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SYNTHESIS OF ENZYME-BOUND ATP BY MITOCHONDRIAL SOLUBLE F_1 -ATPASE
AND ITS MECHANISM

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GENERAL INTRODUCTION

In oxidative phosphorylation of mitochondria, almost all the energy required for ATP synthesis from ADP and P_i is derived from the transfer of electron from respiratory substrates (e.g. NADH and succinate) to oxygen through a series of carrier molecules located in the inner membrane of mitochondrion.

It had been unclear how the electron transfer is coupled to the synthesis of ATP until Mitchell (1-3) proposed a hypothesis for coupling mechanism of ATP synthesis to electron transfer (Fig. 1). He suggested that the electron flow through the mitochondrial electron transfer system extrudes protons from the matrix side to the cytoplasmic side. As a result, an electrochemical potential gradient of proton, $\Delta\mu_{H^+}$, is established across the membrane. Then, chemical reaction of ATP formation is driven by a reverse flow of protons down the gradient. This chemiosmotic theory of Mitchell is also widely accepted as the coupling mechanisms of oxidative phosphorylation by bacteria and of photophosphorylation of chloroplasts and chromatophores. (for review, see 4-8).

The apparatus which synthesizes ATP from ADP and P_i has been discovered by Racker's group (9-11). Vigorous sonication of mitochondria gives submitochondrial particles (SMP), which are inside-out vesicles of fragmented inner membrane of mitochondria. SMP still retains the capability to form ATP in an electron transfer-dependent manner. This activity is diminished by removal of a soluble protein, which has been named F_1 . The phosphorylating

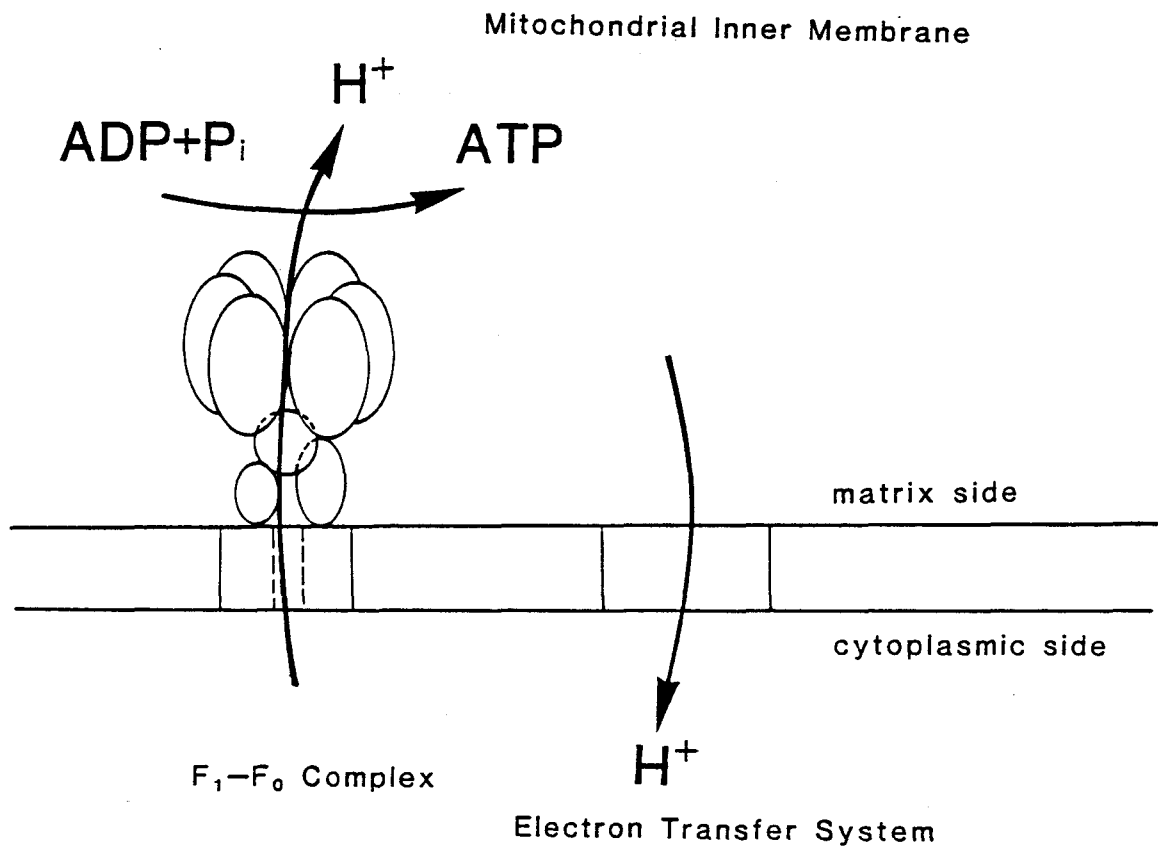


Fig.1. Diagram of the energy coupling mechanism of oxidative phosphorylation in mitochondria.

activity can be reconstituted by adding back of F_1 to the lipid vesicles. F_1 is attached to the membrane through another factor designated F_0 , which is an integral protein embedded in the membrane. The F_1 - F_0 complex is the entire system for ATP synthesis. Being coupled with proton influx through the F_0 sector, ATP synthesis from ADP and P_i occurs on the F_1 sector.

The isolated soluble F_1 catalyzes ATP hydrolysis into ADP and P_i . The ATP synthesis by F_1 is considered to occur as a reversal of ATP hydrolysis by F_1 since F_1 - F_0 complex can generate $\Delta\mu_{H^+}$ when ATP is hydrolyzed by F_1 - F_0 complex. Recently, Matsuoka et al. (12,13) and Penefsky and his colleagues (14-17) performed kinetic studies on the mechanism of ATP hydrolysis by soluble F_1 and suggested that ATP is hydrolyzed via at least two intermediates, enzyme-ATP complex and enzyme-ADP- P_i complex, and that the liberation of P_i occurs prior to the release of ADP from F_1 .

Though, it has not yet been elucidated in which step of the ATP synthesis energy of $\Delta\mu_{H^+}$ is required. Several hypotheses have been postulated for the energy coupling mechanism. Mitchell (18) advocated the hypothesis that the free energy of $\Delta\mu_{H^+}$ is used directly in the step where ADP and P_i are bound covalently to form ATP with elimination of water. On the other hand, Boyer and coworkers (19,20) proposed that the free energy is used for the release of ATP from F_1 and for the binding of ADP and P_i to F_1 . The evidences from studies of the enzyme reaction has not yet been sufficient to decide among them.

Recently, I have found that the soluble F_1 isolated from bovine heart mitochondria synthesized ATP when the enzyme was

incubated with ADP and P_i in the presence of dimethylsulfoxide (DMSO). Furthermore, I found that the resulting ATP remains bound on the enzyme. These findings indicated that the enzyme can synthesize enzyme-bound ATP without energy supply. In chapter I, the optimal condition for the net synthesis of ATP by F_1 and several properties of this reaction are discussed. Chapter II deals with the effects of DMSO on the ATP synthesis by F_1 . The elementary steps of this reaction was also discussed.

The next problem is which subunit participates the ATP synthesis. F_1 consists of five distinct subunits, α , β , γ , δ , and ϵ , in order of decreasing molecular weight (Fig. 2). Accumulating evidences support the subunit stoichiometry as $\alpha_3\beta_3\gamma\delta\epsilon$ (7,8). Each of the larger two subunits, α and β has a nucleotide binding site. One mol of F_1 have 6 mol of nucleotide binding sites; 3 mol out of 6 mol of nucleotides are bound tightly to F_1 and are not easily released nor exchanged with externally added nucleotides while the residual 3 mol are bound exchangeably (21). The tight binding site and the exchangeable binding site are suggested to be located on α and β subunit respectively, from the experiment using the isolated subunit of *E. coli* (22) and from the experiments of photoaffinity labelling to mitochondrial F_1 (23,24). However, it has not yet been clarified at which subunit ATP is synthesized. In chapter III, we found that ATP is synthesized at exchangeable binding site of F_1 , suggesting that the site for ATP synthesis locates on β subunit.

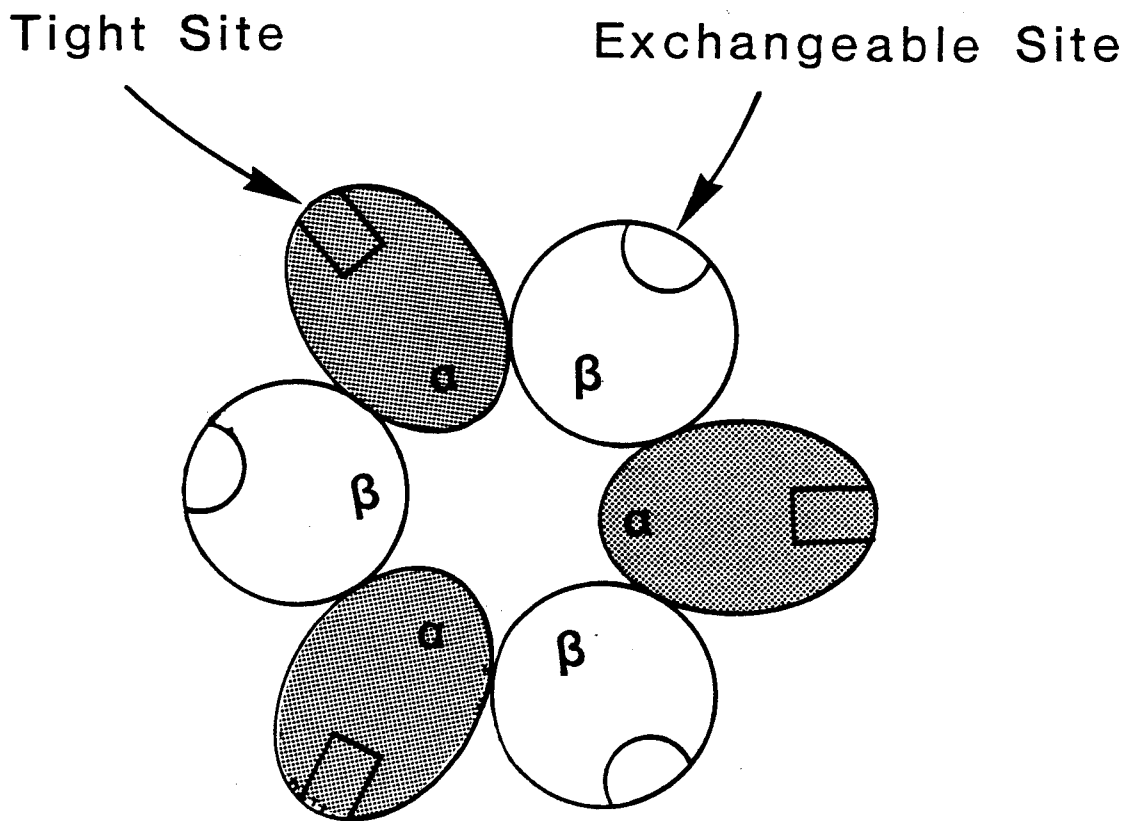
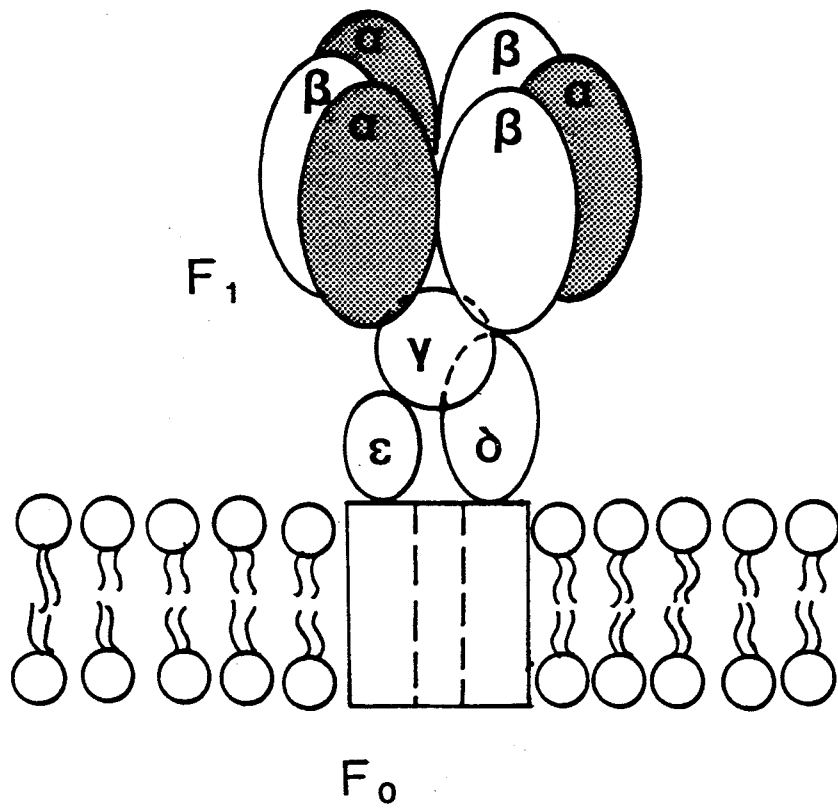


Fig.2. Diagram of the subunit structure of F₁-ATPase.

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ABBREVIATIONS

F ₁	coupling factor 1 (soluble mitochondrial ATPase)
F ₀	coupling factor 0
SMP	submitochondrial particles
ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
CDP	cytidine 5'-diphosphate
GTP	guanosine 5'-diphosphate
IDP	inosine 5'-diphosphate
UDP	uridine 5'-diphosphate
AMPPNP	adenylyl 5'-imidodiphosphate
DNS-ATP	2'-(5-dimethylaminonaphthalene-1-sulfonyl)amino-2'-deoxy-ATP
DNS-ADP	2'-(5-dimethylaminonaphthalene-1-sulfonyl)amino-2'-deoxy-ADP
P _i	inorganic phosphate
DMSO	dimethylsulfoxide
MES	2-(<u>N</u> -morpholino)ethanesulfonic acid
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid
NBD-Cl	7-chloro-4-nitro-2,1,3-benzoxadiazole
DCCD	<u>N,N'</u> -dicyclohexylcarbodiimide
TCA	trichloroacetic acid
SDS	sodium dodecyl sulfate
PP _i	inorganic pyrophosphate

CHAPTER I

Synthesis of Enzyme-Bound ATP by Mitochondrial Soluble F_1 -ATPase
in the Presence of Dimethylsulfoxide

SUMMARY

I found that ATP is synthesized by mitochondrial soluble F_1 -ATPase from medium ADP and P_i in the presence of dimethylsulfoxide (DMSO), with the amount synthesized increasing with the DMSO concentration to a maximum at 30% (w/v) DMSO. In the presence of 35% (w/v) DMSO, ADP was scarcely converted into AMP and much more ATP was formed than AMP. The pH dependence curve of ATP synthesis was bell-shaped with the optimum at 6.7. The amount of synthesized ATP measured after stopping the reaction with trichloroacetic acid was almost equal to that measured after stopping the reaction with sodium dodecyl sulfate or with ethanol. Therefore, we measured the amount of ATP synthesized by F_1 from ADP and P_i in the presence of 4.2 mM Mg^{2+} and 35% (w/v) DMSO at pH 6.7 and 30°C after stopping the reaction with trichloroacetic acid. The following results were obtained.

1. The rate and extent of $[\alpha\text{-}^{32}P]$ ATP synthesis from $[\alpha\text{-}^{32}P]$ ADP and P_i were equal to those of $[\gamma\text{-}^{32}P]$ ATP synthesis from ADP and $^{32}P_i$.
2. The ATP synthesized was inaccessible to hexokinase, and its amount was proportional to that of F_1 . The ATP synthesis was inhibited by sodium azide, but not by 7-chloro-4-nitro-2,1,3-benzoxadiazole, or by N,N' -dicyclohexylcarbodiimide.
3. No nucleoside 5'-triphosphate was synthesized by F_1 , when GDP, IDP, CDP, or UDP was used as a substrate.
4. Both the dependence on ADP concentration of the amount of ATP formed in the presence of a sufficient concentration of P_i and the

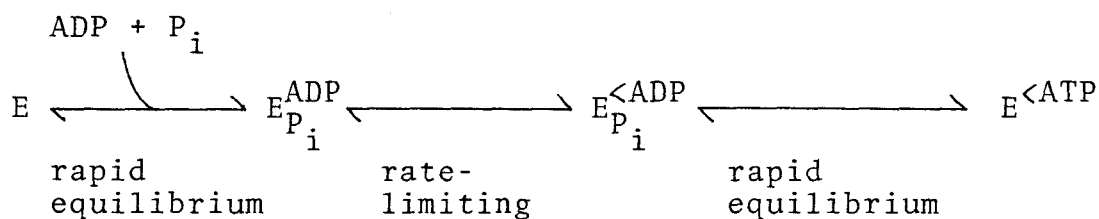
dependence on P_i concentration of the amount in the presence of a sufficient concentration of ADP were given by the following equation:

$$[\text{ATP formed}] = [\text{ATP formed}]_{\text{max}} / (1 + K_{\text{ADP}} / [\text{ADP}] \text{ or } K_p / [P_i])$$

where $K_{\text{ADP}} = 3 \mu\text{M}$, $K_p = 0.55 \text{ mM}$, and $[\text{ATP formed}]_{\text{max}} = 0.4\text{-}0.6 \text{ mol/mol } F_1$.

5. When the reaction mixture was diluted with the buffer solution after the ATP-synthesis reaction had reached equilibrium, the amount of synthesized ATP decreased monophasically at a higher rate than that of ATP formation. When the pH of the reaction mixture was rapidly increased from 6.9 to 8.0, about half of the synthesized ATP disappeared very rapidly, while the remainder decreased rather slowly.

6. All these findings can be explained by the following reaction scheme in which the catalytic sites in F_1 for ATP synthesis are assumed to function independently:



where the brackets indicate tight binding.

7. However, the dependence on P_i concentration of the initial rate of ATP synthesis, v_f , in the presence of a sufficient amount of ADP was given by the equation, $v_f = v_{f \cdot \text{max}} / [1 + (K_p' / [P_i])^2]$. Furthermore, when AMPPNP was added to the reaction mixture, 60-70% of the formed ATP disappeared very rapidly and the remainder decreased very slowly. These two findings suggest cooperativity

between catalytic or nucleotide-binding sites of F_1 during the ATP-synthesis reaction.

INTRODUCTION

The F_1-F_0 complex catalyzes ATP synthesis from ADP and P_i in mitochondrial oxidative phosphorylation, and the nucleotide and P_i binding sites are located on F_1 (for reviews, see 1,2). It is also widely accepted that ATP synthesis is driven by the electrochemical potential difference, $\Delta\mu_{H^+}$, across the membrane. However, the molecular mechanism of the ATP synthesis by F_1 driven by $\Delta\mu_{H^+}$ remains controversial.

