Characteristics of the Formation of Enzyme-Bound ATP from Medium Inorganic Phosphate by Mitochondrial F_1 Adenosinetriphosphatase in the Presence of Dimethyl Sulfoxide†

Rajendra P. Kandpal, Kerstin E. Stempel, and Paul D. Boyer*

Molecular Biology Institute and Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024

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ABSTRACT: Addition of dimethyl sulfoxide promotes the formation of enzyme-bound ATP from medium P_i by mitochondrial F_1 adenosinetriphosphatase that has tightly bound ADP present. Measurements are reported of medium $P_i \rightleftarrows H^{18}OH$ exchange and of the dependence of formation of enzyme-bound ATP on P_i concentration. Attainment of an apparent equilibrium between medium P_i and bound ATP requires longer than 30 min, even though the rates of P_i binding and release after apparent equilibrium is reached would suffice for a faster approach to equilibrium. Slow protein conformational changes or other unknown modulating factors may be responsible for the slow rate of bound ATP formation. After apparent equilibrium is reached, each P_i that binds to the enzyme reversibly forms ATP about 50 times before being released to the medium. The rate of interconversion of bound ATP to bound ADP and P_i is much slower than that in the absence of dimethyl sulfoxide as measured with sufficiently low ATP concentrations so that single-site catalysis is favored. Although the interconversion rate is slowed, the equilibrium constant for bound ATP formation from bound ADP and P_i is not far from unity. Dimethyl sulfoxide favors the formation of enzyme-bound ATP by promoting the competent binding of P_i to enzyme with ADP bound at a catalytic site rather than by promoting formation of bound ATP from bound ADP and P_i.

Feldman and Sigman (1982) demonstrated that chloroplast F_1 adenosinetriphosphatase (CF_1 ATPase)[1] with a tightly bound ADP present could form tightly bound ATP from medium P_i. This was in contrast to earlier experiments with mitochondrial F_1 ATPase (MF_1 ATPase) in which the formation of bound ATP from medium P_i was not detected (Choate et al., 1979). This difference in behavior can in part be explained by the use of a more acid pH and higher P_i concentrations in the experiments with CF_1 ATPase and, as noted in the present paper, by the lack of an ADP bound at the catalytic site of the mitochondrial enzyme or the presence of excess medium ADP under the conditions used. However, even under favorable conditions, a very high P_i concentration is necessary to detect formation of bound ATP with MF_1 ATPase (Sakamoto, 1984a). A means of enhancing the formation of bound ATP with MF_1 ATPase was suggested by the salient observation of deMeis et al. (1980) that addition of some 20–40% (v/v) of dimethyl sulfoxide (Me_2SO) or other organic solvents greatly increased the extent of phosphorylation of sarcoplasmic reticulum ATPase by P_i. This served as a stimulus for the experiments of Sakamoto and Tonomura (1983) with MF_1 ATPase and Yoshida (1983) with TF_1 ATPase, who showed that Me_2SO promoted considerable formation of bound ATP from medium P_i.

The MF_1 ATPase catalyzes a slow exchange of medium P_i oxygens with water oxygens (Choate et al., 1979), and it seemed likely that a similar exchange might be detected in the presence of Me_2SO. Such detection is reported here. The oxygen exchange data together with data on P_i concentration dependency and $[^{32}P]ATP$ formation from $[^{32}P]P_i$ allow approximation of rates of P_i binding and release and of reversal of bound ATP hydrolysis. Earlier results on the slowness of attaining apparent equilibrium of bound ATP formation from medium P_i in the presence of Me_2SO suggested very slow rates of P_i binding and release (Sakamoto & Tonomura, 1983; Sakamoto, 1984a). Our results show that in the presence of Me_2SO, other factors account for the slow initial rate of bound ATP formation, that the reversal of bound ATP hydrolysis to bound ATP and P_i is much faster than P_i binding and release, and that the equilibrium of enzyme-bound reactants is not far from 1.

Experimental Procedures

Materials. $[^{32}P]P_i$ was obtained from ICN Radiochemicals and purified on an anion-exchange column, AG 1-X4 (Bio-Rad). ADP, ATP, and yeast inorganic pyrophosphatase were obtained from Sigma Chemical Co. Glass-distilled Me_2SO was purchased from Burdick and Jackson, Muskegan, MI. MF_1 ATPase was purified from beef heart mitochondria by the method of Penefsky (1979) and was stored as an ammonium sulfate precipitate at 4 °C. An aliquot of the ammonium sulfate suspension was pelleted, dissolved in buffer, and desalted on a Sephadex–centrifuge column (Penefsky, 1977) equilibrated with an appropriate buffer. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard; for this ATPase, the Lowry color equivalent is 1.18 times that of bovine serum albumin (Kasahara & Penefsky, 1977). The molecular weight of the

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[1] Abbreviations: MF_1 ATPase, mitochondrial F_1 adenosinetriphosphatase; TF_1 ATPase, F_1 ATPase from thermophilic bacteria; CF_1 ATPase, F_1 ATPase from chloroplasts; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Me_2SO, dimethyl sulfoxide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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ATPase was taken as 347 000 (Knowles & Penefsky, 1972).

