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Fluorometric Evidence for Control of the Activity of F_1-Adenosinetriphosphatase by Ligand-Induced Conformation Change[1]

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Abstract

The effect of ATP on the fluorescence intensity of bovine heart F_1-adenosinetriphosphatase labeled at its essential Lys with 7-chloro-4-nitro-2,1,3-benzoxadiazole (N-NBD-F_1) has been examined in solutions containing different concentrations of ADP. The fluorescence of N-NBD-F_1 is unaffected by ATP in the absence of ADP. But when increasing amounts of ATP are added to a solution of N-NBD-F_1 containing 0.37 or 1.0 mM ADP, the fluorescence of N-NBD-F_1 first decreases and then increases continually as the concentration of ATP is further raised. Parallel measurements of the suppression of the fluorescence of N-NBD-F_1 and the inhibition of the ATPase activity of the unlabeled enzyme by ADP in the presence of ATP show a quantitative correlation between the changes in fluorescence and in ATPase activity. The data are consistent with the model for F_1-ATPase with one principal catalytic β' subunit for ATP hydrolysis and synthesis, and two auxiliary β'' subunits which control the conformation and hence the catalytic activity of β' through interaction between all the subunits.

Key Words: F_1-Adenosinetriphosphatase; F_1-ATPase; N-NBD-F_1; fluorescence change; conformation change; regulation of ATPase activity.

Introduction

Since the binding of ADP to F_1-ATPase changes both the chemical properties of the enzyme (Adolfsen and Moudrianakis, 1976; Kayalar *et al.*, 1977; Harris *et al*, 1978; Cross *et al.*, 1982) and its conformation (Chang and Penefsky, 1973; Cantley and Hammes, 1975; Ferguson *et al.*, 1975b; Hatefi *et al.*, 1982; DiPietro *et al.*, 1983; Roux *et al.*, 1984; Hirano *et al.*, Wang, 1984), it is generally believed that adenine nucleotides bound to regulatory sites of F_1-ATPase control the conformation and thereby regulate the

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reaction at the catalytic site. The reported suggestive evidence would be more convincing, if a quantitative correlation could be found between the observed changes in conformation and in catalytic activity. In the present work, the effect of varying ADP to ATP ratio on the fluorescence of N-NBD-F_1[2] and on the catalytic efficiency of F_1-ATPase has been examined. The two sets of data correlate quantitatively, if it is assumed that F_1-ATPase has one catalytic β' subunit and two auxiliary β'' subunits which control the conformation and hence the catalytic activity of β' through interaction between all the subunits.

Materials and Methods

Materials

ATP, ADP, NADH, phosphoenolpyruvate, pyruvate kinase (Type II), L-lactic dehydrogenase (Type III), and Sephadex G-50–80 were purchased from Sigma Chemical Co. The [^{14}C]NBD chloride, which was supplied by Research Products International with a stated specific radioactivity of 109 mCi/mmole, was found by titration (Ferguson *et al.*, 1975a) to have a specific radioactivity of 77 mCi/mmol. Aquasol II and [^{14}C]toluene standard for radioactive assays were purchased from New England Nuclear.

F_1-ATPase was prepared and stored according to the procedure of Knowles and Penefsky (1972). N-[^{14}C]NBD-F_1 was prepared by first labeling F_1-ATPase with [^{14}C]NBD chloride in Buffer A (25% glycerol, 2 mM EDTH, 50 mM Hepes-NaOH, ph 7.0) at the essential Tyr of the enzyme in the dark for about an hour at 25°C. Small aliquots of the reaction mixture was taken at 10 min intervals for ATPase assay. In order to avoid nonspecific labeling, the reaction was terminated by centrifugal gel-filtration (Penefsky, 1977) when 15–20% of the initial ATPase activity was still left. After gel-filtration, the O-NBD-labeled F_1-ATPase was usually found to carry 0.75–0.80 mol of [^{14}C]NBD-label per mole of F_1 and 25–20% of the initial ATPase activity. The pH of this solution of O-NBD-F_1 in Buffer A was then raised to 8.95 by mixing with 1/5 of its volume of 1 M triethanolamine solution, and the mixture was then kept in the dark for 4–5 hours at 25°C to allow the spontaneous transfer of the radioactive label from the essential Tyr to the essential Lys to take place. The resulting N-[^{14}C]NBD-F_1 was again centrifugally gel-filtered through Sephadex G-50-80 which had been