Mitchell (3) advocated the hypothesis that the free energy of $\Delta\mu_{H^+}$ is used directly in the step where ADP and P_i bind covalently to form ATP with dehydration. On the other hand, Boyer and coworkers (4) proposed that the free energy is used for the cooperative release of formed ATP from and the binding of ADP and P_i to F_1 . The latter hypothesis was proposed initially by analogy to the reverse reaction of myosin ATPase (5,6). Recently, Matsuoka et al. (7,8) investigated the elementary steps of ATP hydrolysis by mitochondrial and bacterial F_1 -ATPase [EC 3.6.1.3], using a fluorescent ATP analog, 2'-(5-dimethylaminonaphthalene-1-sulfonyl) amino-2'-deoxyATP (DNS-ATP), and found that the reaction sequence of F_1 -DNS-ATPase is similar to that of myosin ATPase. More recently, Grubmeyer et al. (9) measured the rate constants of various partial reactions of F_1 -ATPase and also proposed a reaction sequence which is essentially equivalent to that of myosin ATPase. However, there are several lines of evidence indicating that the F_1 -ATPase reaction involves at least two different routes (7,10). Therefore, the reaction mechanism of

ATP synthesis by mitochondria should be clarified to see whether ATP bound to F_1 is formed from medium ADP and P_i without $\Delta\mu_H^+$ across the membrane.

Feldman and Sigman (11) reported that enzyme-bound ATP is synthesized by chloroplast F_1 from added P_i and tightly bound ADP. However, this reaction might not be a partial reaction of photo-phosphorylation, since medium ADP is not utilized by the reaction. On the other hand, efforts to detect ATP synthesis from medium ADP and P_i by mitochondrial soluble F_1 have been unsuccessful (e.g., 12).

Recently, we succeeded in synthesizing ATP with mitochondrial soluble F_1 from medium ADP and P_i in the presence of dimethylsulfoxide (DMSO), which is known to promote phosphorylation of Ca^{2+}, Mg^{2+} -ATPase in the sarcoplasmic reticulum by P_i in the presence of Mg^{2+} (13). ATP synthesis by F_1 was observed in the presence of more than 10% (w/v) DMSO. Therefore, we measured the amount of ATP formed in the presence of 35% (w/v) DMSO and 4.2 mM Mg^{2+} at pH 6.7 and 30°C.

The amount of synthesized ATP measured after stopping the reaction with trichloroacetic acid (TCA) was almost equal to that measured after stopping the reaction with sodium dodecyl sulfate (SDS) or with ethanol. The rate and extent of [α - ^{32}P]ATP synthesis from [α - ^{32}P]ADP and P_i were equal to those of [γ - ^{32}P]ATP synthesis from ADP and $^{32}P_i$. Furthermore, the ATP formed was inaccessible to hexokinase, and its amount was proportional to the amount of F_1 . The maximal amount of ATP synthesized was 0.4-0.6 mol/mol F_1 . The reaction was inhibited by sodium azide, but not by 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) or N,N'-dicyclohexylcarbodiimide

(DCCD). No nucleoside 5'-triphosphate was synthesized by F_1 when GDP, IDP, CDP, or UDP was used as a substrate. The dependence on ADP and P_i concentrations of the amount of ATP formed was given by an equation for a first-order binding reaction, and the apparent affinity for ADP was much higher than that for P_i .

EXPERIMENTAL PROCEDURES

Materials — $^{32}\text{P}_i$ was purchased from the Japan Radioisotope Association, Tokyo and purified on a Dowex 1 x 4 anion-exchange column to reduce radioactive impurities to below 0.05%. $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ was obtained by F_1 -catalyzed hydrolysis of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$, which was purchased from Amersham International Ltd., Amersham, and used after purification on a Dowex 1 x 4 column to reduce contaminating $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ to below 0.4% and other radioactive impurities to about 1.2%.

ADP and ATP were purchased from Kohjin Co., Ltd., Tokyo. IDP, GDP, CDP, and UDP were obtained from Yamasa Shoyu Co., Choshi. Phosphoenol pyruvate and adenylyl-5'-imidodiphosphate (AMPPNP) were purchased from Sigma Chemicals Co., St. Louis. DMSO was obtained from Wako Pure Chemical Industries, Ltd., Osaka.

Pyruvate kinase was prepared according to the method of Tietz and Ochoa (14), and hexokinase was purchased from Sigma Chemicals Co., St. Louis.

Submitochondrial particles were prepared from bovine heart mitochondria as described by Beyer (15). F_1 was extracted from submitochondrial particles with chloroform, purified by means of ammonium sulfate fractionation and gel filtration on a Sephacryl S-300 Superfine column, and concentrated by ultrafiltration on an Amicon Diaflo XM-100A membrane, as described previously (7). F_1 was stored at 0°C in a solution containing 4 mM EDTA, 50-55% (w/v) glycerol, 20 μM phenylmethylsulfonyl fluoride, 0.2 mM dithiothreitol, and 0.1 M Tris-HCl at pH 8.0. A unit of ATPase activity is defined as the activity which liberates 1 μmol of P_i per min.

The specific activity of purified F_1 was 70-110 units/mg in the presence of 3 mM ATP and 5 mM $MgCl_2$ at pH 8.0 and 30°C. The protein concentration of purified F_1 was determined by the biuret method (16) with bovine serum albumin ($A_{279\text{ nm}} = 0.667\text{ cm}^2\cdot\text{mg}^{-1}$) as a standard. The molecular weight of F_1 used in all calculations was 360,000 (17). We confirmed that the F_1 preparation used here contained only five subunits of F_1 , α , β , γ , δ , and ξ (7), by SDS-polyacrylamide gel electrophoresis (18).

ATP Synthesis — ATP synthesis was started by adding 20 μl of F_1 solution to 80 μl of a reaction mixture at 30°C, unless otherwise stated. The final reaction mixture usually contained 10-11% (w/v) glycerol, 35% (w/v) DMSO, 5 mM $MgCl_2$, 0.8 mM EDTA, and 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES)-Tris at pH 6.7 (referred to hereafter as the "standard DMSO-buffer solution"), and various concentrations of F_1 , ADP, and P_i . The standard DMSO-buffer solution without DMSO is referred to as the "standard buffer solution." Either $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ or $^{32}\text{P}_i$ was used in each experiment. The reaction was stopped by adding 0.9 ml of cold 5% TCA, along with carrier ATP, ADP, and P_i . The resulting mixture was centrifuged to remove the denatured protein. A 0.9-ml aliquot of the supernatant was passed through an activated charcoal column (ϕ 0.6 cm x 0.5 cm), which was washed with 1 ml of 5% TCA with carrier P_i , followed by 1 ml of H_2O . Nucleotides were eluted with 1 ml of ethanol:1 M ammonia (40:60). The eluent was evaporated, and the nucleotides were dissolved in water and chromatographed on a polyethyleneimine-cellulose thin-layer plate using 2.5 M sodium formate at pH 3.4 as the developer according to

the method of Randerath and Randerath (19). Radioactivity was measured with a Beckman LS-9000 liquid scintillation counter.

F_1 at 1.9 mg/ml was allowed to react with 0.5 mM $^{32}P_i$ and 30 μ M ADP in the standard DMSO-buffer solution for 30 min, and 90- μ l aliquots were taken and mixed with 10 μ l of 50% TCA, 2.5, 5, or 10% SDS or with 90 μ l of ethanol. The amounts of synthesized [^{32}P]ATP measured after stopping the reaction with several concentrations of SDS or ethanol were equal to those measured after stopping the reaction with TCA. Thus, the amount of synthesized ATP was independent of the procedure for stopping the reaction.

F_1 at 1.82 mg/ml was allowed to react with 0.2 mM ADP and 0.2 mM $^{32}P_i$ in the standard DMSO-buffer solution for 30 min, and the reaction was stopped with 5% perchloric acid. The mixture was neutralized with KOH and centrifuged to remove the denatured protein and potassium perchlorate. When 5 mM glucose and 30 μ g/ml hexokinase were added to the supernatant, [^{32}P]ATP disappeared rapidly, indicating that [γ - ^{32}P]ATP was formed.

ATPase Reaction — The ATPase reaction was started by adding F_1 to a reaction mixture containing 3 mM ATP, 2 mM phosphoenol pyruvate, 66-260 μ g/ml pyruvate kinase, 5 mM $MgCl_2$, 50 mM KCl, and 50 mM Tris-HCl at pH 8.0 and 30°C, and stopped with 5% TCA, unless otherwise stated. The amount of P_i liberated was determined by the method of Youngburg and Youngburg (20).

Determination of [^{32}P]Glucose-6-Phosphate — The hexokinase reaction was stopped with 5% TCA, and the resulting mixture was boiled for 20 min to cleave the remaining [γ - ^{32}P]ATP. $^{32}P_i$ was

extracted with n-butanol:benzene (1:1), and the radioactivity of the water phase was measured as that of [³²P]glucose-6-phosphate.

RESULTS

ATP Synthesis in the Presence of DMSO — Figure 1 shows the dependence on DMSO concentration of the amount of [γ - 32 P]ATP formed by F_1 and the F_1 -ATPase activity. For [γ - 32 P]ATP synthesis, F_1 at 0.82 mg/ml was allowed to react with 2 mM ADP and 1 mM $^{32}P_i$ in the presence of 5 mM $MgCl_2$, 0.8 mM EDTA, 10% (w/v) glycerol, and 0.1 M MES-Tris at pH 5.8 and 30°C for 5 min, while the F_1 -ATPase activity was measured in the presence of 4 mM ATP, 10 mM $MgCl_2$, 0.1 M KCl, and 0.1 M MES-Tris at pH 6.5 and 30°C. The [^{32}P]ATP synthesized was found to be [γ - ^{32}P]ATP, as described in EXPERIMENTAL PROCEDURES. The amount of synthesized [γ - ^{32}P]ATP increased sharply when the DMSO concentration was increased over 10% (w/v), reached the maximal level at 30% (w/v), then decreased gradually. On the other hand, the ATPase activity increased gradually with increasing DMSO concentration until 30% (w/v), then decreased sharply. The following experiments were performed in the presence of 35% (w/v) DMSO, where the ATPase activity was almost equal to that in the absence of DMSO.

To examine the stability of F_1 in 35% (w/v) DMSO, F_1 at 2.1 mg/ml was incubated in the standard DMSO-buffer solution containing 30 μ M ADP and 0.2 mM P_i at 30°C for various periods of time, and the ATPase activity was measured in the standard DMSO-buffer solution containing 2 mM ATP, 4 mM phosphoenol pyruvate, and 0.13 mg/ml pyruvate kinase. The ATPase activity of F_1 was found to be constant for at least 60 min.

Figure 2 compares the time course of [γ - ^{32}P]ATP formation

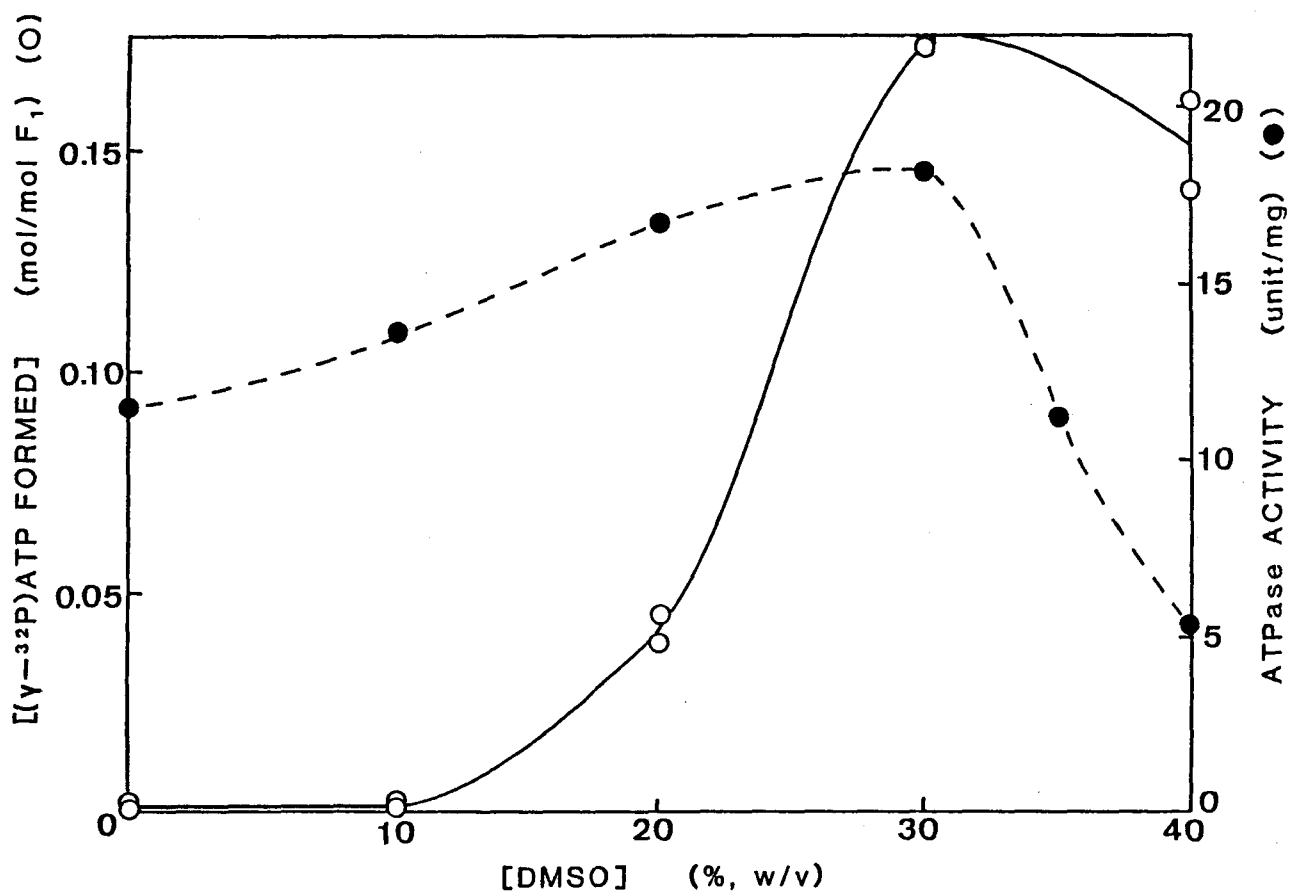


Fig. 1. Dependence on DMSO concentration of the amount of ATP synthesized by F_1 and the F_1 -ATPase activity. For [γ - 32 P]ATP synthesis (O), F_1 at 0.82 mg/ml was allowed to react with 2 mM ADP and 1 mM $^{32}P_i$ in the presence of 5 mM $MgCl_2$, 0.8 mM EDTA, 10% (w/v) glycerol, and 0.1 M MES-Tris at pH 5.8 and 30°C for 5 min. For the ATPase activity (●), F_1 at 12.8 μ g/ml was allowed to react with 4 mM ATP in the presence of 10 mM $MgCl_2$, 5 mM phosphoenol pyruvate, 0.26 mg/ml pyruvate kinase, 0.1 M KCl, and 0.1 M MES-tris at pH 6.5 and 30°C. A unit of ATPase activity is defined as the activity at which 1 μ mol of P_i is liberated per min.

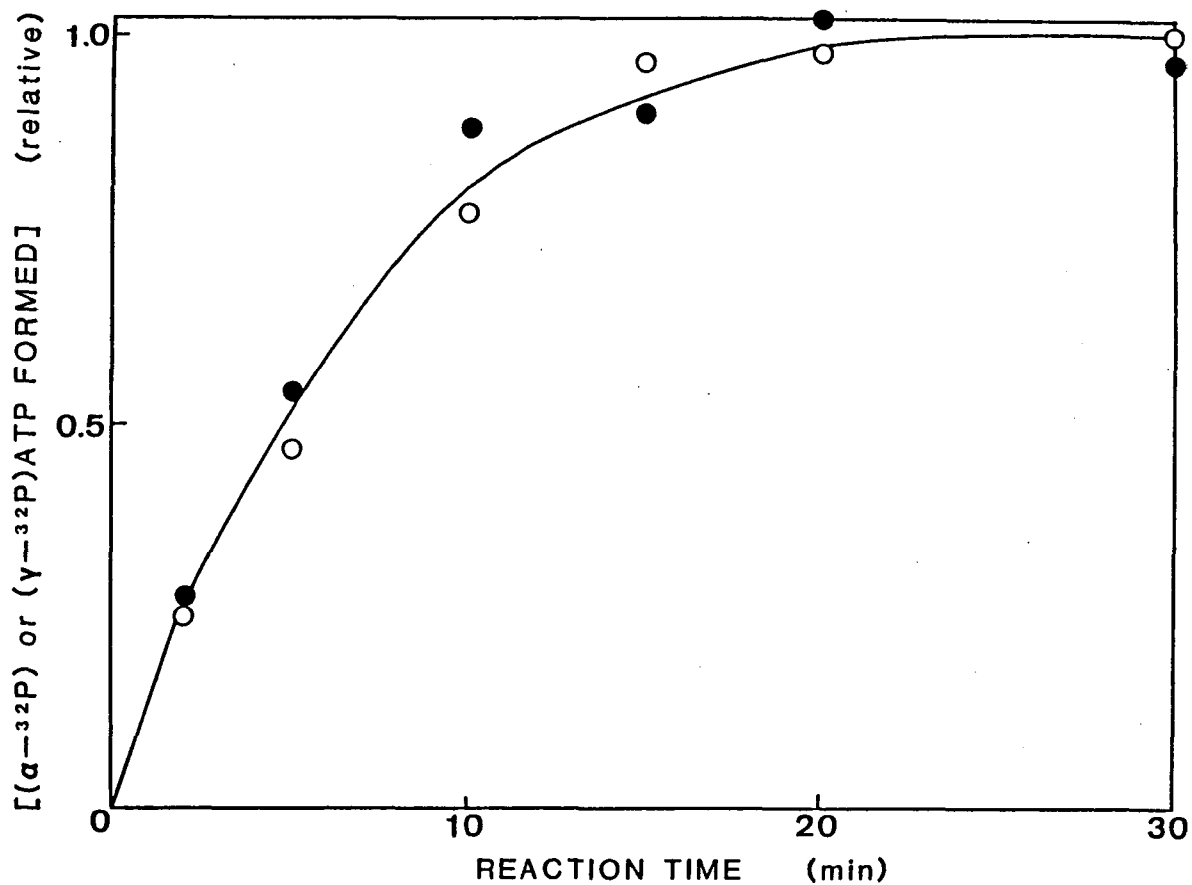


Fig. 2. Comparison of the time course of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ formation from ADP and $^{32}\text{P}_i$ with that of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formation from $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and P_i by F_1 . F_1 at 1.7 mg/ml was allowed to react with 30 μM ADP and 0.33 mM $^{32}\text{P}_i$ (○) or with 30 μM $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and 0.33 mM P_i (●) in the standard DMSO-buffer solution [5 mM MgCl_2 , 0.8 mM EDTA, 35% (w/v) DMSO, 11% (w/v) glycerol, and 0.1 M MES-Tris at pH 6.7] at 30°C. The amounts of formed $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ are expressed relative to that of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ formed at 30 min, which was 0.042 mol/mol F_1 .

from 30 μM ADP and 0.33 mM $^{32}\text{P}_i$ with that of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formation from 30 μM $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and 0.33 mM P_i by 1.7 mg/ml F_1 in the standard DMSO-buffer solution. The rate and extent of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formation were equal to those of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ formation. The amount of $[\text{}^{32}\text{P}]\text{ATP}$ synthesized reached the maximal value at about 20 min after the start of the reaction.

