and the single low affinity, nonexchangeable ADP binding site. Fig. 4 shows the circular dichroism difference spectra obtained for the 1:1 TF\(_1\)-ADP complex and for the complexes formed between ADP and the isolated \(\alpha\) or \(\beta\) subunits in the presence and absence of Mg\(^{2+}\). By comparison of Fig. 4b with 4c it is clear that the 1:1 stable TF\(_1\)-ADP complex results in a difference spectrum which is characteristic of ADP binding to the \(\beta\) subunit. It is interesting that the addition of ADP to TF\(_1\) in the absence of Mg\(^{2+}\) gave a \(\beta\) type circular dichroism difference spectrum (Fig. 4d) which indicates that ADP binds only to the \(\beta\) subunit in the absence of Mg\(^{2+}\). The magnitude of the difference spectrum of this mixture was 3 times that of the complex of the isolated subunit with ADP (Fig. 4b) and

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**Figure 3. Characteristics of nonexchangeable binding of [\(^3\)H]ADP to TF1.** A, the reaction mixtures contained 8.9 \(\mu\)M TF\(_1\) and 500 \(\mu\)M [\(^3\)H]ADP in 60 mM triethanolamine sulfate, pH 7.3, containing 10 \(\mu\)M CDTA (0) or 10 \(\mu\)M TF\(_1\) and 500 \(\mu\)M [\(^3\)H]ADP in 50 mM triethanolamine sulfate, pH 7.3, containing 1 mM MgSO\(_4\) (C). At the times indicated, 20-\(\mu\)l samples were withdrawn and subjected to a first gel filtration on 1-ml centrifuge columns of Sephadex G-50 which were equilibrated with 50 mM triethanolamine sulfate, pH 7.3, containing 0.1 \(\mu\)M CDTA. The effluents were incubated for 5 h at 20 °C and then were subjected to a second gel filtration on 1-ml centrifuge columns of Sephadex G-50 equilibrated with the same buffer as used in the first centrifugal elution. These effluents originating from samples incubated in the absence of Mg\(^{2+}\) (0) were used for protein determinations and HPLC analysis for bound ADP. Those effluents originating from samples which were incubated in the presence of 1 mM MgSO\(_4\) (C) were incubated for an additional 12 h at 20 °C and were then subjected to a third gel filtration on 1-ml centrifuge columns equilibrated with 50 mM triethanolamine sulfate, pH 7.3, containing 0.1 \(\mu\)M CDTA. The ADP content and protein concentrations of the effluents were determined as described under "Experimental Procedures." B, the reaction mixtures contained 7.1 \(\mu\)M TF\(_1\) and the concentrations of [\(^3\)H]ADP indicated in 50 mM triethanolamine sulfate, pH 7.3, containing either 10 \(\mu\)M CDTA (C) or 1 mM MgSO\(_4\) (0).
ADP Binding Sites of the TF₁-ATPase

| Conditions                | Kₐ (μM) | kᵣ (M⁻¹ s⁻¹) | kₑ (s⁻¹) |
|---------------------------|---------|---------------|-----------|
| Membrane filter assay     |         |               |           |
| +Mg²⁺ exchangeable        | 3       | 10            | 3 × 10⁻²  |
| +Mg²⁺ nonexchangeable     | 0.8     | 100           | 2 × 10⁻⁴  |
| −Mg²⁺ nonexchangeable     | 0.7     | 39            | 4.3 × 10⁻⁴|
| Centrifuge column assay   | 0.9     | 100           | 2 × 10⁻⁴  |
| +Mg²⁺                    | 1.0     | 30            | 4.2 × 10⁻⁴|