Loading of MF_1 ATPase with ADP. MF_1 ATPase was loaded with ADP by incubating it with 250 μM MgATP in 100 mM MOPS–Tris buffer, pH 7.0, at room temperature (22–25 °C) for a few seconds. The reaction mixture was then passed through a centrifuge–Sephadex column equilibrated with 100 mM MOPS–Tris buffer, pH 6.7. The enzyme eluted off the column retained bound ADP and was used for the synthesis of enzyme-bound ATP.

Synthesis of Enzyme-Bound ATP. Reaction mixtures containing the components indicated in the legends to the figures were incubated at 30 °C and then quenched with equal volumes of 1 M perchloric acid. The denatured protein was removed by centrifugation, the supernatants were neutralized with Tris, and 0.5 μmol of carrier ATP was added. The volumes were made up to 1.0 mL, and the ATP was separated from [^{32}P]P_i by employing charcoal and AG 1-X4 colums (Vinkler et al., 1978). The neutralized solution was applied to a column (1.0 × 0.5 cm) of acid-washed charcoal previously washed with 2 mL of 0.025 M PP_i, 0.1 M H_3PO_4, and 0.3 M perchloric acid. The column was further washed with 10 mL of a solution containing 0.025 M PP_i, 0.1 M H_3PO_4, and 0.3 M perchloric acid and then with 5 mL of water. The charcoal-bound nucleotides were eluted with 6.0 mL of 95% EtOH–1 M NH_4OH (40:60 v/v). The eluent was applied to a column (0.7 × 4.5 cm) of AG 1-X4 anion-exchange resin, which had been washed with several column volumes of 1 M HCl followed by water to neutrality. After the sample was applied, the column was washed with 5 mL of water, 15 mL of 30 mM HCl, and 15 mL of 60 mM HCl. ATP was eluted with 7 mL of 1 M HCl, and the ^{32}P present was determined by Cerenkov counting. The recovery of ATP off the column was calculated by determining the absorbance of the 1 M HCl eluate at 259 nm. A control with denatured enzyme was run to determine nonspecific radioactivity eluting with ATP fractions. This was essential because only a small amount of the total ^{32}P added formed bound ATP. The control counts were usually about equal to the background counts.

Medium $P_i \rightleftarrows H^{18}OH$ Exchange. Oxygen exchange was measured in a medium containing 100 mM MOPS–Tris buffer, pH 6.7, 30% (v/v) Me_2SO, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM P_i, 52.3–52.7 atom % (final average enrichment) $H^{18}OH$, and MF_1 preloaded with ADP. Other experiments not reported here show that Me_2SO oxygens do not exchange with water oxygens. The 100-μL reaction mixture was incubated at 30 °C for 3 and 6 h and then an equal volume of quench solution containing 1 M perchloric acid and 5 mM EDTA was added. The quenched reaction mixture was neutralized with Tris base and the volume made up to 12 mL. A trace of carrier-free [^{32}P]P_i was added to track the recovery of P_i off the column. P_i was separated by applying the sample to an AG 1-X4 anion-exchange column (0.7 × 1 cm) which had been washed with several column volumes of 1 M HCl and then with water until the eluate was neutral. After the sample was applied, the column was washed with 5 mL of water and then with 10 mM HCl until the eluate had reached pH 2, and P_i was eluted with 4.5 mL of 30 mM HCl. The eluate was lyophilized and used for [^{18}O]P_i analysis.

Determination of Average Enrichment of $H^{18}OH$ by Inorganic Pyrophosphatase Equilibration. Preliminary experiments with [^{18}O]P_i suggested that the bound P_i underwent extensive oxygen exchange before release. However, the results were ambiguous because it was not possible to distinguish between the presence of a small amount of extensively exchanged P_i and contaminating natural-abundance P_i. This ambiguity was resolved by running the reactions in $H^{18}OH$ and looking for the presence of $P^{18}O_2$, $P^{18}O_3$, and $P^{18}O_4$ species which could only come from P_i that had exchanged extensively with the labeled medium water. Measurement of the ^{18}O content of the medium water by reaction of small volumes of $H^{18}OH$ with PCl_5 (Stempel & Boyer, 1986) gave results that appeared slightly low. We thus used the pyrophosphatase-induced $P_i \rightleftarrows H^{18}OH$ exchange for ^{18}O measurement (Hackney et al., 1980).

Equilibration reaction mixtures at 30 °C contained 30 mM Hepes buffer, pH 7.5, 15 mM KCl, 5 mM $MgCl_2$, 6 mM KH_2PO_4, $H^{18}OH$, and 50 μg of pyrophosphatase in a volume of 100 μL, with an aliquot of toluene (5 μL) to prevent microbial growth. The reaction was quenched with 1 M perchloric acid, and P_i was isolated as described in the previous section.