Nelson and Racker (21) previously reported that PP_i is formed from γ -phosphate of ATP and P_i in the presence of a high concentration of DMSO, and that PP_i is also formed from β -phosphate of ADP and P_i at a much slower rate. However, this phosphorolysis of ATP and ADP was observed only in the presence of DMSO at concentrations higher than 50% (21). As shown in Fig. 3, the amount of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ synthesized by F_1 was much larger than that of formed $[\alpha\text{-}^{32}\text{P}]\text{AMP}$. When F_1 at 1.9 mg/ml was allowed to react with 30 μM $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and 10 mM P_i in the presence of 35% (w/v) DMSO. Furthermore, the amount of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formed was almost constant from 30 min to 100 min after initiation of the reaction (data not shown).

Effect of pH on ATP Synthesis — Figure 4 shows the pH dependence of the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ formed by the reaction of 0.82 mg/ml F_1 with 2 mM ADP and 0.2 mM $^{32}\text{P}_i$ in the presence of 5 mM MgCl_2 , 0.8 mM EDTA, 35% (w/v) DMSO, 10% (w/v) glycerol, and a pH buffer at 0.1 M at 30°C for 20 min. MES-Tris was used at pH 5.3-6.7 and N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid-Tris at pH 7.1-8.1 as a pH buffer. The pH dependence was bell-shaped with the optimal pH at 6.7-6.8. The amount of $[\gamma\text{-}^{32}\text{P}]=\text{ATP}$ formed at each pH under the conditions used was given by the

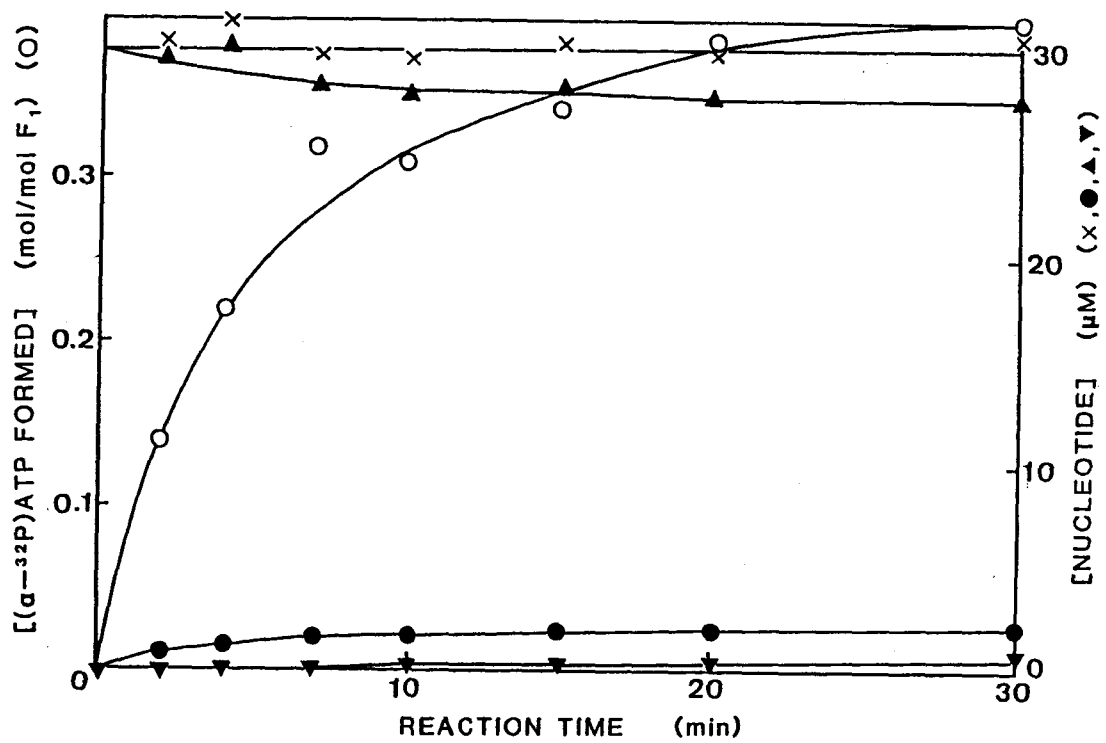


Fig. 3. Time courses of change in the amounts of adenine nucleotides during the reaction of ATP synthesis by F_1 . F_1 at 1.9 mg/ml was allowed to react with $30 \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and 10 mM P_i in the standard DMSO-buffer solution at 30°C . \circ , \bullet , $[\alpha\text{-}^{32}\text{P}]\text{ATP}$; \blacktriangle , $[\alpha\text{-}^{32}\text{P}]\text{ADP}$; \blacktriangledown , $[\alpha\text{-}^{32}\text{P}]\text{AMP}$; \times , total $[\alpha\text{-}^{32}\text{P}]$ adenine nucleotide.

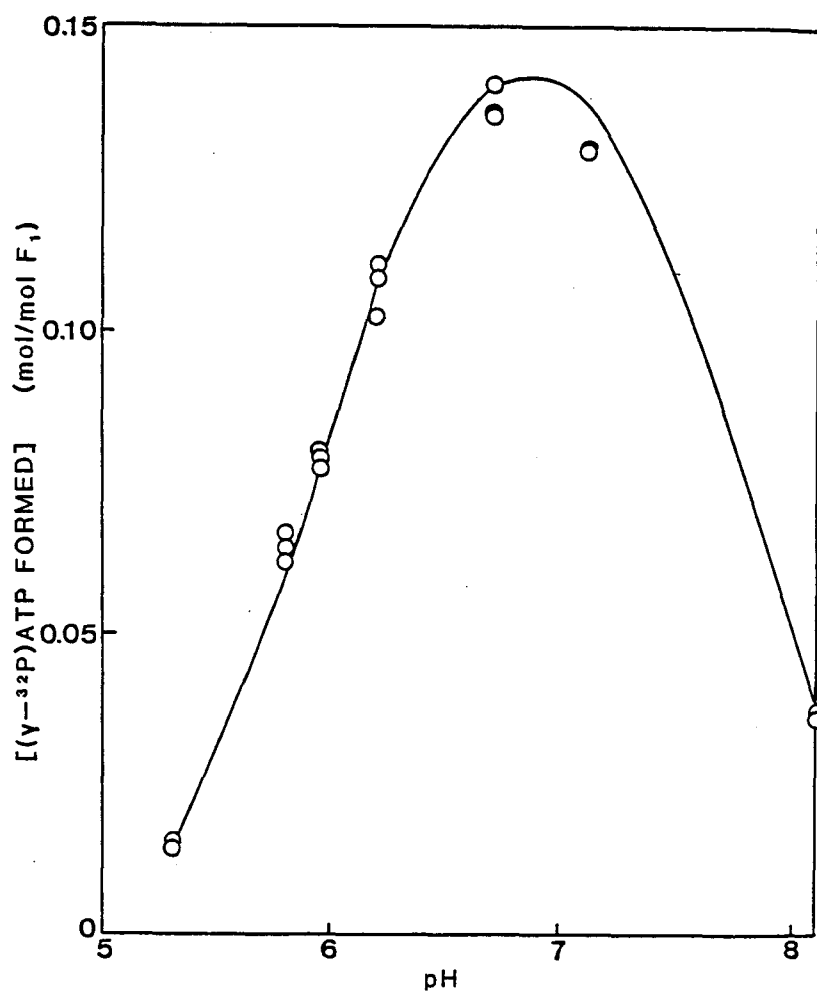


Fig. 4. pH dependence of the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ synthesized by F_1 from ADP and $^{32}\text{P}_i$. F_1 at 0.82 mg/ml was allowed to react with 2 mM ADP and 0.2 mM $^{32}\text{P}_i$ in the presence of 5 mM MgCl_2 , 0.8 mM EDTA, 35% (w/v) DMSO, and 10% (w/v) glycerol at 30°C for 20 min. We used 0.1 M MES-Tris and N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid-Tris at pH 5.3-6.7 and 7.1-8.1, respectively, as pH buffers.

following equation:

$$[\text{ATP formed}] = 0.156(\text{mol/mol } F_1) / [1 + ([\text{H}^+]/1.1\mu\text{M})^{1.5} + (18\text{nM}/[\text{H}^+])^{1.5}]$$

The solid line in the figure is the calculated one. This finding suggests that amino acid residues with pK values of about 6.0 and 7.7 participate in the reaction of ATP synthesis (22).

Requirement of F_1 for ATP Synthesis — Figure 5 shows the dependence of the concentration of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formed on F_1 concentration. F_1 at various concentrations was allowed to react with $20\ \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and $0.2\ \text{mM}$ P_i in the standard DMSO-buffer solution at 30°C for 30 min. The concentration of synthesized $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ increased in proportion to that of F_1 . It was negligible in the absence of F_1 or in the presence of heat-denatured F_1 . The dependence of the concentration of synthesized $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ on F_1 concentration deviated downward from the linear relationship when the F_1 concentration was higher than $4\ \mu\text{M}$, probably because the concentration of free $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ was decreased by the binding of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ to F_1 .

Figure 6 shows the effects of inhibitors on the F_1 -ATPase activity and the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ synthesis by F_1 from ADP and $^{32}\text{P}_i$. F_1 at $2.1\ \text{mg/ml}$ was preincubated with DCCD, NBD-Cl, or sodium azide in the presence of $33\ \mu\text{M}$ ADP (no ADP in the case of sodium azide), $0.9\ \text{mM}$ EDTA, 35% (w/v) DMSO, 12% (w/v) glycerol, and $0.1\ \text{M}$ MES-Tris at pH 6.7 and 30°C for 80 min. The concentration of each inhibitor used for preincubation is given on the abscissa. The preincubation mixture was mixed with 1/9 volume of a solution containing $0.3\ \text{mM}$ ADP, $2\ \text{mM}$ $^{32}\text{P}_i$, 35% (w/v) DMSO, and $0.1\ \text{M}$ MES-

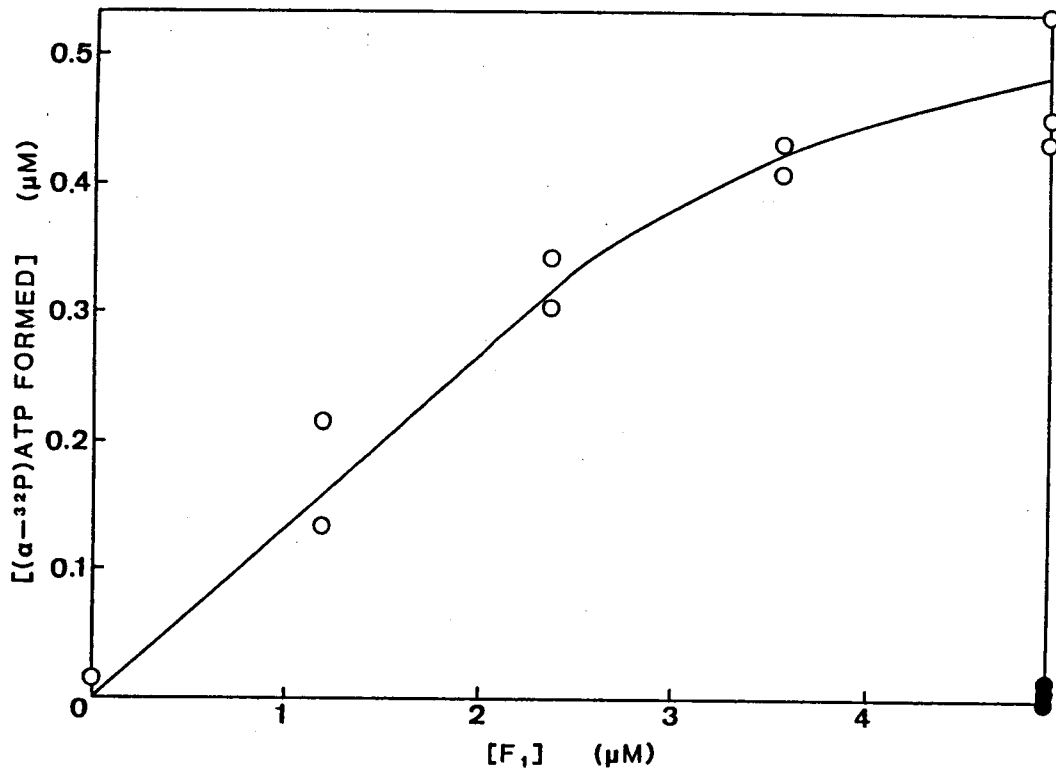


Fig. 5. Dependence of concentration of formed ATP on that of F_1 . F_1 at various concentrations was allowed to react with $20 \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and 0.2 mM P_i in the standard DMSO-buffer solution at 30°C for 30 min (O). Closed circles (●) show the amount of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formed when F_1 denatured by boiling for 10 min was used.

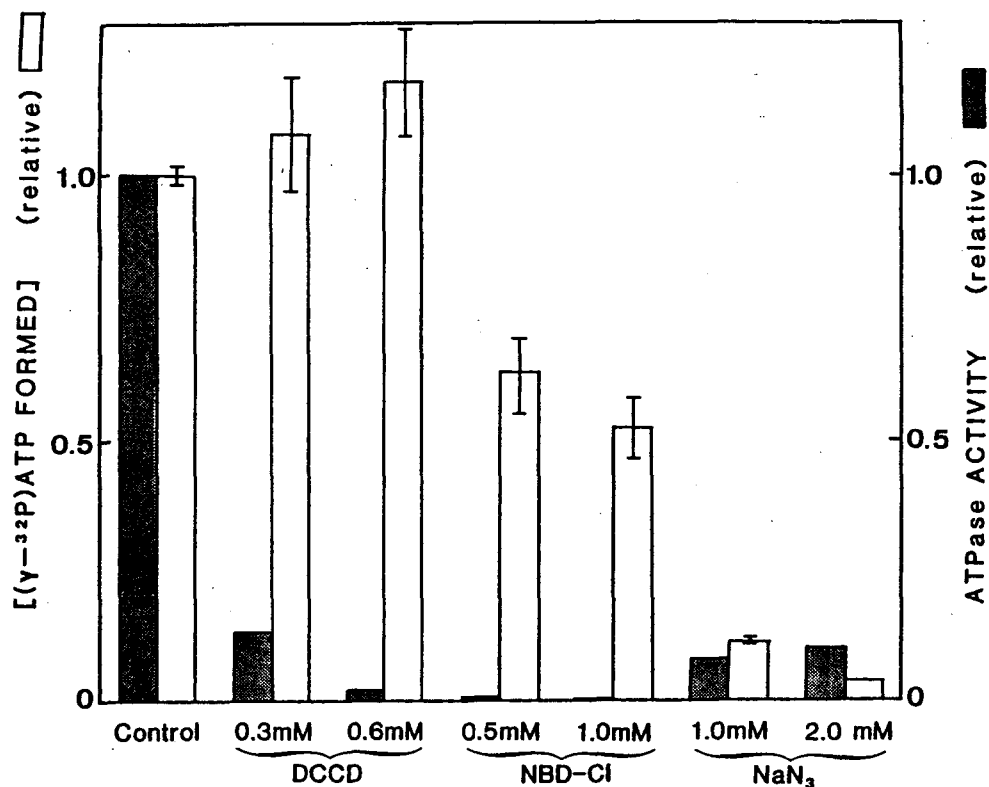


Fig. 6. Effects of inhibitors on the F_1 -ATPase activity and the ATP synthesis by F_1 . F_1 (2.1 mg/ml) was preincubated with DCCD, NBD-Cl or sodium azide in the presence of $33 \mu\text{M}$ ADP (no ADP in the case of sodium azide), 0.9 mM EDTA, 35% (w/v) DMSO, 12% (w/v) glycerol, and 0.1 M MES-Tris at pH 6.7 and 30°C for 80 min. The concentration of each inhibitor used for the pretreatment is given on the abscissa. The preincubation mixture was mixed with 1/9 volume of a solution containing 0.3 mM ADP, 2 mM $^{32}\text{P}_i$, 35% (w/v) DMSO, and 0.1 M MES-Tris at pH 6.7, and the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ formed was measured 30 min after the start of the reaction. The ATPase activity was measured as described in EXPERIMENTAL PROCEDURES. Values are expressed relative to those of the control, where F_1 was preincubated with no inhibitors. The control value of ATPase activity was 68 unit/mg and that of the amount of ATP formed was $0.036 \text{ mol/mol } F_1$.

Tris at pH 6.7, and the amount of [γ - 32 P]ATP formed was measured 30 min after the start of the reaction. The ATPase activity was measured as described in EXPERIMENTAL PROCEDURES. Sodium azide at 1 and 2 mM inhibited both the [γ - 32 P]ATP synthesis and the ATPase reaction. DCCD and NBD-Cl inhibited the ATPase activity almost completely, while DCCD did not affect [γ - 32 P]ATP synthesis and NBD-Cl inhibited it only partially.

Figure 7 shows the substrate specificity of the ATP-synthesis reaction. F_1 at 1.9 mg/ml was allowed to react with 1 mM $^{32}P_i$ and 30 μ M ADP, IDP, GDP, UDP, or CDP in the standard DMSO-buffer solution. Nucleoside 5'-triphosphate was synthesized only when ADP was used as the substrate, being insignificant when GDP, IDP, UDP, or CDP was used.