Fig. 4. Difference circular dichroism spectra induced by ADP binding to the isolated α or β subunits or TF₁, in the presence of Mg²⁺. The buffers used were: a–d, 20 mM triethanolamine sulfate, pH 7.3, containing 10 mM EDTA; and e–h, 20 mM triethanolamine sulfate, pH 7.3, containing 1 mM MgSO₄. The difference spectra were generated from the following solutions: a, (190 μM α subunit + 180 μM ADP) − (190 μM β subunit) − (180 μM ADP); b, (62 μM β subunit + 250 μM ADP) − (62 μM β subunit) − (250 μM ADP); c, (9 μM TF₁, ADP complex) − (9 μM TF₁) − (7.6 μM ADP), the complex contained 0.84 mol of bound ADP; d, (6.3 μM TF₁ + 250 μM ADP) − (6.3 μM TF₁) − (250 μM ADP), the CD spectrum of TF₁ + ADP was measured 50 min after mixing TF₁ with ADP; e, (190 μM β subunit + 180 μM ADP + Mg²⁺) − (190 μM β subunit + Mg²⁺) − (180 μM ADP + Mg²⁺); f, (62 μM α subunit + 250 μM ADP + Mg²⁺) − (62 μM α subunit + 250 μM ADP); g, (250 μM ADP + Mg²⁺) − (250 μM ADP + Mg²⁺); h, (6.3 μM TF₁ + 10 μM ADP + Mg²⁺) − (4.5 μM TF₁ + 10 μM ADP) − (4.5 μM TF₁ + Mg²⁺) − (10 μM ADP + Mg²⁺); and i, (6.3 μM TF₁ + 250 μM ADP + Mg²⁺) − (6.3 μM TF₁ + 250 μM ADP). In this experiment MgSO₄ was added to the solution used to generate spectrum d to a final concentration of 1 mM. The difference spectrum shown was obtained from the circular dichroism spectra before and after addition of Mg²⁺ with the use of a data processor.

Three times that of the isolated 1:1 TF₁-ADP complex (Fig. 4c). Provided that the circular dichroism changes associated with ADP binding to the isolated β subunits represents the whole population of the β subunits and that the magnitude of the circular dichroism change for the isolated subunit is the same as that for the β subunit in the intact enzyme, these data suggest that each β subunit binds ADP when TF₁ is incubated with 250 μM ADP, but that ADP dissociates from two of them during the membrane filter assay or during gel filtration on centrifuge columns.

When TF₁ was incubated with Mg²⁺ and low concentrations of ADP, the circular dichroism spectrum characteristic of the α subunit developed as shown in Fig. 4g. A difference spectrum between TF₁ in the presence of ADP and Mg²⁺ and TF₁ in the presence of ADP alone is shown in Fig. 4h. This shows that in the presence of Mg²⁺ and ADP, TF₁ develops a circular dichroism spectrum characteristic of the complex of the α subunit with Mg²⁺ plus ADP. The magnitude of the spectra shown in Fig. 4g and h per mol of TF₁ is greater than three times on a molar basis than the spectrum generated by the interaction of ADP with the α subunit shown in Fig. 3e. This might reflect that the isolated α subunit might have been partially denatured during preparation.

From these results it is concluded that the Mg²⁺-dependent, exchangeable sites are located on each of the three α subunits and that the single, nonexchangeable binding site which is stable to membrane filtration and repeated gel filtration on centrifuge columns is located on one of the β subunits.

Properties of the 1:1 TF₁-ADP Complex—Once isolated, the 1:1 TF₁-ADP complex is stable in the absence of medium ADP for at least three days at 4°C as described earlier (20). Bound [³H]ADP in this complex is not released by exchange with medium ADP in the presence or absence of Mg²⁺. Of the ligands (ATP ± Mg²⁺, AMP-PNP ± Mg²⁺, Pi ± Mg²⁺, ADP + Pi ± Mg²⁺, or Mg²⁺) added to the 1:1 TF₁-³H]ADP complex, only ATP in the presence of Mg²⁺ displaced [³H]ADP from the enzyme. Since neither ATP alone nor AMP-PNP plus Mg²⁺ affected the stability of the complex, it appears that enzyme turnover is required to release ADP from the 1:1 complex. AMP-PNP is a strong, competitive inhibitor of the hydrolytic reaction catalyzed by TF₁,² presumably by occupying at least one of the catalytic sites of the enzyme.