[2] Abbreviations: DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LDH, L-lactic dehydrogenase; NBD chloride, 7-chloro-4-nitro-2,1,3-benzoxadiazole; N-NBD-F_1, F_1-ATPase labeled by NBD chloride at an essential Lys in its β subunit; O-NBD-F_1, F_1-ATPase labeled by NBD chloride at a single essential Tyr in its β subunit; PEP, phosphoenolypruvate; PK, pyruvate kinase; TNP-ATP, 2′,3′-O-(2,4,6-trinitrocyclohexadienylidene)-adenosine-5′- triphosphate.

preequilibrated with Buffer A and assayed for specific ATPase activity and radioactivity.

Fluorescence Measurements

A Hitachi Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A, was used to measure the fluorescence emission of N-NBD-F_1 with 440-nm exciting light under various experimental conditions. Calibration measurements show that after correction for Raman Scattering by the medium the intensity of fluorescence emission is proportional to the concentration of N-NBD-F_1. A 2-ml solution containing 0.08–0.10 mg/ml of N-NBD-F_1 in Buffer A was used for each set of fluorescence measurements. Calculated volumes of 25 or 250 mM ADP or ATP solution were added successively to the same sample and mixed gently but thoroughly before each fluorescence measurement. The observed fluorescence intensities were corrected by dilution factors due to a small change in volume as the titration was continued. Most of the loosely bound nucleotides had been removed by gel-filtration during the preparation of N-NBD-F_1. Since the concentration of F_1 in the fluorescence samples was only 0.29 μM, the trace amount of residual endogenous nucleotides left on its β subunits was negligible as compared to the ADP and/or ATP added to the sample. The samples were found to have the same partially inhibited ATPase activity before and after the fluorescence measurements.

Assay of ATPase

The rate of F_1-catalyzed ATP hydrolysis was determined by an ATP-regenerating system coupled to the oxidation of NADH. The assay medium contained 50 mM Hepes-NaOH, pH 8.0, 3 mM $MgCl_2$, 2 mM PEP, 0.4 mM NADH, 21 units/ml PK, and 11 units/ml LDH. A 2-ml solution containing 0.7–1.0 mg of F_1 per ml in Buffer A was used for each set of hydrolysis measurements. Calculated amounts of 0.20 or 0.25 M ADP or ATP stock solution were added successively to the same F_1 solution as in the fluorescence measurements and preincubated for 5 min after each addition before a 2-μl aliquot was withdrawn for ATPase assay. In order to minimize possible artifacts due to ADP in the preincubated sample, each assay was started by injecting only 2 (or 3) μl of the preincubated mixture into 2 ml of the above assay medium without ATP (a 1000-fold dilution), waiting for 1–1.4 min for the small amount of ADP from the preincubated mixture to be converted to ATP by PEP and PK, and then injecting 16 μl of 0.25 M ATP stock solution to start the assay reaction. The rate of hydrolysis was computed from the linear decrease of A_{340} with time due to the oxidation of NADH, which was monitored in a Gilford Spectrophotometer with a sample compartment

thermostated at 30°C, by using 6220 $M^{-1} cm^{-1}$ as the molar extinction coefficient of NADH. Small corrections for the dilution of F_1 due to volume changes were also made when necessary.

Protein concentrations were determined by the Coomassie blue binding method (Bradford, 1976). Radioactivity was assayed by liquid scintillation counting. A 10- or 20-μl sample was first mixed with 250 μl of water, then shaken with 5 ml of Aquasol II and assayed after 5 min or longer. The counting efficiency was found to be 92% by comparison with [^{14}C]toluene radioactive standard injected into the same counting medium.