Identification of the ATP Formed as Enzyme-bound ATP — Figure 8 shows the inaccessibility of [γ - 32 P]ATP synthesized by F_1 to hexokinase. [γ - 32 P]ATP was formed from 0.1 mM ADP and 0.2 mM $^{32}P_i$ by 1.8 mg/ml F_1 in the standard DMSO-buffer solution containing 5 mM glucose. When 1/10 volume of 0.55 or 5.5 mg/ml hexokinase was added to the reaction mixture 30 min after the start of the reaction, the amount of [γ - 32 P]ATP remained constant after a slight decrease, which was due to the dilution effect, since it was also observed when the buffer or heat-denatured hexokinase at 1/10 volume was added. As shown in the inset, all the radioactivity of [γ - 32 P]ATP was transferred to glucose within 30 s, when 1/10 volume of 0.55 or 5.5 mg/ml hexokinase was added to a solution containing 0.6 μ M [γ - 32 P]ATP and 5 mM glucose in the standard

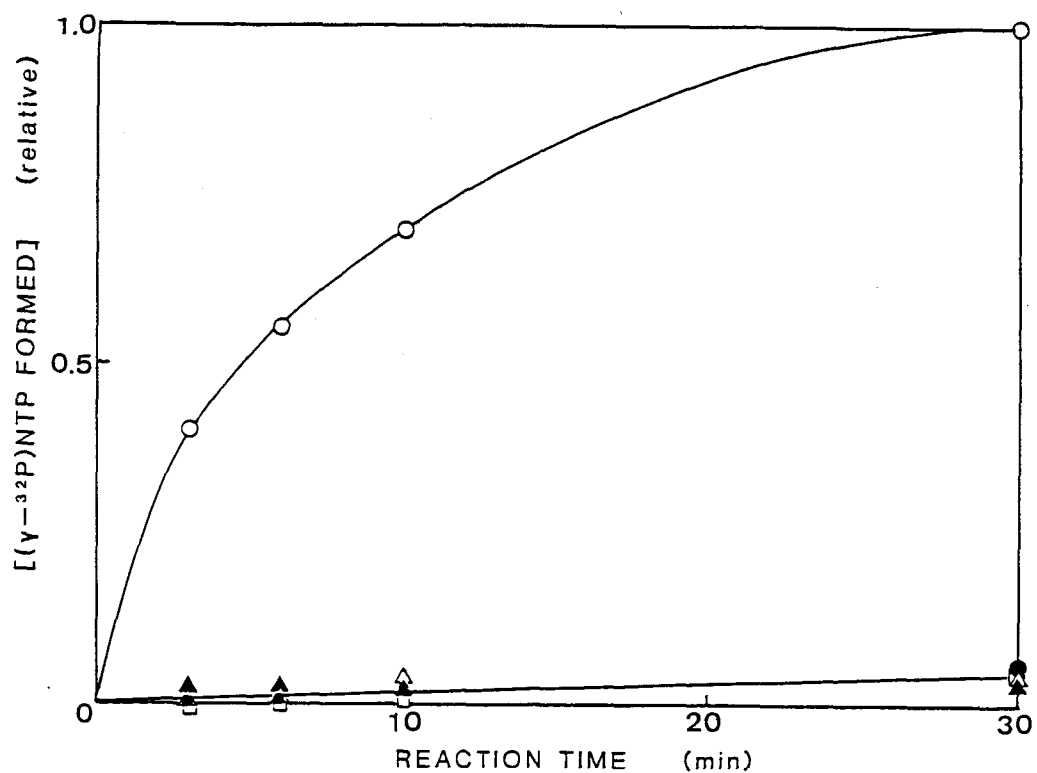


Fig. 7. Substrate specificity of nucleoside 5'-triphosphate synthesis by F_1 . F_1 at 1.9 mg/ml was allowed to react with 1 mM $^{32}P_i$ and 30 μ M ADP, GDP, IDP, UDP or CDP in the standard DMSO-buffer solution at 30°C. Nucleoside 5'-diphosphate used: ○, ADP; △, GDP; □, IDP; ●, UDP; ▲, CDP. The amount of $[\gamma\text{-}^{32}P]$ nucleoside 5'-triphosphate formed is expressed relative to that of $[\gamma\text{-}^{32}P]$ ATP formed at 30 min, which was 0.064 mol/mol F_1 .

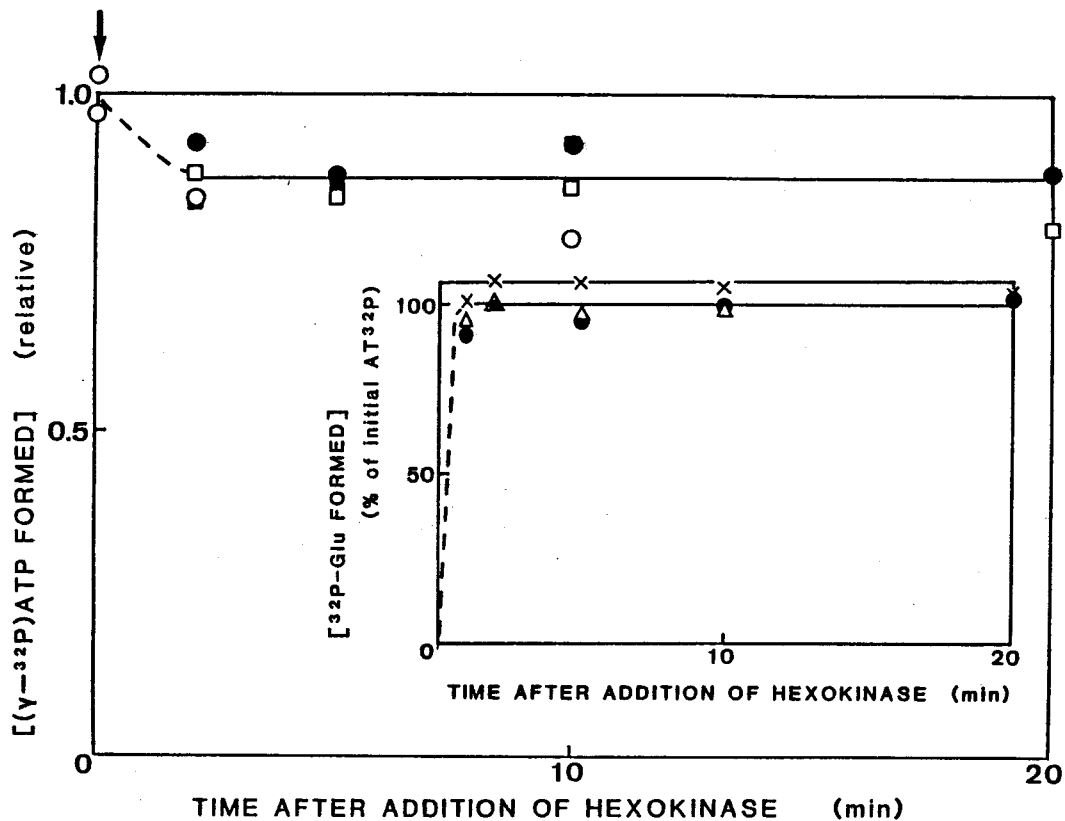


Fig. 8. Inaccessibility of ATP synthesized by F_1 to hexokinase. F_1 at 1.82 mg/ml was allowed to react with 0.1 mM ADP and 0.2 mM $^{32}P_i$ in the standard DMSO-buffer solution containing 5 mM glucose at 30°C. 1/10 volume of 0 (○), 0.55 (●), 5.5 (■) mg/ml hexokinase or 5.5 mg/ml heat-denatured hexokinase (□) was added to the reaction mixture 30 min after the start of the reaction. The amounts of formed $[\gamma-^{32}P]ATP$ are expressed relative to that of $[\gamma-^{32}P]ATP$ at time zero, which was 0.039 mol/mol F_1 . Inset shows the time courses of $[^{32}P]$ glucose-6-phosphate formation when hexokinase was added to 0.6 μM $[\gamma-^{32}P]ATP$ in the standard DMSO-buffer solution (X, Δ) or in the standard buffer solution (●) containing 5 mM glucose. Final concentration of hexokinase: X, ●, 0.05; Δ , 0.5 mg/ml.

DMSO-buffer solution or in the standard buffer solution in the absence of F_1 .

Dependence of the Amount and Rate of ATP Synthesis on P_i

Concentration — Figure 9 shows a double reciprocal plot of the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ formed against $^{32}\text{P}_i$ concentration, when F_1 was allowed to react with 2 mM ADP and various concentrations of $^{32}\text{P}_i$ in the standard DMSO-buffer solution for 60 min. The straight line in the figure was obtained from the following equation:

$$[\text{ATP formed}] = [\text{ATP formed}]_{\text{max}} / (1 + K_p / [P_i])$$

where $[\text{ATP formed}]_{\text{max}} = 0.61 \text{ mol/mol } F_1$ and $K_p = 0.55 \text{ mM}$.

Figure 10 shows the dependence on $^{32}\text{P}_i$ concentration of the initial rate of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ formation, v_f , under the same conditions as for Fig. 9. The abscissa represents the reciprocal of the square of P_i concentration and the ordinate represents the reciprocal of v_f . The v_f values calculated from the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ formed at 1 min and 2 min after the start of the reaction were equal within experimental error. The straight line in the figure was obtained from the following equation:

$$v_f = v_{f \cdot \text{max}} / [1 + (K_p' / [P_i])^2]$$

where $v_{f \cdot \text{max}} = 0.105 \text{ min}^{-1}$ and $K_p' = 0.58 \text{ mM}$. The same preparation of F_1 was used in the experiments shown in Figs. 9 and 10, since the dependence of the amount of ATP formed on P_i concentration differed from one preparation to another, while the amount of ATP formed at sufficient amounts of ADP and P_i was constant (0.4-0.6 mol/mol F_1).

Dependence of the Amount of ATP Synthesized on ADP Concentra=

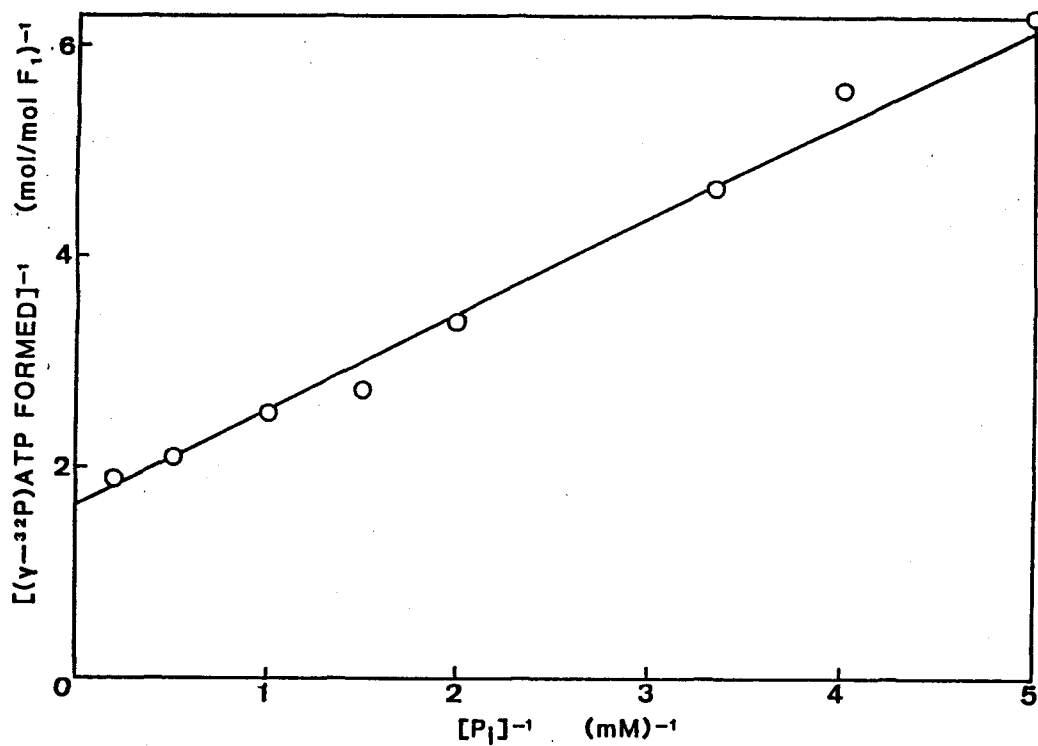


Fig. 9. Double reciprocal plot of the amount of ATP synthesized by F_1 against P_i concentration. F_1 at 0.82 mg/ml was allowed to react with 2 mM ADP and various concentrations of $^{32}P_i$ in the standard DMSO-buffer solution at 30°C for 60 min.

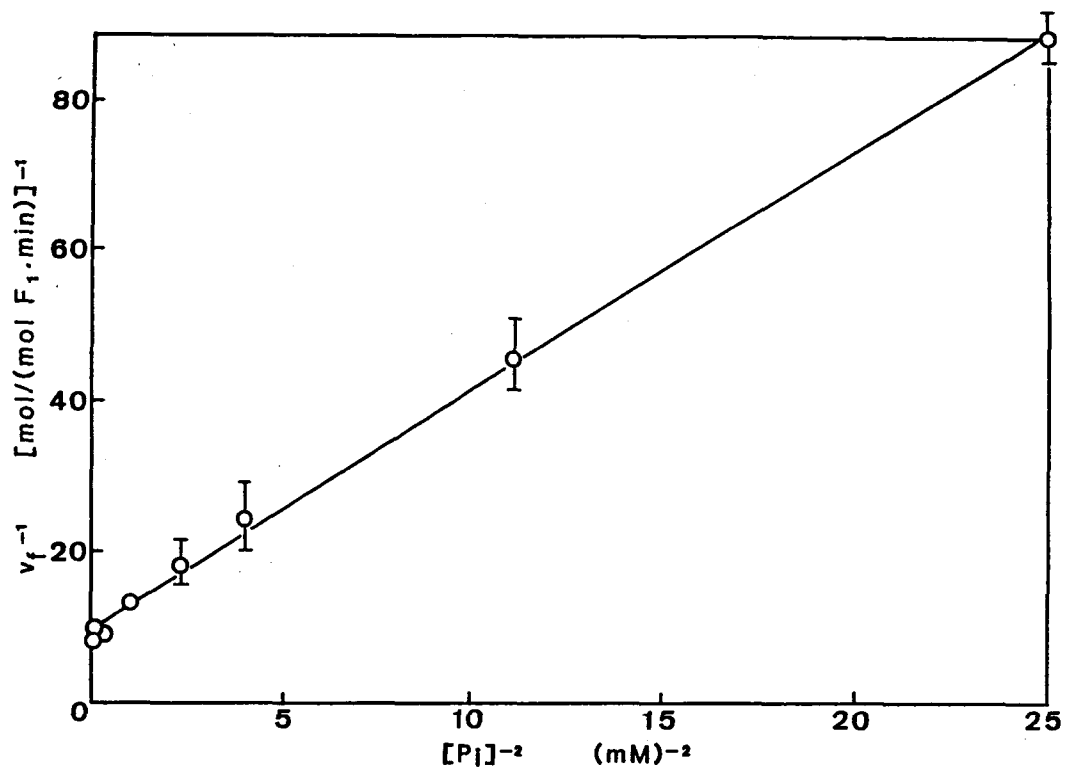


Fig. 10. Double reciprocal plot of the initial rate of ATP formation, v_f , against the square of P_i concentration. F_1 (0.82 mg/ml) was allowed to react with 2 mM ADP and various concentrations of $^{32}P_i$ under the same conditions as for Fig. 9. The ends of the bars indicate the v_f values calculated from the amounts of $[\gamma\text{-}^{32}P]\text{ATP}$ formed at 1 min and 2 min, and the open circles (O) indicate the means of the v_f values.

tion — Figure 11A shows the dependence on the concentration of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ added of the amount of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ synthesized when F_1 at 1.9 mg/ml was allowed to react with 10 mM P_i and various concentrations of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ in the standard DMSO-buffer solution for 30 min. The inset shows a double reciprocal plot of the amount of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formed against the concentration of free $[\alpha\text{-}^{32}\text{P}]\text{ADP}$. The concentration of free $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ was calculated on the following two assumptions. (i) The sum of the amounts of bound ADP and ATP is 2 mol/mol F_1 at an infinite concentration of ADP (7,23) and (ii) the amount of bound ADP is proportional to that of the ATP formed. The solid line in the inset was obtained from the following equation:

$$[\text{ATP formed}] = [\text{ATP formed}]_{\text{max}} / (1 + K_{\text{ADP}} / [\text{free ADP}])$$

where $[\text{ATP formed}]_{\text{max}} = 0.43$ mol/mol F_1 and $K_{\text{ADP}} = 3.05 \mu\text{M}$.

Figure 11B shows the dependence on ADP concentration of the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ formed, when the P_i concentration was reduced to 0.2 mM. The amounts of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at 60 min were almost equal to those at 100 min. The amount of $[\text{}^{32}\text{P}]\text{ATP}$ without added ADP was very small. The K_{ADP} value was found to be 2-3 μM , when the concentration of free ADP was calculated as described for Fig. 11A.