Under these conditions, the $t_{1/2}$ for the $P_i \rightleftarrows HOH$ exchange was about 30 min. Equilibrations were made for 50 h to ensure that the exchange was complete. The pyrophosphatase retained most of its activity during this period of incubation. A small correction was made for the isotope effect on the equilibrium of the reaction (Longinelli & Nutti, 1973). There is a bias for ^{18}O oxygens toward phosphate such that at equilibrium the ratio of ^{18}O in P_i to ^{18}O in H_2O is approximately 1.022.

^{18}O Measurements. [^{18}O]P_i was derivatized to triethyl phosphate with diazoethane and analyzed with a Hewlett-Packard 5995 gas chromatograph/mass spectrometer (Stempel & Boyer, 1986). P_i species containing zero to four atoms of ^{18}O were determined by specific ion monitoring of m/e ratios 155, 157, 159, 161, and 163 corresponding to the diethyl phosphate fragments. Calculations were made as described previously (Hackney, 1980; Hackney et al., 1980; Stempel & Boyer, 1986).

To use a computer algorithm, originally derived for generating the distribution of ^{18}O in the γ-PO_3 of ATP of known initial distribution undergoing intermediate exchange during hydrolysis at different P_c values (Hackney, 1980; Hackney et al., 1980), the following assumptions were made: (a) the initial enrichment of the γ-PO_3 would be 100% if the solvent water were 100% enriched and exchange was complete; (b) the distributions resulting from solvent water exchange could be taken as representing only one cycle of exchange because as shown in Table I, the fractional approach to isotopic equilibrium was small; i.e., molecules of P_i that have bound to the enzyme and undergone exchange with enriched water are unlikely to have been rebound and undergone further exchange after being released into the medium following the first cycle of exchange. The distributions generated by the algorithm could then be modified for the known actual enrichment of water using the appropriate binomial expansions for four, three, two, and one labeled oxygens to give the theoretical distributions of the P_i released from the enzyme. From these data, the $^{18}O_2/^{18}O_3$ and $^{18}O_3/^{18}O_4$ ratios for different P_c values were calculated and compared with the experimental ratios.

Results

Bound ATP Synthesis Induced by Dimethyl Sulfoxide and Its Inhibition by Medium ADP. The promotion of bound ATP synthesis and of net catalytic capacity of MF_1 ATPase by Me_2SO (Schuster, 1979; Sakamoto & Tonomura, 1983; Kandpal et al., 1985) was readily confirmed. We observed an increase in the amount of enzyme-bound ATP formed on increasing the Me_2SO concentration up to 50% (v/v) Me_2SO. However, the yields were low, and this was traced to an inhibitory effect of added ADP. The effect of medium ADP

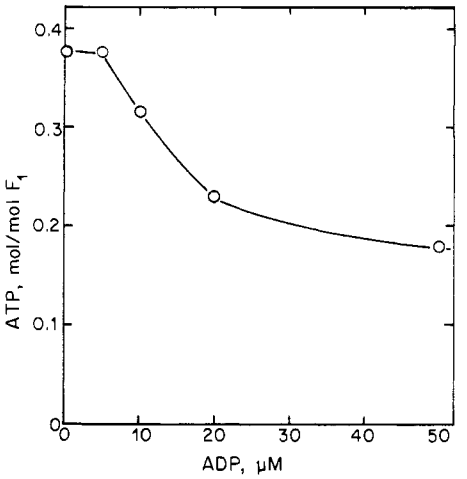

FIGURE 1: Effect of medium ADP concentration on the synthesis of enzyme-bound ATP by MF_1 ATPase in the presence of Me_2SO. Each assay mixture containing 100 mM MOPS–Tris buffer, pH 6.7, 1 mM EDTA, 5 mM $MgCl_2$, 5 mM [^{32}P]P_i, 40% (v/v) Me_2SO, 3.05 μM MF_1 ATPase, and varying concentrations of ADP was incubated at 30 °C for 60 min. The MF_1 ATPase was preloaded with ADP by incubation with 250 μM MgATP for a few seconds and then passage through a centrifuge–Sephadex column. The [γ-^{32}P]ATP formed was separated and determined as described under Experimental Procedures.

concentration was thus evaluated.

Sakamoto and Tonomura (1979) reported that the synthesis of enzyme-bound ATP in the presence of Me_2SO was dependent upon medium ADP concentration. They observed an $S_{0.5}$ of 3 μM for ADP. With our MF_1 ATPase preparations not preloaded with ADP, we observed maximum synthesis when no medium ADP was added (Figure 1). When medium ADP concentration was increased, the yield of enzyme-bound ATP progressively decreased; the yield was decreased by more than 50% at 50 μM ADP and considerably further by higher ADP concentrations.