The 1:1 TF₁-ADP complex has the same Vₘₐₓ and Kₐ values as free TF₁, for the steady state hydrolysis of ATP. A short lag time precedes the attainment of the steady state Vₘₐₓ for TF₁, as shown in Fig. 5. This lag time increased when the 1:1 TF₁-ADP complex was assayed, again as shown in Fig. 5. The length of the lag is dependent on the ATP concentration in the assay mixtures as is also illustrated by comparison of the rates observed with 1.1 mM ATP or 0.27 mM ATP as substrate shown in Fig. 5.

Synthesis of Bound ATP from ADP Bound by TF₁, in the Presence of 50% Dimethyl Sulfoxide—It has been demonstrated that TF₁ will synthesize enzyme-bound ATP from ADP-bound ATP in the presence of P₃, Mg²⁺, and 60% 

² M. Yoshida, unpublished results.
dimethyl sulfoxide. Although it was not determined directly, the 1:3:3 TF₁, ADP-Mg⁺ complex was probably present under the conditions used in the earlier experiments. Table II shows the results of experiments in which enzyme-bound ATP was synthesized on the 1:1 TF₁, ADP complex and on the 1:1:1 TF₁, ADP-Mg⁺ complex. It is clear from the data presented in Table II that significant amounts of enzyme-bound ATP is converted to bound ATP. It is interesting that the formation of enzyme-bound ATP from the 1:1:1 TF₁, ADP-Mg⁺ complex does not depend on Mg⁺⁺. Therefore, the components of this complex are directly involved in enzyme-bound ATP formation. These results strongly suggest that ADP and Mg⁺⁺ in these complexes are bound to one of the catalytic sites of TF₁, which in the presence of bound P₁ and appropriate other conditions can be converted to a TF₁, ATP-Mg⁺ complex.

To test the possibility that the other class of ADP binding sites which are dependent on Mg⁺⁺ might also form enzyme-bound ATP in the presence of 50% dimethyl sulfoxide, these sites were filled at low ADP concentration (1.12 μM) in the presence of Mg⁺⁺ where the single, stable site remained unoccupied. Under these conditions, ADP bound only to the α subunits as determined by a difference circular dichroism spectrum. When this complex was incubated with P₁, Mg⁺⁺, and 50% dimethyl sulfoxide, an insignificant amount of ATP was synthesized. Therefore, ADP bound to the α subunits cannot be converted to ATP in the presence of Mg⁺⁺, P₁, and 50% dimethyl sulfoxide.

The Effect of ATPase Inactivators on Enzyme-bound ATP Formation—Sakamoto and Tonomura (27) have reported that when the hydrolytic reaction catalyzed by MF₁ is nearly completely inactivated by DCCD or Nbfl-CI, enzyme-bound ATP formation in the presence of dimethyl sulfoxide still proceeds (27). The results of similar experiments with TF₁ are presented in Table III. Of the ATPase inactivators tested, only treatment with DCCD produced a nearly complete loss in the capacity of the enzyme to promote enzyme-bound ATP formation. Different carboxyl groups are modified when TF₁ modified by DCCD does not carry out enzyme-bound ATP synthesis, while MF₁ modified by DCCD does, suggests that the DCCD-reactive carboxyl group in TF₁, unlike the one in MF₁, might have a direct role in the reversible reaction catalyzed by the enzyme.