Decrease in the Amount of ATP after Dilution, pH Change or Nucleotide Addition — Figure 12A shows the time course of decrease in the amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ after 5-fold dilution of the reaction mixture with the standard DMSO-buffer solution. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was formed from 0.2 mM ADP and 0.2 mM $^{32}P_i$ by 1.82 mg/ml F_1 in the standard DMSO-buffer solution for 30 min. The amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ decreased monophasically upon dilution to a low constant

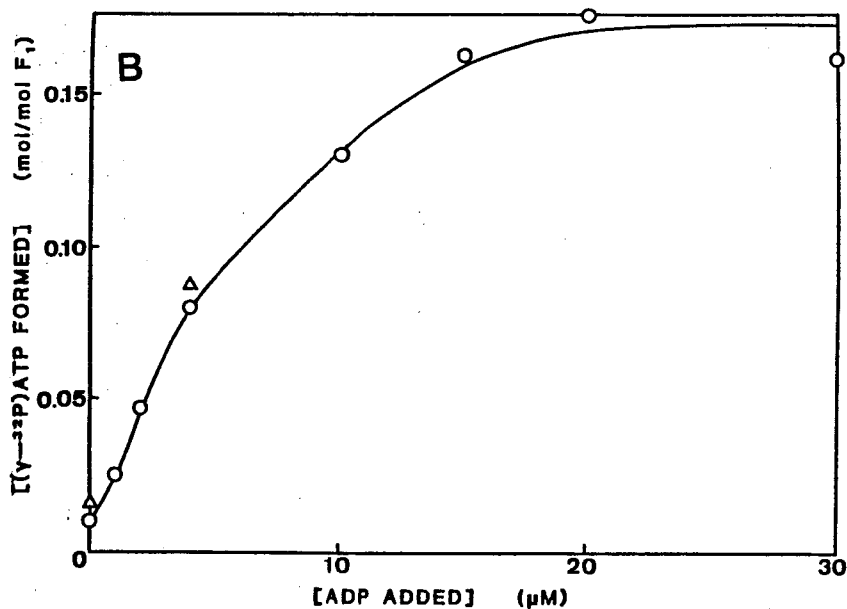
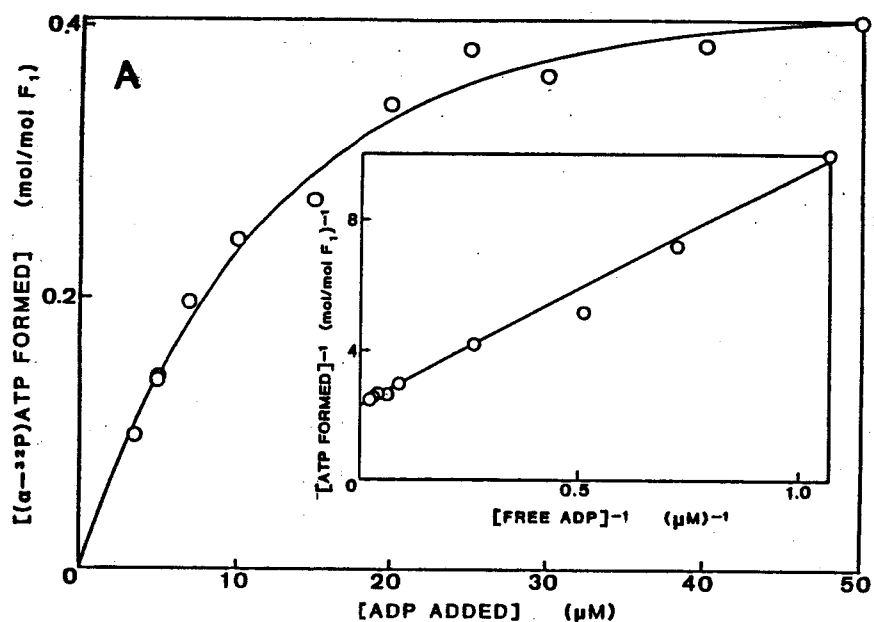


Fig. 11. Dependence on concentration of added ADP of the amount of ATP formed by F_1 . A: F_1 at 1.9 mg/ml was allowed to react with 10 mM P_i and various concentrations of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ in the standard DMSO-buffer solution at 30°C for 30 min. The inset shows a double reciprocal plot of the amount of formed ATP against the concentration of free ADP calculated as described in the text. B: F_1 at 0.82 mg/ml was allowed to react with 0.2 mM $^{32}\text{P}_i$ and various concentrations of ADP in the standard DMSO-buffer solution at 30°C for 60 (\circ) or 100 min (Δ).

level. The rate of decrease in the amount of [γ - 32 P]ATP was larger than that of its formation.

Figure 12B shows the time course of decrease in the amount of [γ - 32 P]ATP after the pH jump from 6.9 to 8.0 induced by adding 1/10 volume of 0.2 M Tris to the reaction mixture. [γ - 32 P]ATP was formed from 90 μ M ADP and 0.2 mM 32 P_i under the same conditions as for Fig. 12A with the exception of 20 mM MES-Tris at pH 6.9. After the pH jump, 50% of the [γ - 32 P]ATP disappeared very rapidly and the remainder decreased slowly to reach a low constant level, which was equal to the level of [γ - 32 P]ATP synthesized when Tris was added before starting the reaction to adjust the pH of the reaction mixture to 8.0. When 1/10 volume of water was added, the amount of [γ - 32 P]ATP remained constant after a small decrease due to the dilution.

Figure 12C shows the time courses of decrease in the amount of [γ - 32 P]ATP after addition of 1/10 volume of the standard DMSO-buffer solution containing 11 mM AMPPNP or 11 mM ATP to the reaction mixture. [γ - 32 P]ATP was formed by the reaction of 1.24 mg/ml F₁ with 0.1 mM ADP and 0.2 mM 32 P_i for 30 min. After the addition of AMPPNP, 60-70% of [γ - 32 P]ATP disappeared very rapidly and the remainder decreased rather slowly. On the other hand, when nonradioactive ATP was added, the amount of [γ - 32 P]ATP decreased very rapidly at first and then increased again. The increase was probably due to the formation of [γ - 32 P]ATP from 32 P_i and ADP which was produced by rapid cleavage of the added ATP by F₁-ATPase. In this experiment, radioactive 32 P_i was diluted with nonradioactive P_i produced from added ATP. Therefore, the actual value of [γ - 32 p]=

Fig. 12. Time courses of decrease in the amount of formed ATP after dilution, pH change, and nucleotide addition. F_1 was allowed to react with ADP and $^{32}P_i$ in the standard DMSO-buffer solution at 30°C. Reaction conditions were rapidly changed 30 min after the start of the reaction (↓). The amount of [γ - ^{32}P]ATP formed is expressed relative to that formed at 30 min. A: F_1 at 1.82 mg/ml was allowed to react with 0.2 mM ADP and 0.2 mM $^{32}P_i$ (○), and the reaction mixture was diluted 5-fold with the standard DMSO-buffer solution (●). The amount of [γ - ^{32}P]ATP formed at 30 min was 0.040 mol/mol F_1 . B: F_1 at 1.82 mg/ml was allowed to react with 0.09 mM ADP and 0.2 mM $^{32}P_i$ for 30 min under the same conditions as for Fig. 12A, except that 20 mM MES-Tris at pH 6.9 was used as a pH buffer (○). Then 1/10 volume of 0.2 M Tris (▲) was added to change the pH of the reaction mixture from 6.9 to 8.0 (↓). Closed circles (●) show the time course after addition of 1/10 volume of water. Open triangles (▲) show the time course when the Tris was added before the start of the reaction to adjust the pH of the reaction mixture to 8.0. The amount of [γ - ^{32}P]ATP formed at 30 min was 0.047 mol/mol F_1 . C: F_1 at 1.24 mg/ml was allowed to react with 0.1 mM ADP and 0.2 mM $^{32}P_i$ for 30 min, and then 1/10 volume of water (○), 11 mM AMPPNP (■) or 11 mM ATP (▲) was added (↓). The amount of [γ - ^{32}P]ATP formed at 30 min was 0.029 mol/mol F_1 .

ATP formed after the addition of ATP should be larger than that given in the figure, which is not corrected for the dilution effect.

Figure 13 shows the time courses of decrease in the amount of [γ - 32 P]ATP after addition of various concentrations of AMPPNP to the reaction mixture. [γ - 32 P]ATP was formed by reaction for 30 min under the same conditions as for Fig. 12C. After addition of 1/10 volume of the standard DMSO-buffer solution containing 3.3, 11, or 33 mM AMPPNP, 60-70% of [γ - 32 P]ATP disappeared very rapidly and the remainder decreased slowly to reach 0. The same effect of AMPPNP was observed when we used AMPPNP which had been pretreated with 0.32 mg/ml F_1 -ATPase to hydrolyze any contaminating ATP.

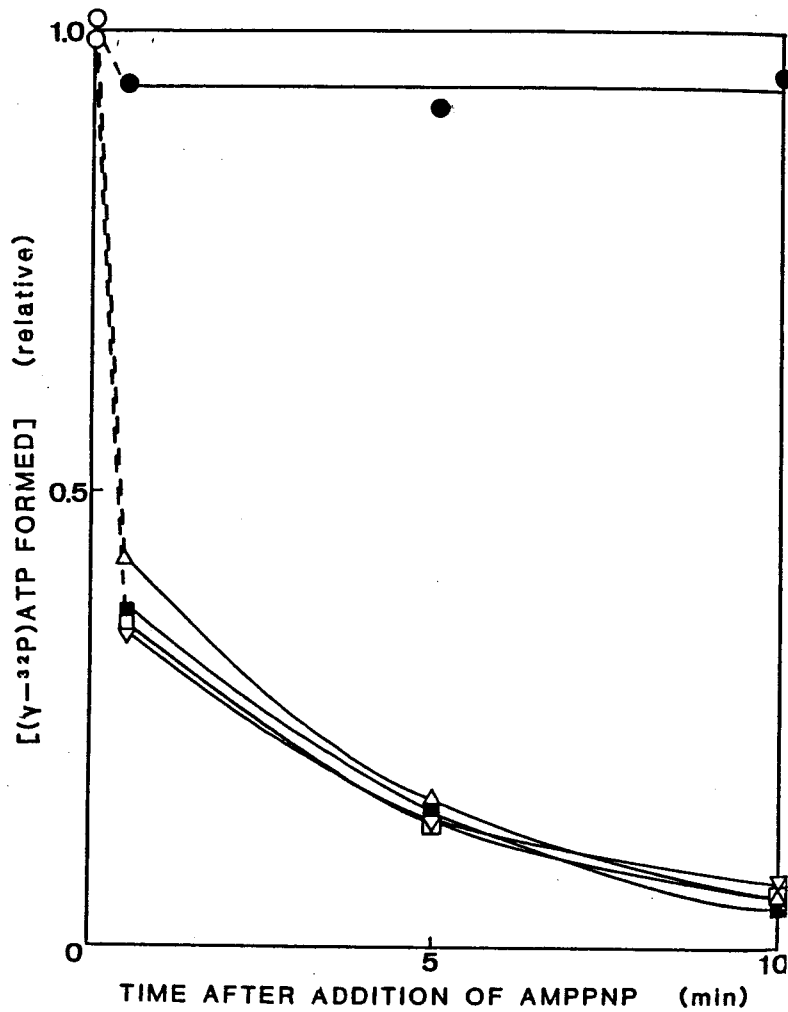


Fig. 13. Time courses of decrease in the amount of formed ATP after addition of various concentrations of AMPPNP. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was produced under the same conditions as for Fig. 12C. At 30 min after the start of the reaction, 1/10 volume of the standard DMSO-buffer solution containing 0 (●), 3.3 (Δ), 11 (□), 33 mM AMPPNP (▽) or 11 mM AMPPNP pretreated by 0.32 mg/ml $F_1\text{-ATPase}$ (■) was added to the reaction mixture. The amount of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is expressed relative to that formed at 30 min, which was 0.036 mol/mol F_1 .

DISCUSSION

The following findings clearly indicate that F_1 catalyzes the formation of ATP from externally added ADP and P_i in the presence of DMSO. (i) The amount of ATP formed was proportional to that of F_1 and was negligible in the absence of F_1 or in the presence of heat-denatured F_1 (Fig. 5). Nelson and Racker (21) previously reported that $^{32}P_i$ radioactivity is not incorporated into ATP in the absence of enzymes although PP_i is formed from ATP and P_i in the presence of DMSO at concentrations higher than 50%. (ii) SDS-Polyacrylamide gel electrophoresis showed that the F_1 preparation used here contained only five subunits of F_1 (7). On the other hand, the maximal amount of ATP synthesized was 0.4-0.6 mol/mol F_1 (Figs. 9 & 11A), and the ATP formed was inaccessible to hexokinase (Fig. 8). (iii) The ATP synthesis was inhibited by sodium azide, an inhibitor of F_1 (Fig. 6). (iv) The ATP formation was highly specific for ADP (Fig. 7), and the specificity is similar to that of ATP synthesis by F_1 on the membrane driven by $\Delta\mu_{H^+}$ (10, 24). (v) The ATP formation was not due to adenylate kinase activity, because $[\gamma\text{-}^{32}P]\text{ATP}$ was synthesized from ADP and $^{32}P_i$, and because the amount of ATP synthesized was almost equal to the decrease in ADP and was much larger than the amount of AMP formed (Fig. 2). Furthermore, it seems highly unlikely that a reaction intermediate could be formed and converted into ATP after addition of TCA to stop the reaction, since the amount of ATP synthesized was independent of whether the reaction was stopped with TCA, ethanol or with SDS, as described in EXPERIMENTAL PROCEDURES.

Purified mitochondrial and chloroplast F_1 have tightly bound, intrinsic nucleotides (2), and the F_1 preparation used here has about 2 mol tightly bound ATP per mol (7). The tightly bound nucleotides are not easily released or exchanged with medium nucleotides (2) and do not directly participate in the reaction of ATP synthesis by $F_1.F_0$ driven by $\Delta\mu_{H^+}$ (4,25). We excluded the possibility that ^{32}P -labeled ATP was produced by an exchange reaction between the tightly bound, intrinsic ATP and medium $^{32}P_i$ or $[\alpha\text{-}^{32}P]ADP$ without net synthesis of $[^{32}P]ATP$ on the following bases. (i) The formed $[\gamma\text{-}^{32}P]ATP$ decreased rapidly after dilution (Fig. 12A), unlike the tightly bound, intrinsic ATP. Furthermore, the high sensitivity of the formed $[\gamma\text{-}^{32}P]ATP$ to pH (Fig. 12B) and to added nucleotides (Figs. 12C & 13) was also different from that of the intrinsic ATP. (ii) If the $[^{32}P]ATP$ had been formed by an exchange reaction of the tightly bound, intrinsic ATP with medium ADP and $^{32}P_i$ or with $[\alpha\text{-}^{32}P]ADP$ and P_i , 2 mol $[^{32}P]ATP$ per mol F_1 should have been formed at equilibrium in the presence of sufficient concentrations of ADP and $^{32}P_i$ or of $[\alpha\text{-}^{32}P]ADP$ and P_i . However, the maximum amount of ATP formed was 0.4-0.6 mol/mol F_1 (Figs. 9 & 11A). (iii) The rate and extent of $[\gamma\text{-}^{32}P]ATP$ formation from ADP and $^{32}P_i$ were equal to those of $[\alpha\text{-}^{32}P]ATP$ formation from $[\alpha\text{-}^{32}P]ADP$ and P_i (Fig. 2). Furthermore, the dependence of the amount of ATP formed at equilibrium on ADP concentration was very different from that on P_i concentration (Figs. 9 & 11A). These findings are inconsistent with the assumption that $[^{32}P]ATP$ is formed by the exchange reaction between the tightly bound, intrinsic ATP and medium ADP or P_i without net synthesis of $[^{32}P]ATP$.

The formation of a "tight enzyme-ATP complex" as a reaction intermediate was first shown in the case of myosin ATPase (6). Boyer and his colleagues (4) suggested that a similar intermediate is formed during the reaction of ATP synthesis by F_1 , and that the free energy of $\Delta\mu_{H^+}$ is used to release this tightly bound ATP. This study clearly shows that a tight F_1 -ATP complex can be formed by the reaction of soluble F_1 with medium ADP and P_i , without $\Delta\mu_{H^+}$ across the membrane.

The formation of tightly bound ATP is not inhibited by DCCD and is inhibited only partially by NBD-Cl (Fig. 6). While these reagents inhibit the F_1 -ATPase activity by binding to the β subunit of F_1 (26,27). Steinmeier and Wang (28) recently demonstrated that when F_1 is labeled by NBD-Cl, almost all the ATPase activity is inhibited while the ATP synthesis activity can be reconstituted efficiently from urea-washed submitochondrial particles and NBD-Cl-treated F_1 -ATPase. Our finding is consistent with this report.

Most of our findings can be explained by the tentative reaction scheme shown in Fig. 14, where the catalytic sites for ATP synthesis on F_1 are assumed to be independent of each other. The brackets indicate tight binding. Both of the double reciprocal plots of the amount of formed ATP against P_i and ADP concentrations showed a linear relationship (Figs. 9 & 11A). When the pH of the reaction mixture was increased from 6.9 to 8.0, 50% of the synthesized ATP disappeared very rapidly and the remainder decreased rather slowly. The latter finding can easily be explained as follows: the equilibrium of the step $E_{P_i}^{<ADP} \rightleftharpoons E^{<ATP}$ is rapidly shifted to the left by the pH jump, then the equilibrium

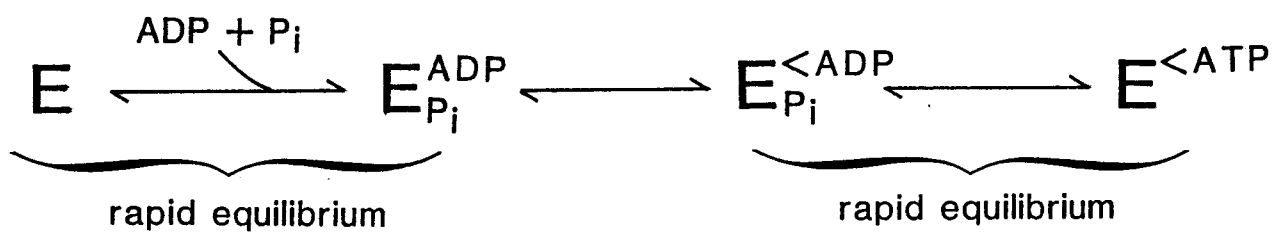


Fig. 14. Tentative reaction mechanism for formation of a tight F_1 -ATP complex by the reaction of F_1 with medium ADP and P_i in the presence of DMSO. Brackets indicate tight binding.

of the step $E_{P_i}^{ADP} \rightleftharpoons E_{P_i}^{<ADP}$ is slowly shifted to the left.

However, the finding that the dependence on P_i concentration of the initial rate of ATP synthesis, v_f , was described by a second-order reaction equation suggests positive cooperativity between active sites for ATP synthesis. Furthermore, the finding that when AMPPNP was added to the reaction mixture, 60-70% of the formed ATP disappeared at a much higher rate than that of ATP formation (Figs. 12C & 13) is not compatible with a hypothesis of simple competition of ADP and AMPPNP binding to a single site, and suggests interaction between nucleotide binding sites (4,7).

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CHAPTER II

Effect of Dimethylsulfoxide in ATP Synthesis by Mitochondrial Soluble F₁-ATPase

SUMMARY

F_1 -ATPase isolated from bovine heart mitochondria catalyzes a synthesis of enzyme-bound ATP from externally added ADP and P_i in the presence of dimethylsulfoxide (DMSO) (Sakamoto, J. and Tonomura, Y. (1983) J. Biochem. 93, 1601-1614). When the concentration of DMSO in the reaction medium decreased from 40% (w/v) to 10% (w/v), the maximal amount of ATP formed decreased from 0.50 to 0.14 mol/mol F_1 and the P_i concentration required for the half maximal amount of ATP formed increased from 0.7 to 11 mM. On the other hand, the ADP concentration required for the half maximal value and the rate of ATP formation were unaffected by the decrease in the concentration of DMSO. These results suggest that DMSO increases the affinity of F_1 for P_i and shifts the equilibrium from enzyme-ADP- P_i complex to enzyme-ATP complex during the course of the ATP synthesis.