It seemed plausible that preparations of MF_1 ATPase might retain various amounts of ADP at catalytic sites, and thus the formation of bound ATP might be promoted if the enzyme were freshly exposed to MgADP or to MgATP to increase the amount of bound ADP initially present. Therefore, experiments were conducted with enzyme which had been loaded with ADP by incubation with MgATP as described under Experimental Procedures. When our preparations of MF_1 were exposed to [^{3}H]ATP as outlined under Experimental Procedures, and then passed through centrifuge–Sephadex columns, 0.8–0.9 mol of bound ^{3}H-nucleotide was retained per mole of enzyme. Bound P_i formed by cleavage of ATP at the catalytic site falls off, and tightly bound ADP is retained.

The enzyme could also be loaded with ADP by incubating it for 1 h with 1 mM MgADP. However, results were more variable with this method of loading, and the use of short exposure to ATP gave better assurance that catalytic sites were being filled by ADP arising from ATP cleavage. Further experiments reported here were performed with enzyme freshly loaded by exposure to MgATP.

Estimations of the time course of bound ATP formation showed that at 10 mM P_i 5–10 min was required to reach the half-maximal value (Figure 2). Detailed appraisal of the time course was not attempted because we encountered variability, which may reflect slow conformational changes in the enzyme.

P_i Dependence of Enzyme-Bound ATP Synthesis. The dependence of ATP synthesis on medium P_i concentration is shown in Figure 3. A double-reciprocal plot for the saturation curve indicates an $S_{0.5}$ of about 1.2 mM for P_i.

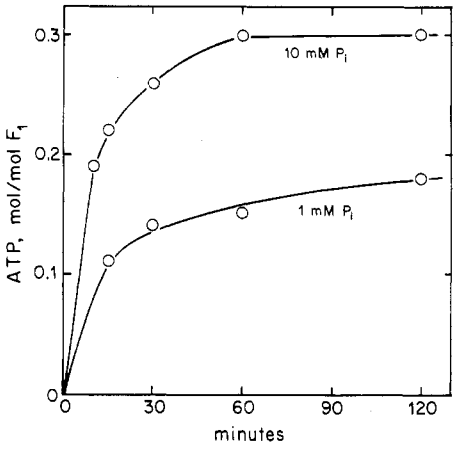

FIGURE 2: Time course of enzyme-bound ATP synthesis by MF_1 ATPase in the presence of Me_2SO. Each reaction mixture containing 100 mM MOPS–Tris buffer, pH 6.7, 30% (v/v) Me_2SO, 1 mM EDTA, 5 mM $MgCl_2$, 1.0 or 10.0 mM [^{32}P]P_i, and 2.0 μM MF_1 ATPase was incubated for one of the times indicated. The [γ-^{32}P]ATP formed was separated and determined as described under Experimental Procedures. The MF_1 ATPase was preloaded with ADP by incubation with 250 μM MgATP for a few seconds and then passage through a Sephadex–centrifuge column.

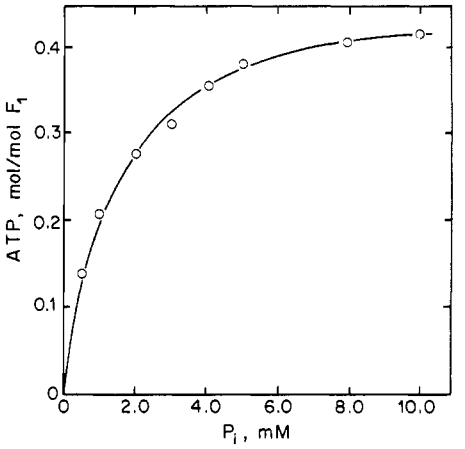

FIGURE 3: Phosphate dependence of enzyme-bound ATP synthesis by MF_1 ATPase in the presence of Me_2SO. Each assay mixture containing 100 mM MOPS–Tris buffer, pH 6.7, 30% (v/v) Me_2SO, 1 mM EDTA, 5 mM $MgCl_2$, 1.31 μM MF_1 ATPase, and varying concentrations of [^{32}P]P_i was incubated at 30 °C for 60 min. The MF_1 ATPase was preloaded with ADP by incubation with 250 μM MgATP for a few seconds and then passage through a centrifuge–Sephadex column. The enzyme-bound [γ-^{32}P]ATP was separated and determined as described under Experimental Procedures.

Although the yields of ATP varied to some degree in different experiments and with different enzyme preparations, the nature of the saturation curve was essentially the same. The P_i dependency with ADP-loaded enzyme was similar to that with the enzyme which contained bound ADP as it was isolated. Our observed $S_{0.5}$ value for P_i is somewhat higher than the 0.55 mM obtained by Sakamoto and Tonomura (1983). Some variability in the saturation curves for P_i dependence was observed, possibly reflecting varying amounts of bound nucleotides present or other parameters that affected the response of enzyme-bound ATP synthesis over time as noted in the next section. It is of interest that Sakamoto and Tonomura (1983) also reported a variable P_i dependency under their conditions, as well as variations in the yield of bound ATP.