**TABLE II**

| Complex | Additions | ADP/TF₁ | ATP/TF₁ |
|---------|-----------|---------|---------|
| 1:1 TF₁, ADP | None | 0.96 | 0.00 |
| | MesSO, P₁ | 0.98 | 0.06 |
| | MesSO, P₁, Mg⁺⁺ | 0.77 | 0.20 |
| 1:1:1 TF₁, ADP-Mg | None | 0.94 | 0.00 |
| | MesSO, P₁ | 0.73 | 0.26 |
| | MesSO, P₁, Mg⁺⁺ | 0.71 | 0.23 |

**TABLE III**

| Inhibitor | Residual Catalyzed ATP Synthesis | ATPase Before MesSO₄ Treatment | ADP | ATP | After MesSO₄ Treatment | ADP | ATP |
|-----------|--------------------------------|-------------------------------|-----|-----|-----------------------|-----|-----|
| None | 100 | 0.82 | 0.15 | 0.29 |
| NaN₃ | 7.3 | 0.73 | 0.18 | 0.32 |
| Nbfl-CI (Try) | 16 | 0.61 | 0.26 | 0.40 |
| Nbfl-CI (Lys) | 16 | 0.47 | 0.19 | 0.33 |
| DCCD | 6 | 0.69 | 0.18 | 0.06 |
| EEDQ | 10 | 0.52 | 0.17 | 0.22 |
| Quinacrine mustard | 5 | 0.57 | 0.17 | 0.28 |

The TF₁, ADP-Mg⁺⁺ complexes were prepared as described under "Experimental Procedures," except that the last centrifuge column was equilibrated with 100 mM MES-NaOH and 40 mM sodium phosphate, pH 6.4. Enzyme-bound ATP synthesis was carried out in 50 μM reaction mixtures which contained 50% MesSO₄, 50 mM MES-NaOH, and 20 mM sodium phosphate, pH 6.3, 48 g of the complexes, and 5 mM MgSO₄. The reaction mixtures were incubated for 1 h at 23°C at which time 50 μl were removed and placed in a microfuge tube. Then 2 μl of 70% perchloric acid were added to precipitate the protein. The protein was removed by centrifugation. After neutralization with K₂CO₃ and removal of KClO₃, the supernatants were analyzed for adenine nucleotides by HPLC and the precipitated protein was assayed as described under "Experimental Procedures."
Whether the asymmetry is induced on the binding of ADP to that observed when ADP binds to the isolated subunits, it suggested that the catalytic site resides at the interface of the active site analogs of ATP containing nitrene (10) or carbene (11). Incorporation into the enzyme-bound ATP in the presence of Mg²⁺ and 50% dimethyl sulfoxide at pH 7.3, containing 1 mM MgSO₄, and then centrifuged under the conditions described above. The washed pellet was homogenized in 1 ml of triethanolamine sulfate, pH 7.3, containing 1 mM MgSO₄. The adenine nucleotide and protein concentrations were determined with 150 ml of 20 mM sodium phosphate, pH 6.3, 2 mM MgSO₄, and 50% Me₂SO. This mixture was incubated for 1 h at 23 °C, at which time it was diluted with 8 ml of 50 mM triethanolamine sulfate, pH 7.3, which contained 1 mM MgSO₄. The diluted sample was centrifuged and the pellet was suspended in 100 μl of 50 mM triethanolamine sulfate, pH 7.3, containing 1 mM MgSO₄ and the protein concentration and adenine nucleotide content were determined as described under “Experimental Procedures.”

### Table IV

| Sequential treatments of TF₁₋F₁ | ADP | ATP |
|---------------------------------|-----|-----|
| A. Native TF₁₋F₁               | 0.03| 0.04|
| B. Incubate A with ADP + Mg²⁺; washed twice | 3.0  | 0.08 |
| C. Incubate B 16 h; washed once | 1.6 | 0.06 |
| D. Incubate C with 20 mM NaPi, 50% Me₂SO, 2 mM MgSO₄; washed once | 1.3 | 0.22 |

This table shows the results of sequential treatments for ADP and ATP binding sites of TF₁₋F₁ complex.