Results and Discussion

Characterization of N-[^{14}C]NBD-F_1

In a typical preparation, the F_1-ATPase had an original specific activity of 78 μmol of ATP hydrolyzed per min per mg (78 units/mg) when dissolved in 0.25 M sucrose solution containing 2 mM EDTA, 5 mM ATP, and 50 mM Hepes-NaOH at pH 8.0. When dissolved in Buffer A and centrifugally gel-filtered to remove free ATP and ammonium sulfate, its specific activity became 51.0 units/mg. After the labeling reaction in the dark at pH 7.0, the isolated O-[^{14}C]NBD-F_1 was found to have a specific activity of 11.2 units/mg, which was sensitive to 2.5 mM DTT, and a label-to-F_1 ratio of $n = 0.77$. After the transfer reaction, the isolated N-[^{14}C]NBD-F_1 was found to have a specific activity of 16.3 units/mg, which was no longer sensitive to 2.5 mM DTT, and the same molar ratio of $n = 0.77$. A control F_1-ATPase sample, which was put through all the operations in the preparation of N-[^{14}C]NBD-F_1 but without NBD chloride, was found after the final gel-filtration to have a specific activity of 52.8 units/mg.

The resulting N-[^{14}C]NBD-F has 16.3/52.8 or 31% of the specific activity of the control F_1, which corresponds to 69% inhibition. But the enzyme with 0.77 NBD-label per F_1 should show 77% inhibition if all covalent labels were attached to the essential Lys. consequently we may conclude that 69/77 or about 90% of the N-[^{14}C]NBD-label are attached to the essential Lys. This distribution seems sufficiently specific for the N-NBD-label to be used as a fluorescent probe for monitoring conformation change at or near the essential Lys in F_1-ATPase.

Complementary Binding of ATP and ADP and Induced Conformation Change

New data on the effect of ADP or ATP on the fluorescence of N-NBD-F_1 in Buffer A at 25°C are summarized in Figs. 1 and 2. The addition of

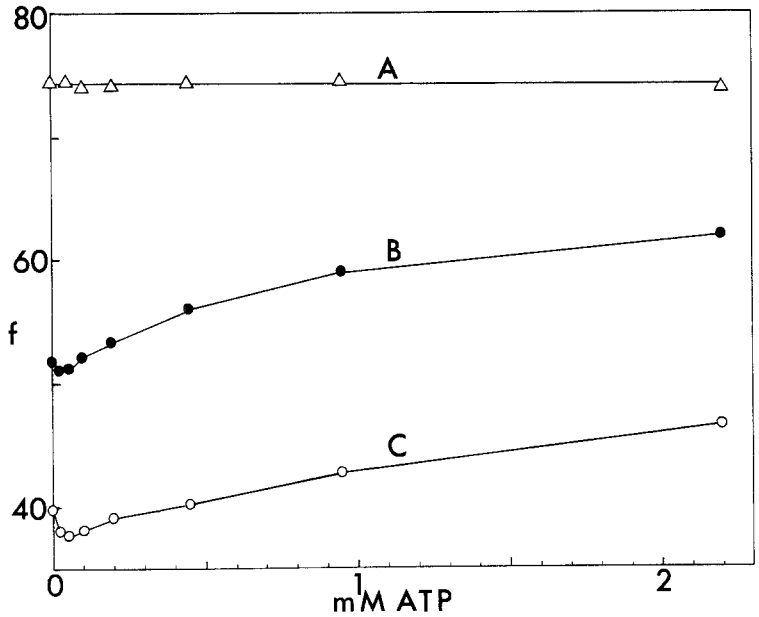

Fig. 1. Effect of added ATP on the fluorescence of N-NBD-F_1 in Buffer A containing constant concentrations of ADP. (A) [ADP] = 0; (B) [ADP] = 0.37 mM; (C) [ADP] = 1.0 mM. Composition of buffer A: 50 mM Hepes-NaOH, 2 mM EDTA, 25% glycerol, pH 7.0. F_1 concentration, 0.099 mg/ml; label-to-F_1 molar ratio n, 0.77. See Materials and Methods section for details.