It is important to note that with a given enzyme preparation and exposure to Me_2SO, good reproducibility of results was obtainable. This is evident in the experiment reported in

Table I: Distribution of $[^{18}O]P_i$ Species after Medium $P_i \rightleftarrows H^{18}OH$ Exchange Catalyzed by MF_1 ATPase in the Presence of Me_2SO[a]

trial	incubation time (h)	$[^{18}O]P_i$ species $P^{18}O_0$	$P^{18}O_1$	$P^{18}O_2$	$P^{18}O_3$	$P^{18}O_4$	av ^{18}O enrichment (%)
1	3	92.90	2.67	2.43	1.59	0.41	3.42
2	3	93.02	2.75	2.27	1.54	0.42	3.33
3	6	88.51	4.28	3.95	2.58	0.68	5.55
4	6	88.15	4.50	4.00	2.67	0.68	5.70
expected distribution at isotopic equilibrium		5.09	22.51	37.31	27.49	7.60	52.50

[a] The reaction mixture at 30 °C contained 100 mM MOPS–Tris buffer, pH 6.7, 30% (v/v) Me_2SO, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM P_i, 52.3–52.7% (final average enrichment) $H^{18}OH$, and 2.68 μM MF_1 preloaded with ADP by brief exposure to MgATP. The P_i was isolated, and $[^{18}O]P_i$ species were determined as described under Experimental Procedures.

Table II: Exchange Parameters[a]

trial	P_c	no. of reversals	R_{en}[b]
1	0.974	37	4.3
2	0.988	82	4.2
3	0.974	37	3.9
4	0.967	29	4.0
mean ± SD	0.975 ± 0.008	46 ± 24	4.1 ± 0.3

[a] The exchange parameters shown were calculated from medium $P_i \rightleftarrows H^{18}OH$ exchange data. The partition coefficient, P_c, is defined as the probability of bound P_i undergoing oxygen exchange prior to release. The number of reversals is defined as how many times the bound ATP was reversibly cleaved to bound ADP and P_i prior to the release of P_i. R_{en} is the rate of entry of medium P_i into the exchange reaction. The values were calculated as described in the text and previously (Hackney, 1980; Hackney et al., 1980) from the distribution of ^{18}O in the P_i formed. [b] Millimoles per mole of enzyme per second.

Figure 2 where a smooth saturation curve was obtained with increasing P_i concentrations.

Oxygen Exchange Measurements. Oxygen exchange measurements and calculations were made as described under Experimental Procedures. From the distribution of ^{18}O in the P_i formed and the extent of oxygen exchange, the rate of exchange and the probability that P_i once bound would undergo an exchange reaction (the P_c) were evaluated. The rate of entry of medium P_i into the exchange reaction, R_{en}, was evaluated from the observed rate of disappearance of the $P^{18}O_0$ species. This rate times $1/F$, where F is the atom fraction of ^{18}O in the water, gives the rate of loss of the $P^{18}O_0$ species that would have occurred if the water had contained 100% ^{18}O, which equals R_{en}. Each time a medium P_i enters the exchange reaction and returns as medium P_i, one or more oxygens must be replaced by water oxygens. This is based on the assumption that the oxygen lost as water when bound ATP is formed mixes with the medium water. Thus, disappearance of the $P^{18}O_0$ species, independent of the extent of exchange after P_i binds, gives a measure of R_{en}.

The results of medium $P_i \rightleftarrows H^{18}OH$ exchange experiments are presented in Table I. Values of P_c, R_{en}, and the number of reversals calculated from these data are given in Table II. The observed oxygen exchange rate of medium P_i was quite slow because the amount of P_i released from the enzyme was small compared to the amount of medium P_i present. This necessitated relatively long periods of incubation. Tests showed that the enzyme retained nearly full catalytic capacity even after the long incubation. The slightly lower rate of exchange in the second 3-h period may reflect a decrease over time in the amount of ADP present at catalytic sites.

Because natural-abundance P_i contributes no $P^{18}O_4$, $P^{18}O_3$, or $P^{18}O_2$ species, the presence of these species and thus the ratios of $P^{18}O_3/P^{18}O_4$ and $P^{18}O_2/P^{18}O_3$ in the exchanged P_i could be determined accurately. The appearance of $P^{18}O_4$ in the medium P_i demonstrates that extensive exchange has occurred with each P_i that binds to the enzyme. Comparisons of the theoretical and experimental ratios showed P_c values for this exchange in the range of 0.97–0.98 (Table II). There is increased uncertainty in the estimated P_c value as it approaches 1, and thus the number of reaction reversals approaches infinity, but it is definitely clear that each P_i that binds to the enzyme undergoes extensive exchange, equivalent to about 50 reversals of step 2 of eq 1 below, before being released again into the medium. The binding and release of P_i are slow compared to the chemical interconversion step.