It is also clear from the circular dichroism difference spectra presented that the high affinity ADP binding sites are on the three α subunits and the low affinity ADP binding site resides on a single β subunit. It was also shown that the ADP bound to a β subunit in the 1:1 TF₁₋F₁ complex is converted to enzyme-bound ATP in the presence of Mg²⁺ and 50% dimethyl sulfoxide at pH 6.3. The ADP in the 1:1 TF₁₋F₁₋ADP-Mg²⁺ complex, which is also bound to a β subunit, is converted to enzyme-bound ATP in the presence of Mg²⁺ and 50% dimethyl sulfoxide in a reaction which does not require additional Mg²⁺. These results provide the least ambiguous evidence collected to date which shows that the site or sites for ATP synthesis do indeed reside in the β subunits.

Based on the observation that both the α and β subunits are labeled when the F₁₋ATPases are inactivated with photoaffinity analogs of ATP containing nitrene (10) or carbene (11) generators esterfied to the ribose moiety, it has been suggested that the catalytic site resides at the interface of α and β subunits. Although not ruled out, this contention is not supported by the observations presented here. The circular dichroism difference spectrum observed when ADP binds to the low affinity site of the intact enzyme is remarkably similar to that observed when ADP binds to the isolated β subunit. This suggests that the circular dichroism difference spectrum observed for the 1:1 TF₁₋F₁₋ADP complex arises from interactions of bound ADP with the β subunit only.

The observation that only one of the three β subunits forms stable 1:1 TF₁₋ADP and 1:1:1 TF₁₋F₁₋ADP-Mg²⁺ complexes reflects an asymmetry of nucleotide binding to β subunits. Whether the asymmetry is induced on the binding of ADP to a single, low affinity site, or whether it pre-exists is not known. Since the isolated 1:1 TF₁₋[³²P]ADP complex did not dissociate when it was incubated with nonradioactive ADP and Mg²⁺ followed by repeated gel filtrations indicates that the asymmetry is retained when all of the ADP binding sites are occupied. Asymmetry of nucleotide binding appears to be characteristic of the F₁₋ATPases (18, 29-35). Penefsky and his colleagues (31) have described a single, high affinity catalytic site in MF₁, which binds Mg-ATP⁻ with a dissociation constant of 10⁻¹² M. Cross and Nalin (18) have shown that AMP-PNP binds to a single, exchangeable site, presumably in a β subunit, with a dissociation constant of 18 nM. Gautheron's laboratory has shown that the “hysteretic” inhibition, which develops slowly when ADP binds to MF₁ in the presence of Mg²⁺, is associated with a single binding site which also appears to reside in a β subunit (35). When the 1:1 TF₁₋ADP complex is assayed with an ATP regenerating system, a slowly developing hysteretic inhibition which is characteristic of MF₁ is not seen but, instead, a transient inhibition or lag is observed which disappears as the assay proceeds. A similar, but much shorter lag, in the absence of Mg-set is observed when MF₁ is assayed with an ATP regenerating system. ADP bound to the low affinity site of TF₁ differs from ADP bound to the hysteric site of MF₁, in another respect. ADP bound to the hysteretic site of MF₁ does not dissociate during enzyme turnover (39), while the ADP bound to the low affinity site dissociates when Mg²⁺ and ATP are added to the enzyme under conditions where turnover occurs.

The noncatalytic ADP binding sites in the α subunits of TF₁ differ considerably from the noncatalytic nucleotide binding sites described for MF₁ (17, 18). While the ADP bound to the high affinity binding sites of TF₁ is removed by gel filtration and exchanges with medium ADP, the nucleotides bound to the noncatalytic binding sites of MF₁, which presumably also reside in the α subunits, are not removed by gel filtration and do not exchange with medium nucleotides in the presence or absence of enzyme turnover (17, 18).