ATP to a solution of N-NBD-F_1 in Buffer A without ADP does not change the intensity of its fluorescence (Fig. 1A), whereas the addition of ADP to a similar solution decreases its fluorescence (Fig. 2B). Clearly the lowering of the fluorescence of N-NBD-F_1 by ADP cannot be due to quenching of fluorescence by the adenine group, but is mainly due to ligand-induced change in protein conformation near the fluorescent label in the labeled β subunit.

The fluorescence of a solution containing N-NBD-F_1 and ATP can be lowered continually by adding increasing concentrations of ADP (Fig. 2A), but the decrease is less than in the absence of added ATP (Fig. 2B). Since all free adenine nucleotides were removed during the preparation of the N-NBD-F_1, the concentration of free ATP in the latter solution is negligible. Similarly, the fluorescence of a solution containing N-NBD-F_1 and moderately high concentrations of ADP can be increased by adding greater than 0.1 mM concentrations of ATP (Fig. 1B and C). These observations show that by itself bound ATP has no effect on the conformation of N-NBD-F_1. But it may displace the ADP bound to β'' which controls the

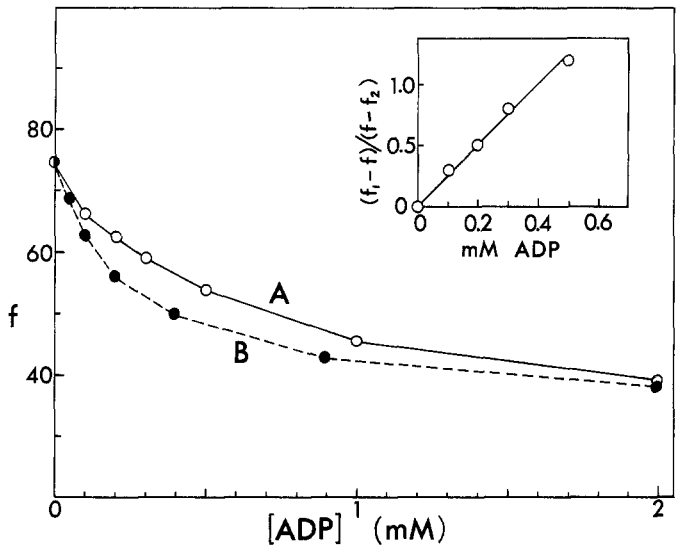

Fig. 2. Effect of added ADP on the fluorescence of N-NBD-F_1 in Buffer A containing constant concentrations of ATP. (A) [ATP = 2 mM; (B) [ATP] = 0. Other experimental conditions are the same as those in Fig. 1.

conformation of N-NBD-F_1 and hence the distribution of the enzyme between its low-fluorescence conformation E_2 and its high-fluorescence conformation E_1. The symbols E_1 and E_2 were used by Hatefi *et al.* (1982) in discussing the conformations of F_1 in submitochondrial particles.

However, the addition of low concentrations of ATP (< 0.1 mM) to N-NBD-F_1 solutions containing 0.37 or 1.00 mM ADP was found unexpectedly to decrease their fluorescence further (Fig. 1B and C). Since this remarkable phenomenon is not observed in the absence of moderately high concentrations of ADP (Fig. 1A), it suggests that ATP (< 0.1 mM) bound to higher-affinity site in the catalytic subunit β' may complement ADP (0.37 or 1.00 mM) bound to lower affinity site(s) of the regulatory β'' subunits in stabilizing N-NBD-F_1 in its low-fluorescence conformation E_2. In other words, the low-fluorescence conformation of N-NBD-F_1 is most stable in the $(ADP)_2E_2(ATP)$ state, where $(ADP)_2$ on the left represents ligand bound to the regulatory sites in β'' subunit and (ATP) on the right represents ligand bound to the catalytic site in β' subunit.