We regard catalysis by the MF_1 ATPase, and not some impurity, to be responsible for the oxygen exchange. Previous studies showed that the ATPase preparation lost its capacity for medium $P_i \rightleftarrows HOH$ exchange upon exposure to cold (Choate et al., 1979). This provides a valuable criterion that other enzymes are not responsible for the exchange because the cold lability of the ATPase is an unusual property. The MF_1 ATPase preparation used was well purified, as judged by suitable gel electrophoresis. Also, the high P_c value observed is not as expected for oxygen exchange catalyzed by phosphatase or pyrophosphatase (Hackney, 1980) but is reasonable for the MF_1 ATPase as it shows a very high P_c when cleaving low concentrations of ATP (O'Neal & Boyer, 1984).

Estimations of Equilibrium Rate Constants. From our observations that exposure of the enzyme to [^{3}H]ATP results in somewhat less than 1 mol of tightly bound [^{3}H]ADP per mole of enzyme, and the observations of Grubmeyer et al. (1982) that enzyme freed of medium nucleotides carries out single-site catalysis at one tight binding site, it is justifiable to assume that only one catalytic site per enzyme molecule participates in bound ATP synthesis. The following reaction scheme for synthesis of enzyme-bound ATP may thus be assumed:

$$E{<}ADP + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{<}^{ADP}_{P_i} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{<}ATP \qquad (1)$$

$$K_1 = k_{-1}/k_1 \qquad (2)$$

$$K_2 = k_{-2}/k_2 \qquad (3)$$

For the sequence of eq 1, the following relationship holds:

$$E_t/[E{<}ATP] = K_1K_2/[P_i] + 1 + K_2 \qquad (4)$$

A plot of $E_t/[E{<}ATP]$ vs. $1/[P_i]$ yields a straight line (Figure 4) and allows estimation of K_2, the equilibrium constant for interconversion of ADP and P_i to ATP, and the dissociation constant of P_i, K_1. The rate of ^{18}O exchange is equal to $k_{-2}[E{<}ATP]$, and the partition coefficient, P_c, equals $k_2/(k_2 + k_{-1})$.

Although the variability in enzyme properties mentioned here makes accurate evaluations of individual rate constants untenable, some useful approximations appear warranted. Large differences in the relative values of rate constants so obtained point out prominent features of the Me_2SO-induced ATP formation.

More than one approach can be used to estimate rate constants from the available data. The best estimates of rate

Table III: Rate Constants Estimated from Different Procedures[a]

part A				part B			
k_1 (s^{-1} M^{-1}) from rate of $P^{18}O_0$ loss	k_{-1} (s^{-1}) from K_1 and k_1	k_2 (s^{-1}) from P_c and k_{-1}	k_{-2} (s^{-1}) from k_2 and K_2	k_{-2} (s^{-1}) from total rate of exchange	k_2 (s^{-1}) from k_{-2} and K_2	k_{-1} (s^{-1}) from k_2 and P_c	k_1 (s^{-1} M^{-1}) from K_1 and k_{-1}
9.3 ± 0.4	0.02 ± 0.0008	0.94 ± 0.5	1.11 ± 0.57	0.57 ± 0.29	0.48 ± 0.25	0.01 ± 0.0006	4.75 ± 0.29

[a] The values are means ± SD calculated from the four experiments of Table II. The equilibrium constants, K_1 and K_2, were calculated from Figure 4.

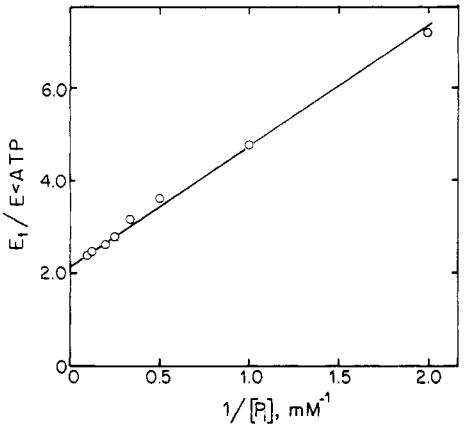

FIGURE 4: E_t/[E<ATP] vs. 1/[P_i] plot for phosphate dependency. The data for this figure are from Figure 3. A least-squares fit gave a correlation coefficient of 0.998, a slope (K_1K_2) of 2.51, and a y intercept (1 + K_2) of 2.18. These data yielded values for K_1 and K_2 of 2.13 mM and 1.18, respectively.

constants may be those from the oxygen exchange data and the amount of bound [γ-^{32}P]ATP formed. The rate of loss of $P^{18}O_0$ species, R_{en}, is given by the following:

$$R_{en} = k_1[P_i][E{<}ADP]P_c$$

thus

$$k_1 = R_{en}/[P_i][E{<}ADP]P_c$$

Of the determinants of this value for k_1, [P_i] is known, R_{en} is a fairly accurate measurement, and the P_c is definitely above 0.9 and can be no greater than 1. An estimate of [E<ADP] from the approximate value of K_2 and the measured E<ATP formed is likely correct within a factor of 2 or less. For example, the [E<ADP] value estimated from the data of Figure 2 agrees well with the difference between the initial bound ADP expected to be present from the loading by cleavage of ATP and the measured level of E<ATP formed from P_i. The value of k_1 estimated in this manner can be used for the sequential calculation of the other rate constants given in part A of Table III.