Recently Khananashvili and Gromet-Elhanan (36, 37) have reported that 2 mol of ADP or ATP bind per mol of the isolated β subunit of the F₁₋ATPase from Rhodospirillum rubrum. One site binds ADP with high affinity (Kₐ = 6.7 μM) in the presence and absence of Mg²⁺, while the second site binds ADP with low affinity (Kₐ = 80 μM) which is absolutely dependent on the presence of Mg²⁺. It has been suggested that this low affinity binding site is the catalytic site. The Kₐ for ADP for the low affinity binding site on the isolated β subunit of F₁ from R. rubrum is similar to that observed in this study for the binding of ADP to both the isolated β subunit of TF₁ and to the catalytic site of intact TF₁. However, while ADP binding to the low affinity site of R. rubrum F₁ is absolutely dependent on the presence of Mg²⁺, the stable binding of ADP to either the isolated β subunit of TF₁, or to a single catalytic site in the intact enzyme does not depend on Mg²⁺. Therefore, it is possible that the high affinity binding site on the isolated β subunit of R. rubrum F₁, which does not depend on the presence of Mg²⁺, is equivalent to the low affinity ADP binding site on TF₁ which we have shown to be a catalytic site which resides on a β subunit. It would be interesting to compare the circular dichroism spectra generated when ADP binds to the high and low affinity sites of the isolated R. rubrum β subunit with the difference spectra shown here when ADP binds to the isolated β subunit of TF₁. It is interesting that, of the inactivators of the TF₁₋ATPase tested, DCCD is the only one which also abolishes the capacity of the soluble enzyme to synthesize enzyme-bound ADP from bound ADP in the presence of medium Pₐ and dimethyl
sulfoxide (27). It has been shown that β-Glu-188 (using the residue numbers of the β subunit of MF₁) is modified when DCCD inactivates the ATPase activity of TF₁ (19) and that DCCD reacts with β-Glu-199 when it inactivates the MF₁-ATPase (28). These observations suggest that β-Glu-188, at least in TF₁, plays a role in both the hydrolysis and synthesis of ATP, while β-Glu-199 in MF₁ does not. The fact that modifications of TF₁ at the residue equivalent to β-Tyr-311 of MF₁ with Nbf-C₁ (38, 39), at the residue equivalent to β-Lys-162 by migration of the Nbf-group (40, 41), or by EEDQ or quinacrine mustard at unknown, essential residues, inactive ATPase activity without altering enzyme-bound ATP capacity of the enzyme to synthesize enzyme-bound ATP from ATP, while @-Glu-199 in MF₁, does not. The fact that modifications of TF₁, at the residue equivalent to β-Tyr-311 of MF₁ with Nbf-C₁ (38, 39), at the residue equivalent to β-Lys-162 by migration of the Nbf-group (40, 41), or by EEDQ or quinacrine mustard at unknown, essential residues, inactive ATPase activity without altering enzyme-bound ATP synthesis is curious. Sakamoto and Tonomura (27) have observed that modification of MF₁ with DCCD or Nbf-C₁ also abolishes ATPase activity without severely affecting the capacity of the enzyme to synthesize enzyme-bound ATP from bound ADP in the presence of medium P₃ and dimethyl sulfoxide. On the basis of different observations, reports have appeared from other laboratories which have proposed that the catalytic site or sites of Fl might exist in two interconvertible conformations, one geared for ATP hydrolysis and the other geared for ATP synthesis (35, 42, 43). It is possible that the reagents which have been observed to abolish the hydrolytic reaction catalyzed by the F₁-ATPases without seriously affecting their capacity for enzyme-bound ATP synthesis do so by locking the catalytic sites in a synthetic mode.

The observation that enzyme-bound ATP synthesis is promoted by the intact TF₁-Fl complex in the presence of dimethyl sulfoxide shows that this reaction is not just an artifact associated with the soluble enzyme. Feldman and Sigman (44, 45) have reached the same conclusion from experiments with isolated CF₁ and thylakoid preparations in which enzyme-bound ATP synthesis was demonstrated by adding high concentrations of P₃ to either CF₁ or the CF₀-P₃ complex, each containing tightly bound ADP.

Acknowledgments—We thank Dr. H. Inoue of the National Institute of Agrobiological Resources and Dr. K. Watanabe of Tokyo University for kindly allowing us to use their circular dichroism spectrometers. We also acknowledge Dr. S. Ohta of the Jichi Medical School for discussion of the circular dichroism spectra.

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