INTRODUCTION

In oxidative phosphorylation of mitochondria, F_1-F_0 complex synthesizes ATP from ADP and P_i , coupled with proton influx across the inner membrane (1-4). However, the molecular mechanism of the ATP synthesis by F_1 driven by the electrochemical potential gradient of proton, $\Delta\mu H^+$, remains controversial. One hypothesis states that the free energy of $\Delta\mu H^+$ is used directly in the formation of covalent bond of ATP from ADP and P_i (5) and the other is that the energy input of $\Delta\mu H^+$ is not required for the covalent bond formation itself but for the release of formed ATP from and the binding of ADP and P_i to F_1 (6,7).

Recently, we (8) succeeded in synthesizing ATP with mitochondrial soluble F_1 from medium ADP and P_i in the presence of dimethylsulfoxide (DMSO). The ATP formed was enzyme-bound and the maximal amount of it was 0.4-0.6 mol ATP/mol F_1 . These findings indicated that F_1 could synthesize enzyme-bound ATP from medium ADP and P_i without free energy input of $\Delta\mu H^+$, thus supporting the latter hypothesis. A similar result has also been obtained by Yoshida (9) using soluble F_1 from thermophilic bacterium PS3. However, it has not been clarified how DMSO affect the elementary steps of ATP synthesis catalyzed by soluble F_1 .

In this paper, we examined the effect of DMSO on ATP synthesis by mitochondrial soluble F_1 -ATPase and found that

DMSO affected the apparent affinity for P_i and the step of enzyme-ATP complex formation from enzyme-ADP- P_i complex during the course of the reaction.

EXPERIMENTAL PROCEDURES

Materials — [α -³²P]ADP was prepared from [α -³²P]ATP as described previously (8). ADP and ATP were obtained from Kohjin Co., Ltd., Tokyo. DMSO was purchased from Wako Pure Chemical Industries, Ltd., Osaka. All other chemicals were of reagent grade purity.

F₁ was extracted from bovine heart submitochondrial particles with chloroform, and was purified as described previously (8). F₁ was concentrated and stored at 0°C in a solution containing 4 mM EDTA, 55% (w/v) glycerol, and 0.1 M Tris-Cl at pH 8.0. The protein concentration of purified F₁ was determined by the procedure of Lowry *et al.* (10), using bovine serum albumin as a standard. The color yield of F₁ was taken as 1.19 times of bovine serum albumin (11). The molecular weight of F₁ used in calculations was 360,000 (12).

ATP Synthesis — ATP synthesis was started by adding 20 μ l of F₁ solution to 80 μ l of a reaction mixture at 20°C, unless otherwise stated. The final reaction mixture usually contained 11% (w/v) glycerol, 5 mM MgCl₂, 0.8 mM EDTA, 0.1 M 2-(N-morpholino)-ethanesulfonic acid (MES)-Tris at pH 6.8, and various concentrations of F₁, [α -³²P]ADP, P_i, and DMSO. The reaction was stopped by adding ice-cold trichloroacetic acid (TCA) solution. [α -³²P]ATP formed was isolated using an activated charcoal column and polyethyleneimine-cellulose thin-layer chromatography as described previously (8). Radioactivity of [α -³²P]ATP was counted with a Beckman LS-9000 liquid scintillation counter.

RESULTS AND DISCUSSION

Figure 1 shows the dependences of the amount of ATP formed on P_i concentration at various concentrations of DMSO. In the presence of 40% (w/v) DMSO, the maximal amount of ATP formed was 0.50 mol/mol F_1 and the P_i concentration required for the half maximal amount of ATP formed (K_{P_i}) was 0.7 mM. As the concentration of DMSO decreased from 40 to 30, 20, and 10% (w/v), the maximal amount decreased from 0.50 to 0.49, 0.37, and 0.14 mol/mol F_1 , respectively, whereas K_{P_i} value increased from 0.7 to 1.9, 4.3, and 11 mM, respectively. In the absence of DMSO, the amount of ATP formed at 1.0 M P_i was 0.07 mol/mol F_1 , which did not appear to reach the maximum. Thus, K_{P_i} value in the absence of DMSO was estimated to be larger than 400 mM. It should be added that the amount of AMP formed in the absence of DMSO was less than 0.006 mol/mol F_1 (data not shown), suggesting that the ATP formation in the absence of DMSO was not caused by an adenylate kinase activity as well as in the presence of DMSO (8).

These results apparently shows that DMSO has dual effects on the ATP synthesis by soluble F_1 ; one is that it increases the maximal amount of ATP formed and the other is that it enhances the apparent affinity of F_1 for P_i in the reaction.

In Figure 2, the dependence of the amount of ATP formed on ADP concentration in the absence of DMSO was compared with that in the presence of 35% (w/v) DMSO. In contrast to the case of K_{P_i} (Fig. 1), DMSO little changed the ADP concentration required for the half maximal amount of ATP formed (K_{ADP}). The

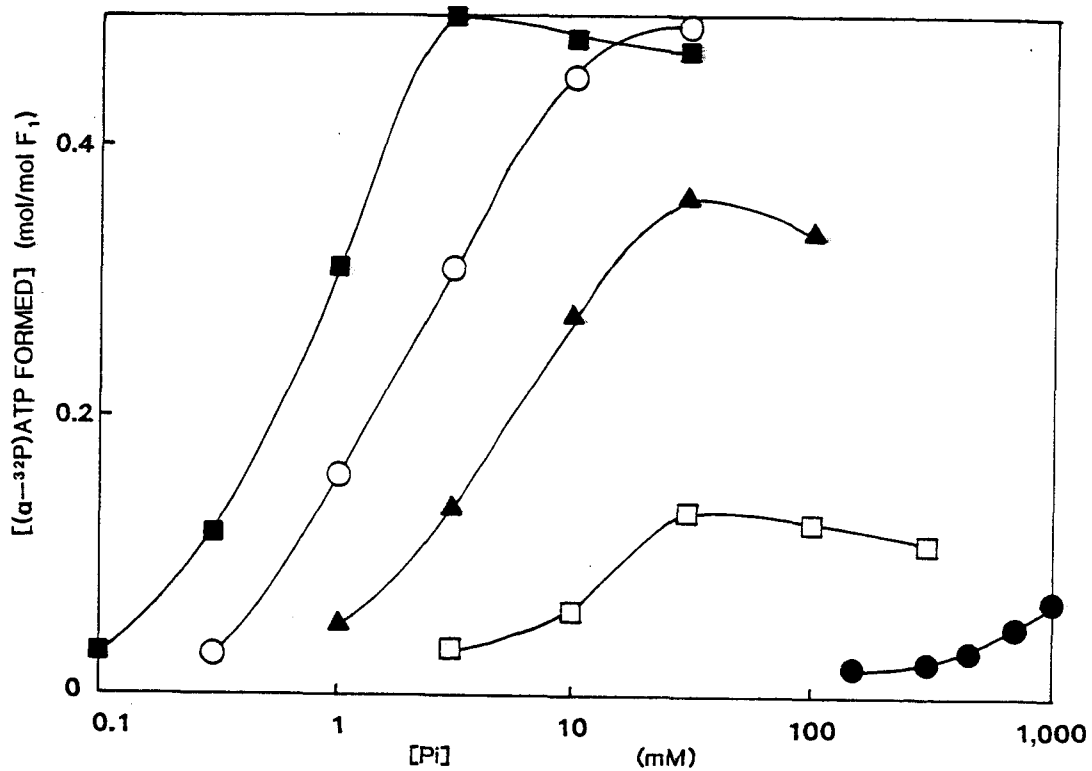


Fig. 1. Dependences on P_i concentration of the amount of ATP synthesized by F_1 at various concentrations of DMSO. F_1 at 2.24 mg/ml was allowed to react with $30 \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and various concentrations of P_i in the presence of 5 mM MgCl_2 , 0.8 mM EDTA, 11% (w/v) glycerol, and 0.1 M MES-Tris at pH 6.8 and 30°C for 30 min. The DMSO concentrations: ●, 0; □, 10; ▲, 20; ○, 30; ■, 40% (w/v).

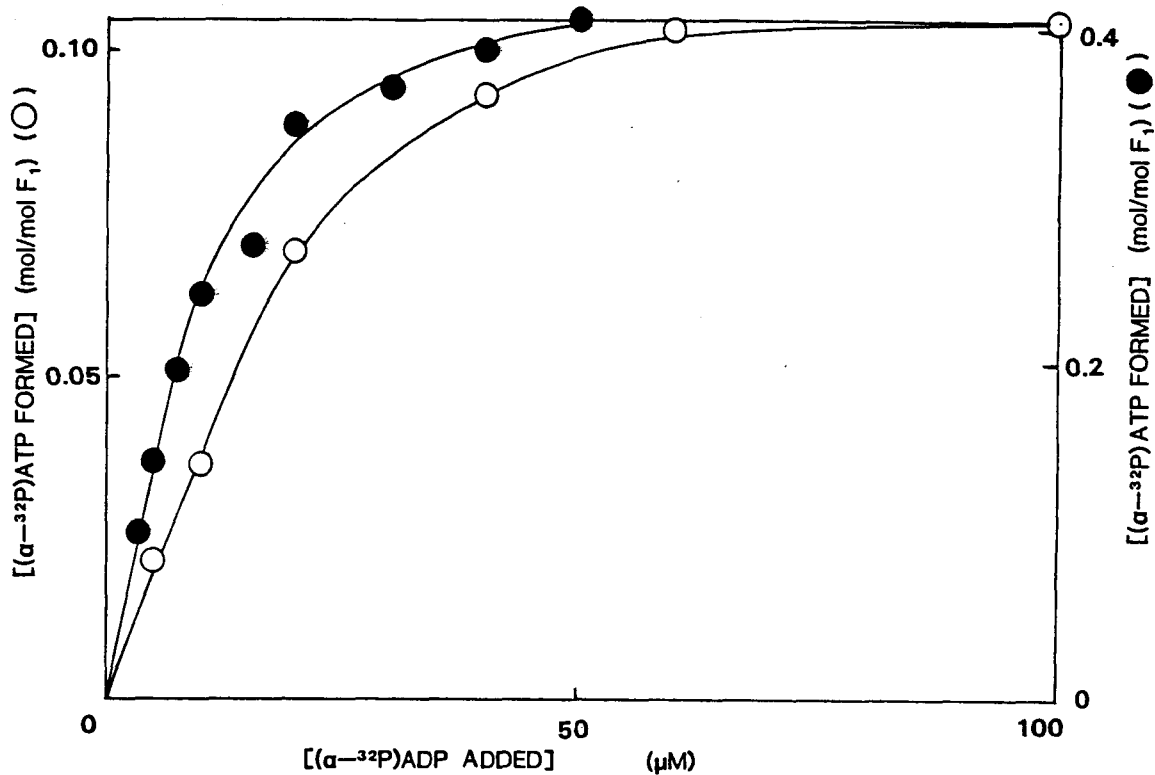
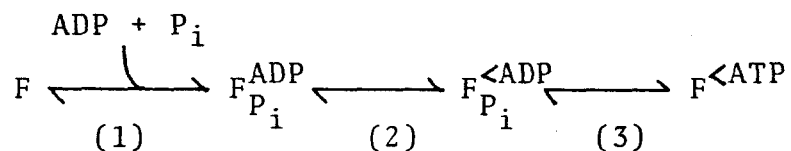


Fig. 2. Dependences on ADP concentration of the amount of ATP synthesized by F_1 in the absence and presence of 35% (w/v) DMSO. In the absence of DMSO (○), F_1 at 1.7 mg/ml was incubated for 30 min with various concentrations of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and 0.9 M P_i . In the presence of 35% (w/v) DMSO (●), F_1 at 1.9 mg/ml was reacted with various concentrations of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and 10 mM P_i . The other conditions are the same as for Fig. 1.

maximal amount of ATP formed was increased about four times by adding 35% (w/v) DMSO.

All the values for the amount of ATP formed shown in figures 1 and 2 are obtained at 30 min after the start of the reaction. To examine whether DMSO accelerates the rate of ATP synthesis, the time course of this reaction was measured both in the presence and absence of DMSO. As shown in Fig. 3, the amount of ATP formed reached half maximal value at 4 min both in the presence and absence of DMSO.

The following is the proposed reaction scheme of the enzyme-bound ATP synthesis by soluble F_1 from medium ADP and P_i in the presence of DMSO (8).



Brackets indicate relatively tight binding. Step (2) is rate limiting step of the reaction. When the concentration of DMSO decreased, the apparent affinity of F_1 for P_i and the maximal amount of ATP formed decreased, while the apparent affinity of F_1 for ADP was virtually unaffected by DMSO. Further, DMSO did not affected the rate of ATP formation. Thus, it is concluded that DMSO affects steps (1) and (3): in step (1), DMSO shifts the equilibrium to the direction of $F_{\text{P}_i}^{\text{ADP}}$ formation and in step (3), DMSO shifts the equilibrium to the direction of ATP synthesis.

These effects of DMSO on F_1 -ATPase are quite similar to those on the Ca^{2+} , Mg^{2+} -ATPase of the sarcoplasmic reticulum (13). DMSO promotes the phosphorylation of the Ca^{2+} , Mg^{2+} -ATPase with

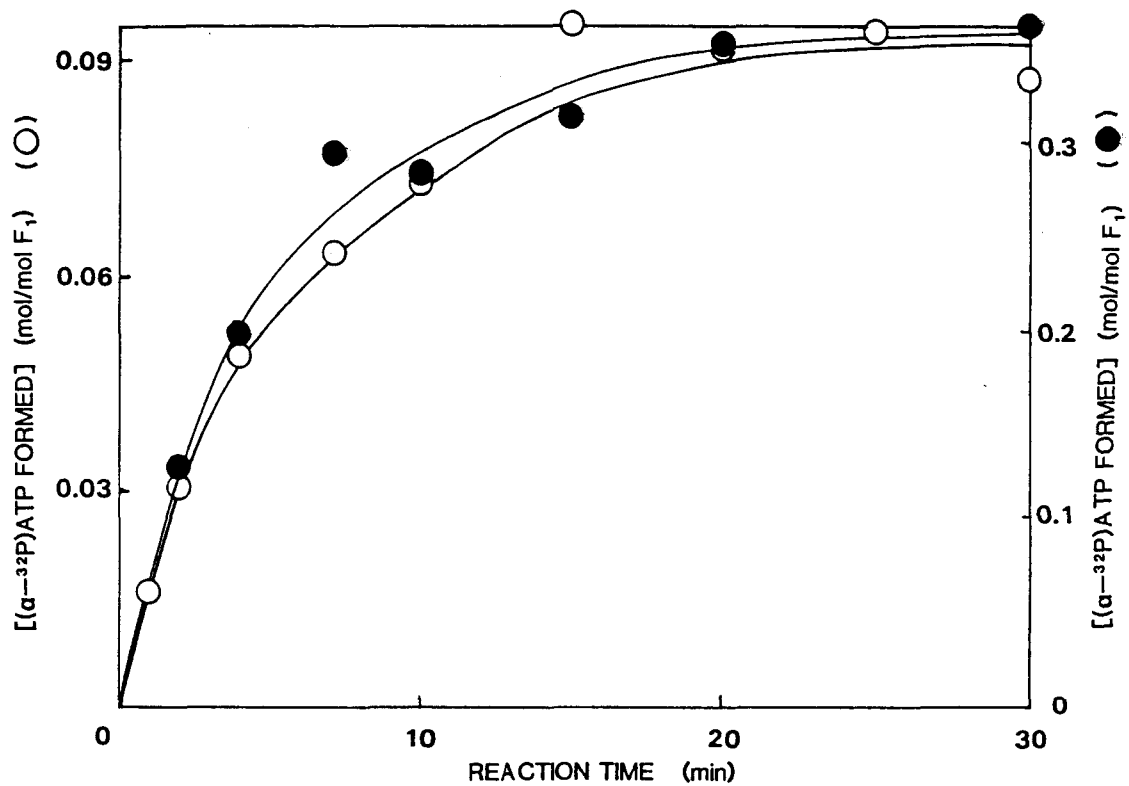


Fig. 3. Time courses of formation of ATP by F_1 in the absence and presence of DMSO. In the absence of DMSO (O), F_1 at 2.1 mg/ml was incubated with $30 \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and 1.0 M P_i in the presence of 5 mM MgCl_2 , 0.8 mM EDTA, 11% (w/v) glycerol at pH 6.8 and 30°C . In the presence of DMSO (●), F_1 at 1.9 mg/ml was incubated with $30 \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and 10 mM P_i in the same conditions as above with 0.1 M MES-Tris.

P_i by increasing the affinity of the ATPase for P_i and shifting the equilibrium between the enzyme-phosphate complex ($E \cdot P_i$) and phosphorylated enzyme (EP) to the right, suggesting that very similar reaction steps involve in the elementary steps of ATPase reaction.

It was reported that soluble F_1 possesses two types of phosphate binding sites (11,14). One shows a saturable high-affinity ($1 \text{ mol/mol } F_1$) and the other is a nonsaturable and low-affinity site. The apparent affinity of F_1 for P_i in the absence of DMSO was larger than 400 mM (Fig. 1). Therefore, the high-affinity P_i binding site of soluble F_1 may not involve in ATP synthesis.

Finally, it should be noted that K_m values for P_i in the oxidative phosphorylation of submitochondrial particles (15-17) are much lower than K_{P_i} value of F_1 observed in the absence of DMSO. The difference can be explained by assuming that the binding of F_1 to F_0 or the energization of the membrane (18,19) induces the increase in the affinity of F_1 for P_i .

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CHAPTER III

Identification of Nucleotide Binding Site for ATP Synthesis and Hydrolysis in Mitochondrial Soluble F₁-ATPase

SUMMARY

The binding of ADP to mitochondrial soluble F_1 -ATPase was studied by a membrane filtration method. One mol of F_1 , which contained about 1 mol of tightly bound adenine nucleotide, bound 4 mol of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ at a saturating concentration of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$. Two mol of the bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ readily exchanged with medium nonradioactive ADP while the remaining 2 mol of the bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ hardly exchanged (tightly bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$). F_1 catalyzed the synthesis of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ from medium $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and P_i . However, when exchangeably bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ was replaced by nonradioactive ADP, no $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formation was observed. Furthermore, tightly bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ was not released from F_1 during ATP hydrolysis catalyzed by the enzyme. These results indicate that both ATP synthesis and hydrolysis catalyzed by F_1 occur at the exchangeable binding site and not at the tight binding site on the enzyme.