An estimate of k_{-2} can be obtained from the total rate of oxygen exchange and the amount of E<ATP formed from P_i that is present. The total flux across step 2 of eq 1 is given by $V_{ex}(4 - 3P_c)/(4 - 4P_c)$, where V_{ex} is the total rate of oxygen exchange (Hackney et al., 1980). This total flux is also equal to k_{-2}[E<ATP]. From this, k_{-2} was evaluated and used for the sequential calculation of other rate constants given in part B of Table III. The rate constants evaluated by the two approaches agree within a factor of 2 or less. Probably the greatest source of error is from measurements of the values for the equilibrium constants K_1 and K_2, because of variability in the primary data and lack of certainty that simple mass action considerations govern the observed values for E<ATP formation. Other factors may also be involved as discussed in the following paragraph.

An apparent value for k_1 can also be approximated from the time course for the formation of enzyme-bound ATP (Figure 2). The oxygen exchange data show that the equilibrium governed by k_2 is adjusted rapidly compared to that governed by k_1 because k_2 is much greater than k_{-1} and, based on the dependency of bound ATP formation on P_i concentration, k_2 and k_{-2} are nearly equal. In this case, the apparent first-order rate constant, k, for formation of bound ATP is equal to $k_1[P_i] + k_{-1}/(1 + 1/K_2)$. Thus, $k_1[P_i] = k - k_{-1}/(1 + 1/K_2)$. Neglecting the second term gives a maximum value for k_1. From the data of Figure 2, at 10 mM P_i this maximum value is 0.16 s^{-1} M^{-1}, considerably less than the values of k_1 reported in Table III. Reasons thus need to be sought for the low value estimated from the rate of appearance of bound ATP. One possibility is that slow changes in rate constants are occurring associated with slow protein conformational changes. The oxygen exchange measurements were made after 3- and 6-h incubations, which would minimize effects such as the time-dependent changes evident in the first part of the incubation shown in Figure 2.

DISCUSSION

Our results show two prominent features of the Me_2SO-promoted formation of enzyme-bound ATP from medium P_i. The first is that the organic solvent does not appreciably change the equilibrium constant governing formation of enzyme-bound ATP from bound ADP and P_i, but markedly promotes the binding of P_i. The second is that the interconversion of bound ATP and bound ADP and P_i is rapid when compared with the binding and release of P_i.

Although the reversible hydrolysis of bound ATP in the presence of Me_2SO is more rapid than P_i binding and release, comparison with other data in the literature shows that the rate of interconversion in the presence of the organic solvent is much slower than that without solvent addition. The interconversion rate measured from single-site catalysis is about 10 per second (Grubmeyer et al., 1982), and that measured from the limits of oxygen exchange as ATP is lowered during steady-state hydrolysis is about 14 per second (O'Neal & Boyer, 1984). In contrast, the data of Table III give an estimated interconversion rate with excess P_i in the presence of Me_2SO of only 0.5–1.0 per second. Even without solvent present, the interconversion rate at low substrate concentration is definitely less than the maximum rate of net ATP hydrolysis with excess substrate.

Comparison of the rate of medium oxygen exchange reported in this paper with that observed for the MF_1 ATPase in the absence of organic solvents (Choate et al., 1979) shows that the rate of bound substrate interconversion in the presence of Me_2SO is relatively slow. Choate et al. (1979) did not detect any bound ATP formation in the presence of the 6 mM P_i used for their oxygen exchange measurements. This is as expected if the K_d for P_i under these conditions is roughly 1 M as suggested by the data of Sakamoto (1984a). If this were the case, then the rate of oxygen exchange with saturating P_i concentration under the conditions of Choate et al. would be about 10 atoms (mol of enzyme)$^{-1}$ s^{-1}. The maximum oxygen exchange rate estimated from our data is 0.29 atom (mol of enzyme)$^{-1}$ s^{-1}, which is thus about 35-fold less than that observed without Me_2SO addition.

The ability of Me_2SO to increase the binding of P_i in a manner competent to make bound ATP is striking. deMeis (1984) has demonstrated that organic solvents markedly shift the equilibrium of PP_i formation from P_i. He presents a convincing case that solvents probably act by decreasing the solvation of P_i. The Me_2SO-induced increase in the phosphorylation of sarcoplasmic reticulum ATPase appears to involve transition of the P_i binding site from a hydrophilic to a more hydrophobic environment (deMeis et al., 1980; deMeis, 1984). These reported effects of Me_2SO, together with our data, suggest that the desolvation of P_i upon binding to a catalytic site with ADP already present is an important step in the subsequent formation of bound ATP.