In a solution of N-NBD-F_1 with 1 mM ADP but no ATP, not all enzyme molecules are in the E_2 conformation because the catalytic site is not occupied by ATP. Accordingly the addition of small amounts of ATP can increase the mole fraction of the enzyme in the E_2 conformation and hence further

decrease the fluorescence (Fig. 1B and C). But as more ATP is added, it can compete with ADP for the regulatory sites, thereby decrease the mole fraction of the enzyme in the E_2 conformation and increase the fluorescence.

The binding of [^{14}C]ATP by F_1-ATPase in the presence of moderately high concentrations of ADP was also measured by the centrifugal gel-filtration and pressure-filtration through Millipore Filter techniques. Much higher concentrations of F_1 (40–60 μM) had to be used so that the equilibrium concentrations of free and bound [^{14}C]ATP were of the same order of magnitude. But the data (not presented) seem too complex for quantitative treatment for the following reasons. The observed [^{14}C]ATP/F_1 ratio includes both the displacement of endogenous bound ADP by [^{14}C]ATP and the isotopic exchange of endogenous bound ATP with added [^{14}C]ATP, although the exchange reaction should have no effect on the conformation of the enzyme. Attempts were made to avoid this complication by removing the loosely bound endogenous nucleotide from F_1 beforehand through repeated precipitation with $(NH_4)_2SO_4$ followed by gel-filtration. But it was very difficult to remove all the nucleotides bound to β subunits without also removing some of the "tightly bound" nucleotide from the α subunits, and consequently the subsequently added ADP and [^{14}C]ATP were bound to empty sites in both the β and α subunits with different dissociation constants. A quantitative study of the binding of [^{14}C]ATP by the N-NBD-F_1 containing 0.77 mol of label per mole of F_1 is even more difficult, because the radioactive assays cannot distinguish the [^{14}C]ATP bound to labeled F_1 from that bound to unlabeled F_1 in the same sample. By contrast, it was not necessary to remove all the loosely bound endogenous nucleotides from F_1 before the fluorescence measurements, because the concentration of F_1 was only 0.29 μM in the experiments of Figs. 1 and 2, and consequently the total concentration of loosely bound nucleotides, if any, was negligible as compared to the concentrations of added ATP ($> 25\,\mu$M) or ADP ($> 50\,\mu$M).

Effect of ADP on the Fluorescence of N-NBD-F_1

The observed suppression of the fluorescence of N-NBD-F_1 by ADP in the absence or presence of 2 mM in Buffer A is shown in Fig. 2. Before the addition of ADP, the N-NBD-F_1 was found to have the same intensity of fluorescence emission in either the absence or the presence of 2 mM ATP. The subsequent addition of increasing concentrations of ADP to the N-NBD-F_1 solution containing 2 mM ATP may cause the displacement of ATP bound at the regulatory sites by ADP. Such a displacement will change the protein conformation as well as the nucleotide bound at the catalytic site according to the chemical reaction

$$(ATP)_2E_1(ADP) + ADP \overset{K}{\rightleftharpoons} (ADP)_2E_2(ATP) + ATP \qquad (1)$$

As an approximation, the change in observed fluorescence intensity f may be treated as follows.

Let C_1 and C_2 denote the concentrations of N-NBD-F_1 in the E_1 and E_2 conformations respectively, and f_1, f_2 denote their fluorescence intensities. We have

$$f = (C_1f_1 + C_2f_2)/(C_1 + C_2)$$

or

$$C_2/C_1 = (f_1 - f)/(f - f_2)$$

Thus the equilibrium constant of reaction (1) may be written as

$$K = \frac{C_2[ATP]}{C_1[ADP]} = \left(\frac{f_1 - f}{f - f_2}\right)\frac{[ATP]}{[ADP]}$$

or

$$\frac{f_1 - f}{f - f_2} = \left(\frac{K}{[ATP]}\right)[ADP] \qquad (2)$$

Values of $(f_1 - f)/(f - f_2)$ for solutions of N-NBD-F_1 in Buffer A containing 2 mM ATP and various concentrations of ADP were calculated from the observed values of f by using $f_1 = 74.5$ arbitrary units (at [ADP] = 0) and $f_2 = 39.3$ (at [ADP] = 2 mM), and plotted vs. [ADP] in the inset of Fig. 2.