INTRODUCTION

In oxidative phosphorylation of mitochondria, ATP is synthesized from ADP and P_i by F_1 - F_0 complex, coupled with proton influx through the mitochondrial inner membrane (1-6). However, the detailed molecular mechanism of the coupling remains to be elucidated. One of the approach to this problem is to decide the catalytic site(s) of F_1 at which ATP is formed from ADP and P_i . Studies on the nucleotide binding sites of F_1 showed that mitochondrial F_1 has five or six adenine nucleotide binding sites (7,8). Three of them are tight binding sites at which bound nucleotides hardly exchanged with medium nucleotides and remaining two or three sites bind nucleotides exchangeably. However, it is still contraversial which kind of nucleotide binding sites participate in ATP synthesis (9,10).

Recently, Feldman and Sigman (11,12) reported that enzyme-bound ATP is synthesized by chloroplast F_1 from endogenous ADP bound tightly to the enzyme and medium P_i . Yoshida (13) also showed that soluble F_1 from thermophilic bacterium PS3 synthesizes ATP from tightly bound ADP and medium P_i . We previously reported that enzyme-bound ATP was synthesized by mitochondrial soluble F_1 from medium ADP and P_i in the presence of dimethylsulfoxide (DMSO) (14). In this paper we studied which class of the nucleotide binding sites in mitochondrial F_1 participate in ATP synthesis and hydrolysis. The results obtained here indicate that both ATP synthesis and hydrolysis occur at the exchangeable binding site.

EXPERIMENTAL PROCEDURES

Materials — [α - 32 P]ADP was prepared from [α - 32 P]ATP as described previously (14). [3 H]glucose was obtained from New England Nuclear, Boston. ADP and ATP were purchased from Kohjin Co., Ltd., Tokyo. DMSO were obtained from Wako Pure Chemical Industries, Ltd., Osaka. Phosphoenol pyruvate and luciferin was purchased from Sigma Chemicals Co., St Louis. Pyruvate kinase was prepared according to the method of Tietz and Ochoa (15). Subfragment-1 of rabbit skeletal muscle myosin was a generous gift from Mr. M. Yasui in our laboratory. Purified luciferase was a generous gift from Dr. T. Watanabe, National Cardiovascular Center Research Institute. All other chemicals were of reagent grade purity.

F₁ was extracted from bovine heart submitochondrial particles with chloroform, and was purified as described previously (16). F₁ was concentrated and stored at 0°C in a solution containing 4 mM EDTA, 55% (w/v) glycerol, and 0.1 M Tris-Cl at pH 8.0. The protein concentration of purified F₁ was measured by the procedure of Lowry et al. (17) with slight modification (18). The molecular weight of F₁ used in calculation was 360,000 (19).

The A₂₈₀/A₂₆₀ ratio of the F₁ preparation was between 1.64 and 1.72, indicating that the amount of adenine nucleotides tightly bound to F₁ was about 1 mol/mol F₁ (7). The amounts of tightly bound nucleotides were also measured by using luciferin-luciferase reaction. Bound nucleotides were extracted

from F_1 with 5% perchloric acid solution, and after neutralizing the solution with $KHCO_3$, the amount of ATP was measured, according to the method of Furukawa et al. (20). The amount of ADP was also estimated after adding pyruvate kinase and phosphoenol pyruvate to the neutralized solution in order to convert ADP into ATP. The amount of ADP remained in the F_1 preparation was between 0.28 and 0.34 mol/mol F_1 and that of ATP was between 0.51 and 0.87 mol/mol F_1 .

Binding of ADP to F_1 — Incubation was started by adding F_1 solution to the incubation mixture containing [α - ^{32}P]ADP at 30°C, unless otherwise stated. The final incubation mixture contained 5 mM $MgCl_2$, 0.8 mM EDTA, 50 mM [3H]glucose, 35% (w/v) DMSO, 11% (w/v) glycerol, and 50 mM 2-(N-morpholino)ethanesulfonic acid (MES)-Tris at pH 6.9 (referred to hereafter as the "standard DMSO-buffer solution"), and various concentrations of F_1 and [α - ^{32}P]ADP. All the solutions containing nonradioactive ADP were pretreated with 0.6 mg/ml subfragment-1 of myosin at 30°C for 30 min to reduce the contaminating ATP.

The amount of [α - ^{32}P]ADP bound to F_1 was measured by the membrane filtration method using an Amicon Diaflo XM-100A membrane filter, as described by Yamaguchi and Tonomura (21).

ATP Synthesis — F_1 was preincubated with [α - ^{32}P]ADP at 30°C in the standard DMSO-buffer solution except that [3H]glucose was replaced by nonradioactive glucose. Reaction was started by adding 10 mM P_i to the incubation mixture. The amount of [α - ^{32}P]ATP formed by F_1 was measured as described previously (14).

ATP Hydrolysis — F_1 was preincubated with 17 μM nonradioactive ADP in the standard DMSO-buffer solution for 15 min at 30°C. Reaction was started by adding 80 μM [γ - ^{32}P]ATP to the preincubation mixture and stopped by adding 5% trichloroacetic acid. The radioactivity of $^{32}\text{P}_i$ liberated was measured as described previously (22).

RESULTS

ADP Binding to F_1 in the Absence and Presence of DMSO —

Figure 1 shows the dependences of the amount of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ bound to F_1 on the concentration of added $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ in the absence and presence of 35% (w/v) DMSO. The amount of bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ was increased with increase in the concentration of added $[\alpha\text{-}^{32}\text{P}]\text{ADP}$, reaching the maximal level of 4 mol/mol F_1 at 100 μM both in the absence and presence of DMSO. As described in the EXPERIMENTAL PROCEDURES, the F_1 preparation used here contained about 1 mol of tightly bound adenine nucleotides per mol of F_1 . Therefore, the total amounts of bound adenine nucleotides were about 5 mol/mol F_1 at a saturating concentration of ADP.

In order to examine the exchangeability of the bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$, we studied the effect of addition of nonradioactive ADP on the amount of bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$. Figure 2A shows the time courses of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ binding to F_1 in the presence of 35% (w/v) DMSO. The amount of bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ reached about 4 mol/mol F_1 at 30s after the addition of F_1 to the incubation mixture containing $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and the level was kept almost constant for up to 25 min. When 1 mM nonradioactive ADP was added to the incubation mixture at 10 min, the amount of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ bound to F_1 rapidly decreased to a constant level of about 2 mol/mol F_1 . These results indicate that there are at least two classes of ADP binding sites on F_1 . The site to which $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ is attached and not released even after addition of nonradioactive ADP is referred to hereafter as the "tight binding site." The

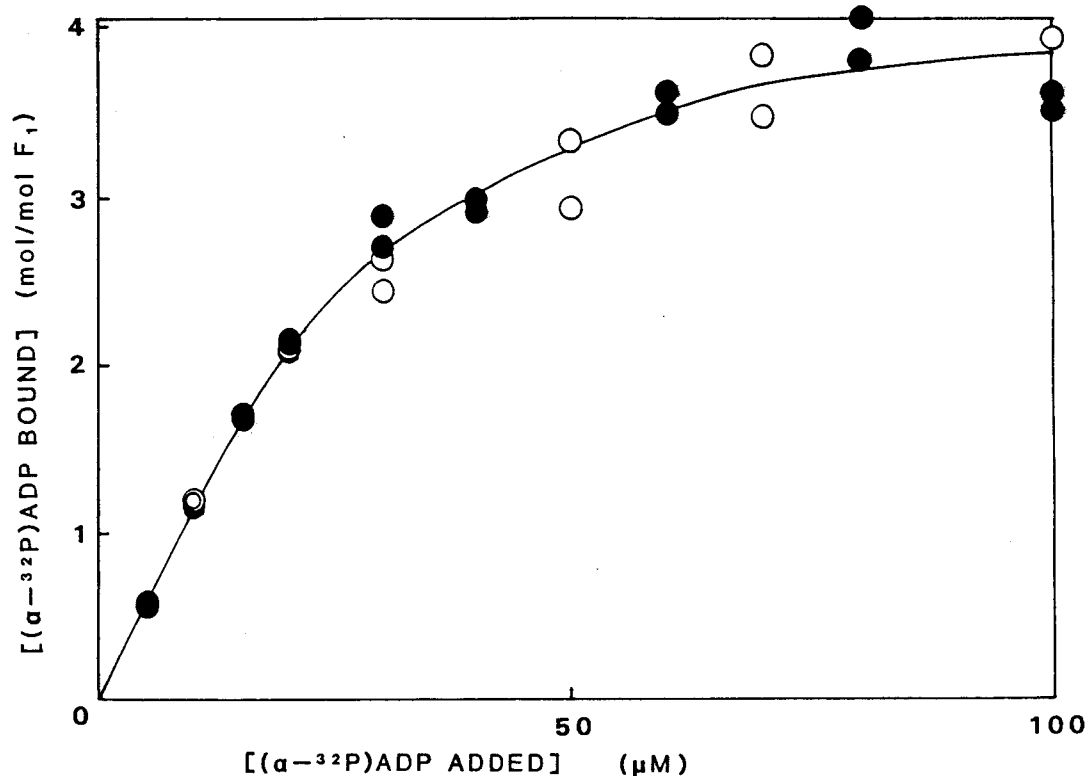


Fig. 1. Dependences of the amount of ADP bound to F_1 on the concentration of added ADP in the absence and presence of DMSO. In the absence of DMSO (●), F_1 at $8.5 \mu\text{M}$ was incubated with various concentrations of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ in the incubation mixture containing 3 mM MgCl_2 , 0.8 mM EDTA, 50 mM $[\text{}^3\text{H}]\text{glucose}$, 150 mM sucrose, 11% (w/v) glycerol, and 50 mM Tris-Cl at pH 8.0 and 18°C for 15 min. In the presence of DMSO (○), F_1 at $8.5 \mu\text{M}$ was incubated with $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ in the standard DMSO-buffer solution [5 mM MgCl_2 , 0.8 mM EDTA, 50 mM $[\text{}^3\text{H}]\text{glucose}$, 35% (w/v) DMSO, 11% (w/v) glycerol, and 50 mM MES-Tris at pH 6.9] at 30°C for 15 min. The amount of bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ was measured as described in EXPERIMENTAL PROCEDURE.

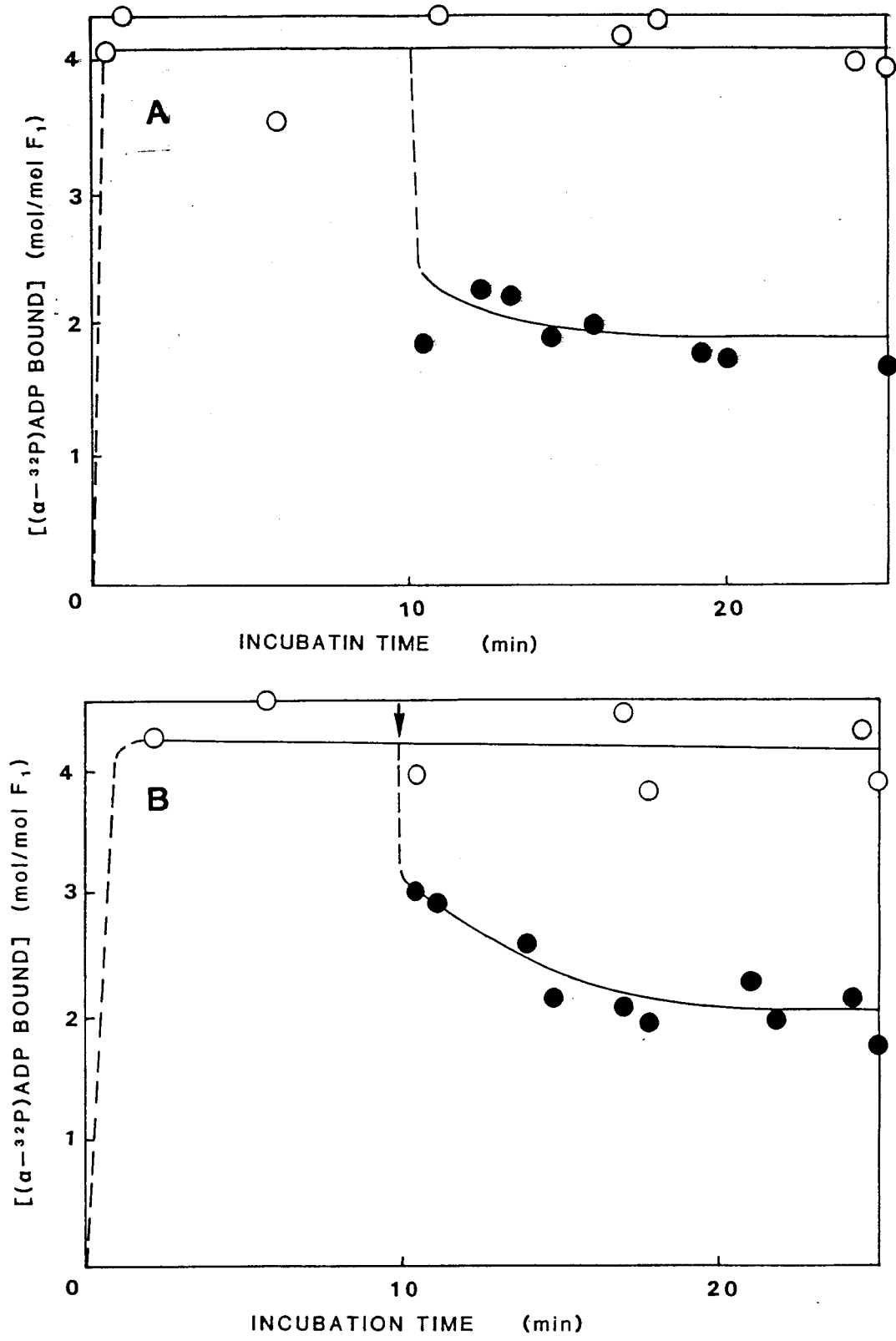


Fig. 2. Time courses of binding of ADP to F₁ in the presence and absence of DMSO. A: F₁ at 8.5 μM was incubated with 80 μM $[\alpha-^{32}\text{P}]\text{ADP}$ in the standard DMSO-buffer solution at 30°C and the amount of bound $[\alpha-^{32}\text{P}]\text{ADP}$ (○) was measured. The closed circles show the amount of $[\alpha-^{32}\text{P}]\text{ADP}$ remaining on F₁ after addition of nonradioactive ADP at the final concentration of 2 mM. B: F₁ was incubated with $[\alpha-^{32}\text{P}]\text{ADP}$ at 18°C in the same solution as for A except that DMSO was omitted but 150 mM sucrose was added. The reaction was chased by 1 mM of nonradioactive ADP.

site to which [α - 32 P]ADP bound readily exchanges with medium nonradioactive ADP is referred to as the "exchangeable binding site." As shown in Fig. 2B, these two classes of ADP binding sites are also observed in the absence of DMSO.

ATP Synthesis from Medium P_i and ADP Bound to the Exchangeable Binding Site — Since soluble F_1 catalyzes ATP synthesis from externally added ADP and P_i , we studied which class of ADP binding sites participates in the ATP synthesis. As shown in Fig. 3, about 0.4 mol of [α - 32 P]ATP/mol of F_1 was synthesized on adding P_i to F_1 which had been preincubated with 80 μ M [α - 32 P]ADP for 15 min under the same condition as for Fig. 2A. On the other hand, when [α - 32 P]ADP bound exchangeably to F_1 was replaced by nonradioactive ADP, negligible amount of [α - 32 P]ATP was synthesized by adding P_i .

In order to further clarify the relationship between the ADP binding sites and ATP synthesis, the dependences of ADP binding and ATP synthesis on the concentration of added ADP were examined. Figure 4 shows the time courses of [α - 32 P]ADP binding to the tight and exchangeable binding sites. 1.7 mol of [α - 32 P]ADP was bound to 1 mol of F_1 in 30s of incubation and then the amount of bound [α - 32 P]ADP increased slowly to 2.3 mol/mol F_1 at 20 min of incubation. When 2 mM of nonradioactive ADP was added at 30s after the start of the incubation, the amount of bound [α - 32 P]ADP rapidly decreased from 1.7 to 1.1 mol/mol F_1 and it remained nearly constant. Though, when nonradioactive ADP was added at 17 min, little decrease in [α - 32 P]ADP level was observed. Therefore, almost all the bound [α - 32 P]ADP was

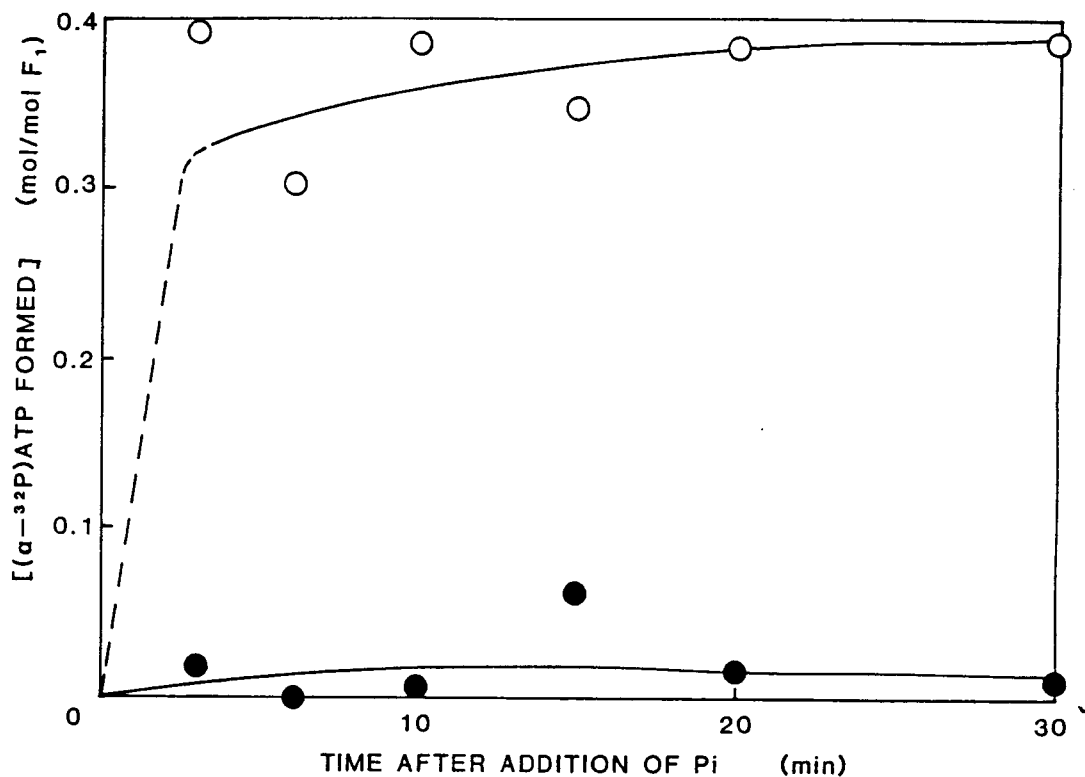


Fig. 3. ATP synthesis from medium P_i and ADP bound to the exchangeable binding site of F_1 . F_1 at $8.5 \mu\text{M}$ was incubated with $80 \mu\text{M}$ [α - ^{32}P]ADP in the same condition as for Fig. 2A except that [^3H]glucose was replaced by nonradioactive glucose. At 10 min, 2 mM nonradioactive ADP was added (●) to displace [α - ^{32}P]ADP from the exchangeable binding site. At 5 min after the addition of nonradioactive ADP, 10 mM P_i was added to start the [α - ^{32}P]ATP synthesis and the amount of [α - ^{32}P]ATP formed (●) was measured as a function of time after addition of P_i . The amount of [α - ^{32}P]ATP formed was also measured without addition of the nonradioactive ADP (O).

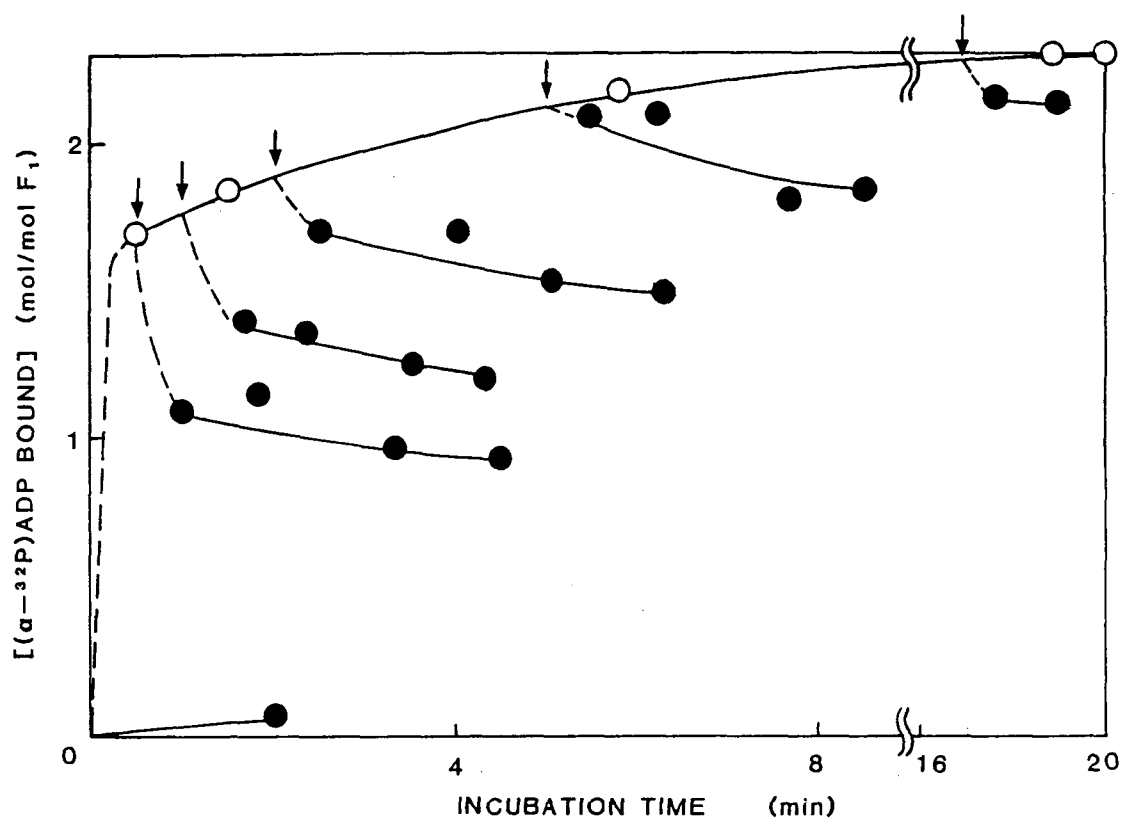


Fig. 4. Conversion of exchangeably bound ADP into tightly bound ADP. F_1 at $8.5 \mu\text{M}$ was incubated with $20 \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ in the standard DMSO-buffer solution and the total amount of bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ (O) was measured at prescribed periods of the incubation time. At indicated times (\downarrow), 2 mM nonradioactive ADP was added to the mixture, and the amount of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ remaining to be bound to F_1 (\bullet) was measured as a function of time.

present as a tightly bound form.

Figure 5 shows the dependences of the amounts of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formed and $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ bound to the tight and exchangeable site of F_1 on the concentration of added $[\alpha\text{-}^{32}\text{P}]\text{ADP}$. Since the total amount of bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and the amount of tightly bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ reached the steady level in 10 min incubation of F_1 with $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ (see Fig. 4), the total amount of bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ was measured after 15 min incubation. The amount of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ tightly bound to F_1 (●) was measured by adding excess amount of nonradioactive ADP to F_1 , which had been preincubated with $[\alpha\text{-}^{32}\text{P}]\text{ADP}$. The amount of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ bound exchangeably to F_1 (○) was estimated as the difference between total amount of bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and the amount of tightly bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$. The amount of tightly bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ increased linearly with increase in $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ concentration, reaching the nearly constant level of 1.7 mol/mol F_1 at 30 μM of added $[\alpha\text{-}^{32}\text{P}]\text{ADP}$. The amount of exchangeably bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ increased sigmoidally with increase in $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ concentration. It was about 0.8 mol/mol F_1 at 30 μM and reached 2.0 mol/mol F_1 at 100 μM $[\alpha\text{-}^{32}\text{P}]\text{ADP}$. The dependence of the amount of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ formed on the concentration of added $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ (Δ) was measured after preincubation of F_1 with various concentrations of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ under the similar conditions as for the binding. The amount of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ increased sigmoidally with increase in $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ concentration and reached a nearly maximal value of 0.39 mol/mol F_1 at 30 μM $[\alpha\text{-}^{32}\text{P}]\text{ADP}$.

ATP Hydrolysis by F_1 Preincubated with ADP — Figure 6 shows

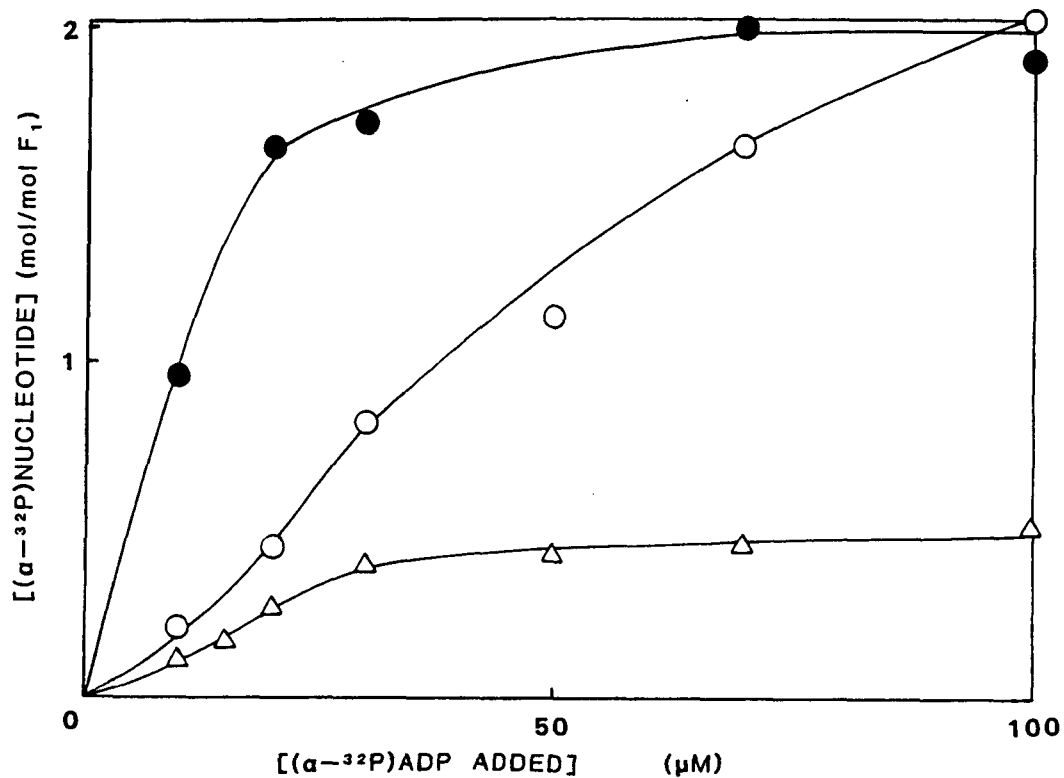


Fig. 5. Dependences of the amount of ADP bound to F₁ and that of the amount of ATP formed by F₁ on the concentration of added ADP. F₁ at 8.5 μM was incubated with various concentrations of [α-³²P]ADP in the standard DMSO-buffer solution at 30°C for 15 min and the total amount of bound [α-³²P]ADP was measured. The amount of tightly bound [α-³²P]ADP (●) was measured at 5 min after addition of 2 mM nonradioactive ADP to the incubation mixture. The amount of exchangeably bound [α-³²P]ADP (○) was estimated as the difference between the total amount of bound [α-³²P]ADP and the amount of tightly bound [α-³²P]ADP (see text). The [α-³²P]ATP synthesis was started by adding 10 mM P_i to F₁, which had been preincubated with [α-³²P]ADP for 15 min as described above, and the amount of [α-³²P]ATP formed (Δ) was measured at 15 min after adding P_i.

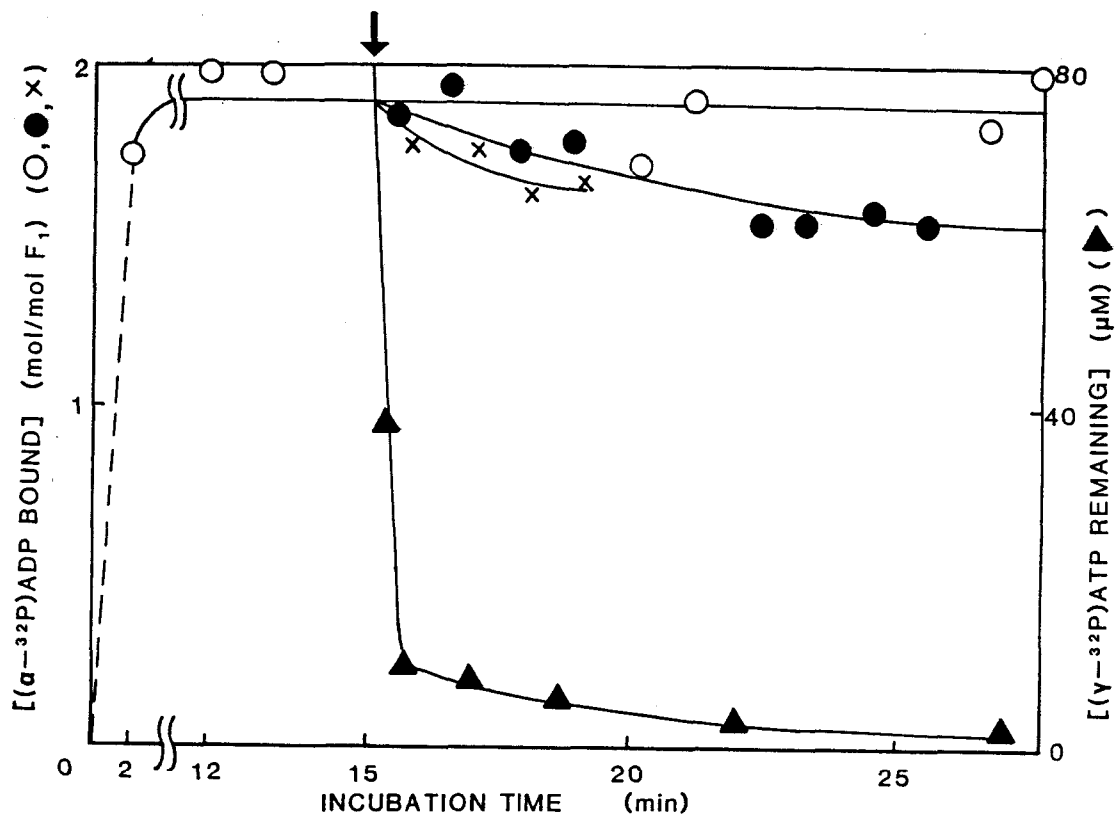


Fig. 6. Effect of addition of ATP on the amount of ADP bound tightly to F_1 . F_1 at $8.5 \mu\text{M}$ was incubated with $17 \mu\text{M}$ $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ in the standard DMSO-buffer solution at 30°C and the amount of bound $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ (O) was measured. At 15 min after the start of the incubation, 2 mM nonradioactive ADP (X) or $80 \mu\text{M}$ nonradioactive ATP (●) was added to the incubation mixture and the amount of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ remaining on F_1 (X, ●) was measured. For the measurement of ATP hydrolysis, $80 \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to F_1 , which had been preincubated with $17 \mu\text{M}$ nonradioactive ADP as described above, and the amount of $^{32}\text{P}_i$ liberated (▲) was measured at indicated time.

the effect of addition of ATP on [α - 32 P]ADP binding to F_1 . When F_1 at 8.5 μ M was incubated with 17 μ M [α - 32 P]ADP, most of the added [α - 32 P]ADP was bound to F_1 . On adding 2 mM nonradioactive ADP at 15 min, less than 15% of bound [α - 32 P]ADP was released. A similar result was obtained when 80 μ M nonradioactive ATP was added instead of nonradioactive ADP. Since it is well known that F_1 -ATPase activity is inhibited when F_1 is incubated with ADP (2), an experiment was carried out to test whether ATP added to F_1 was hydrolyzed during incubation. As clearly shown in the figure, more than 90% of 80 μ M [γ - 32 P]ATP added to the incubation mixture was hydrolyzed within 1 min, and the remaining was hydrolyzed slowly.

DISCUSSION

Using a membrane filtration method, we found that F_1 can maximally bind 4 mol of ADP/mol of F_1 (Fig. 1). 4 mol of bound ADP could be classified into two groups. 2 mol of bound ADP/mol F_1 could be readily exchanged with externally added ADP (Fig. 2), while the remaining 2 mol of ADP was tightly bound to F_1 . Since the F_1 preparation contained about one mol of tightly bound nucleotide as described in EXPERIMENTAL PROCEDURES, it is easily assumed that 3 mol of adenine nucleotide was tightly bound to F_1 . The amount of tightly bound nucleotide/mol of F_1 is in good agreement with the reported value (7,8,23). It has been also reported that the amount of exchangeably bound nucleotide is 2 mol/mol F_1 in the case of ADP although it is 3 mol/mol F_1 in the case of a nonhydrolyzable ATP analogue, 5'-adenylyl- β,γ -imidodiphosphate (8,24). Our result is also consistent with these reports. It should be also noted that DMSO did not affect the binding of ADP to F_1 (Fig. 2). This is consistent with our preceding report (25) which showed that DMSO scarcely affected the affinity of ADP in the reaction of ATP synthesis by soluble F_1 .

We previously showed that ATP is synthesized from medium ADP and P_i by mitochondrial soluble F_1 in the presence of DMSO (14). The following findings clearly indicate that ATP is synthesized on the exchangeable binding sites and not on the tight binding sites. (i) [α - ^{32}P]ATP synthesis was observed when the both class of binding sites were occupied with [α - ^{32}P]ADP, while it was not observed when only the tight binding sites were

occupied with [α - 32 P]ADP (Fig. 3). (ii) [α - 32 P]ATP was also synthesized when the tight binding sites were preoccupied with nonradioactive ADP and the reaction was started by adding [α - 32 P]ADP and P_i (data not shown). (iii) The sigmoidal dependence of the amount of ATP formed by F_1 on the concentration of added ADP was very similar to the dependence of ADP bound exchangeably to F_1 in the low concentration range of ADP (Fig. 5). (iv) ATP formed by F_1 from medium ADP and P_i decreased rapidly when the substrates were diluted (14).

The dependence of the amount of ATP formed by F_1 on the concentration of added ADP was different from that of ADP bound to the exchangeable binding site of F_1 in the concentration range higher than 30 μ M (Fig. 5). The amount of ATP formed was nearly maximal when one mol of the exchangeable binding site was occupied with ADP. This result suggests that ATP was synthesized on one of the two exchangeable sites, which had higher affinity for ADP than the other.

As shown in Fig. 6, tightly bound ADP was not released from F_1 during ATP hydrolysis, suggesting that the ATP hydrolysis also occurred at the exchangeable site. A similar suggestion was made by Cross and Nalin (8). Therefore, we conclude that both ATP synthesis and hydrolysis catalyzed by F_1 occur at the exchangeable binding site and not at the tight binding site on F_1 .

Accumulating evidences support the subunit stoichiometry as $\alpha_3\beta_3\gamma\delta\epsilon$ (5,6). Each of the larger two subunits, α and β has a nucleotide binding site (26,27). It has been suggested

that the catalytic site locates on β subunit, based on the findings that F_1 -ATPase activity is inhibited by some chemical modifiers and photoaffinity analogues of ATP with their binding to β subunit (28-31). On the other hand, Matsuoka et al. (16,32) suggested that α subunit is the catalytic subunit, based on the experiments using a fluorescent ATP analogue, 2'-(5-dimethylaminonaphthalene-1-sulfonyl)amino-2'-deoxy ATP (DNS-ATP). DNS-ATP, which is hydrolyzed by F_1 of mitochondria and E.coli, binds to α subunit but not to β subunit isolated from F_1 of E.coli. Furthermore, N,N'-dicyclohexylcarbodiimide which binds to β subunit of F_1 does not inhibit a single turnover of DNS-ATP hydrolysis by F_1 although it inhibits ATP and DNS-ATP hydrolysis by F_1 in the steady state. Thirdly, it has been also suggested that the catalytic site locates at the interface of α and β subunits (5,6), based on the experiments using photoaffinity labels, 3'-O-{3-[N-(4-azido-2-nitrophenyl)amino]propionyl}adenosine 5'-triphosphate (33), 8-azido derivative of it (34), and 3'-O-(4-benzoyl)benzoyl adenosine 5'-triphosphate (35).

Recently, Dunn (36) reported that a dissociation rate constant of ATP from isolated α subunit is very slow and suggested that nucleotide binding site in α subunit is the tight binding site. Furthermore, Wagenvoord et al. (37,38) showed that β subunit in F_1 is labeled by a photoaffinity analogue of ATP, 8-azido ATP without displacing tightly bound endogenous nucleotides. These two findings indicate that the tight and exchangeable nucleotide binding sites are located on α and β subunit, respectively. If we assume this relationship, our present study supports the

idea that the site for ATP synthesis and hydrolysis locates on β subunit.

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