The plot is sufficiently linear to justify this approximate treatment and gives $K = 5.1$ for the equilibrium constant of the above ligand displacement reaction involving N-NBD-F_1.

Inhibition of F_1-ATPase by ADP-Induced Conformation Change

If we infer from the above fluorometric data on N-NBD-F_1 that ADP also induces a similar conformation change in unlabeled F_1-ATPase, the next question is whether such an induced conformation change is indeed responsible for the well-known inhibition of the initial rate of F_1-catalyzed ATP hydrolysis after the enzyme has been preincubated with ADP. For answering this question, the rates of ATP hydrolysis by F_1 which had been preincubated under identical conditions as in the fluorometric measurements of Fig. 2A were determined. The results are summarized in Fig. 3.

The observed decrease in ATPase activity with increasing [ADP] in the preincubation mixture could not be due to competitive inhibition by ADP, because the assay method (see Materials and Methods) includes an

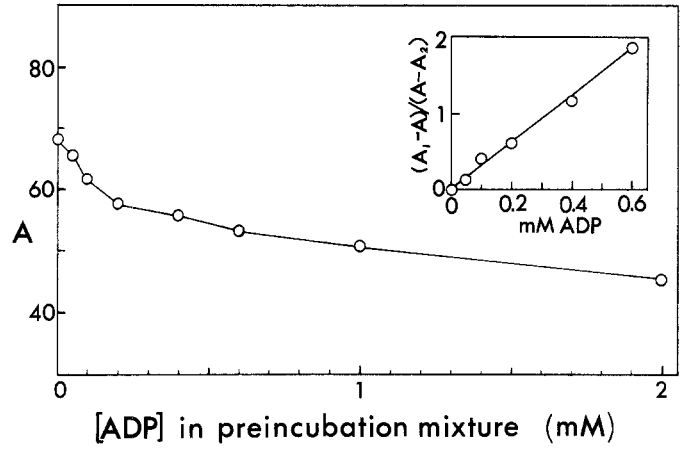

Fig. 3. Specific ATPase activities of F_1 which had been preincubated in Buffer A containing 2 mM ATP and different concentrations of ADP. The specific ATPase activity A is expressed in μmoles of ATP hydrolyzed per minute per mg of F_1. Concentration of F_1, 0.83 mg/ml. Other experimental conditions are the same as in Fig. 1.

immediate 1000-fold dilution followed by the rapid removal of all unbound ADP by PEP + PK. For example, when F_1-ATPase had been preincubated with 2 mM ADP, the starting concentration of free ADP was 2 μM and was rapidly removed by PEP + PK in the assay medium. The concentration of any residual free ADP, if any, would be too low to compete with 2 mM ATP in the assay medium. In fact, when 2 μl of the preincubation medium was injected into 2 ml of the assay medium and followed by the injection of 2 μl of F_1-ATPase in Buffer A, the rate of ATP hydrolysis was found to be indistinguishable from that of the control experiment without 2 μl of the preincubation medium.

The decrease in ATPase activity A with increasing concentration of ADP in the preincubation mixture may be treated in the same way as the fluorometric data. Let A_1 and A_2 be the specific activities of F_1-ATPase which has been preincubated in Buffer A containing 2 mM ATP without ADP and 2 mM ATP + 2 mM ADP respectively. The observed specific activity is given by

$$A = (C_1A_1 + C_2A_2)/(C_1 + C_2)$$

and hence

$$\frac{A_1 - A}{A - A_2} = \left(\frac{K'}{[\text{ATP}]}\right)[\text{ADP}] \tag{3}$$

where K' is the equilibrium constant of reaction (1) for the unlabeled F_1-ATPase. The inset in Fig. 3 shows that a plot of the observed values of

$(A_1 - A)/(A - A_2)$ vs. [ADP] gives a fairly good straight line with $K' = 6.2$, as required by Eq. (3).

The above results support the assumption that ADP-induced conformation change in F_1 is responsible for the inhibition of the enzyme by preincubation with ADP.

Reaction at a Single Catalytic Site Assisted by Nucleotides Bound to Two Regulatory Sites

According to the present model, the substrate bound to the catalytic site should be ATP + H_2O for F_1 in the E_2 conformation and ADP + P_i for F_1 in the E_1 conformation. When the conformation of F_1 is changed from E_2 to E_1 due to the displacement of ADP at regulatory sites by ATP, the ATP + H_2O at the catalytic site automatically becomes ADP + P_i, and *vice versa.*

In unassisted uni-site catalysis with no nucleotide bound to the regulatory sites (Grubmeyer *et al.*, 1982), the F_1-ATPase with its single [^{14}C]ATP bound at the catalytic site may exist in the moderately stable state $E_2(ATP \cdot H_2O)$ or $E_1(ADP \cdot P_i)$ with a very slow rate of product release. But the subsequent addition of excess ATP will drive the system to the $(ATP)_2E_1(ADP \cdot P_i)$ state and facilitate the release of product [^{14}C]ADP + P_i from the catalytic site (assisted multi-site catalysis).

Conversely, preincubation of F_1-ATPase with excess ADP increases the molefraction of the enzyme in the stable and unreactive $(ADP)_2E_2(ATP \cdot H_2O)$ state after the preincubated sample is injected into the assay mixture, and in this way inhibits the initial rate of ATP hydrolysis.

It is interesting to note that although the exchange of bound ATP with medium ADP in the Mg^{2+}-free preincubation mixture reached equilibrium in a few minutes, the subsequent back-exchange of bound ADP with medium ATP in the assay mixture was retarded by Mg^{2+}. For this reason, the recorder trace of each assay showed an inhibited rate which was essentially constant over a period of several minutes. But since the enzyme turned over 10^4 times per minute in most of the assays, the responsible ADP had to be bound to auxiliary or regulatory site(s) rather than to another catalytic site which alternates with the first.

The present model might seem incompatible with the observation that there are at least two catalytic sites per F_1 molecule for the very slow hydrolysis of 2′,3′-O-(2,4,6-trinitrocyclohexadienylidene)-adenosine-5′-triphosphate (TNP-ATP) (Grubmeyer and Penefsky, 1981). However, this apparent discrepancy could be due to a difference in response of F_1 toward normal substrates versus structurally hindered substrates such at TNP-ATP. For example, while the ratio of multi-site rate to uni-site rate for the

hydrolysis of ATP by F_1 is 10^6 (Cross *et al.*, 1982), the corresponding ratio for TNP-ATP is only 15–20. Recently Harris *et al.* (1985) succeeded in isolating a β subunit from the F_1 of *Rhodospirillum rubrum* with an ATPase activity (uni-site catalysis) which is only 10^3-fold lower than the intact enzyme. This discovery suggest the possibility that the intrinsic ATPase activity of β subunit in F_1 may be effectively supressed in the β''-positions as well as in the β'-position for uni-site catalysis, but is greatly enhanced in the β'-position for assisted multi-site catalysis. If protein conformation change due to the binding of exactly fit natural substrates is required for the functional differentiation of the β subunits in F_1, it is possible for all β subunits to have comparable catalytic activity for the hydrolysis of TNP-ATP. There is also the indication that such cooperative conformational control can be relaxed and the suppressed ATPase activity of β'' subunits partially restored by solvent perturbation (Kandpal *et al.*, 1985)

Acknowledgments

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