Our data add to the evidence that MF_1 ATPase readily retains a tightly bound ADP at a catalytic site, based on the criterion that the nucleotide is retained when the enzyme is passed through a Sephadex–centrifuge column. The ease with which this tight site is loaded with ADP by brief exposure to MgATP and subsequently converted to ATP by exposure to Me_2SO and P_i gives convincing evidence that ADP is binding to a catalytic site. An ADP tightly bound to MF_1 (Drobinskaya et al., 1985) and CF_1 (Feldman & Boyer, 1985) has been shown to be at the catalytic site. The MF_1 ATPase may also have tightly bound ADP at noncatalytic sites which shows relatively slow exchange with medium nucleotides (Cross & Nalin, 1982; Esch & Allison, 1979). Sakamoto (1984b) has shown that the Me_2SO-promoted synthesis does not occur with the ADP that is bound to the nonexchangeable sites.

One difference in our findings compared to those of Sakamoto and Tonomura (1983) and Sakamoto (1984a) is that they observed medium ADP dependence of the reaction and estimated a K_d of 3 μM for ADP. Our observations show that any added medium ADP is inhibitory and that the yields are maximum when the enzyme is loaded with ADP first. The effect of medium ADP may arise from differences in the procedures used for enzyme purification. They carried out gel filtration in the presence of 55% glycerol and stored the enzyme without any nucleotides in a solution containing 55% glycerol (Matsuoka et al., 1981). The enzyme thus isolated was likely nearly devoid of catalytic site nucleotides and showed medium ADP dependence for ATP synthesis. However, the enzyme used in our experiments was not exposed to glycerol and was stored as an ammonium sulfate suspension with ATP present; it thus likely had some tightly bound ADP at the catalytic site. The exogenous ADP probably binds to an alternate catalytic site on the enzyme and either perturbs the equilibrium of the reaction of bound ADP + $P_i \rightleftarrows$ ATP more toward the left or, more likely as shown for P_i dissociation (Hutton & Boyer, 1979), promotes dissociation of the bound ADP and/or P_i.

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Registry No. ATP, 56-65-5; P_i, 14265-44-2; ATPase, 9000-83-3; dimethyl sulfoxide, 67-68-5.

REFERENCES

Choate, G. L., Hutton, R. L., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 286–290.
Cross, R. L., & Nalin, C. M. (1982) *J. Biol. Chem. 257*, 2874–2881.
deMeis, L. (1984) *Curr. Top. Cell. Regul. 24*, 15–20.
deMeis, L., Martins, O. B., & Alves, E. W. (1980) *Biochemistry 19*, 4252–4261.
Drobinskaya, I. Ye., Kozlov, I. A., Murataliev, M. B., & Vulfson, E. N. (1985) *FEBS Lett. 182*, 419–424.
Esch, F. S., & Allison, W. S. (1979) *J. Biol. Chem. 254*, 10740–10746.
Feldman, R. I., & Sigman, D. S. (1982) *J. Biol. Chem. 257*, 1676–1683.
Feldman, R. I., & Boyer, P. D. (1985) *J. Biol. Chem. 260*, 13088–13094.
Grubmeyer, C., Cross, R. L., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12092–12100.
Hackney, D. D. (1980) *J. Biol. Chem. 255*, 5320–5328.
Hackney, D. D., Stempel, K. E., & Boyer, P. D. (1980) *Methods Enzymol. 64*, 60–83.
Hutton, R. L., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 9990–9993.
Kandpal, R. P., Melese, T., Stroop, S. D., & Boyer, P. D. (1985) *J. Biol. Chem. 260*, 5542–5547.
Kasahara, M., & Penefsky, H. S. (1977) *J. Biol. Chem. 253*, 4180–4187.
Knowles, A. F., & Penefsky, H. S. (1972) *J. Biol. Chem. 247*, 6624–6630.
Longinelli, A., & Nutti, S. (1973) *Earth Planet. Sci. Lett. 19*, 373–376.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Matsuoka, I., Watanabe, T., & Tonomura, Y. (1981) *J. Biochem. (Tokyo) 90*, 967–989.
O'Neal, C. C., & Boyer, P. D. (1984) *J. Biol. Chem. 259*, 5761–5767.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Penefsky, H. S. (1979) *Methods Enzymol. 55*, 304–308.
Sakamoto, J. (1984a) *J. Biochem. (Tokyo) 96*, 483–487.
Sakamoto, J. (1984b) *J. Biochem. (Tokyo) 96*, 475–481.
Sakamoto, J., & Tonomura, Y. (1983) *J. Biochem. (Tokyo) 93*, 1601–1614.
Schuster, S. M. (1979) *Biochemistry 18*, 1162–1167.
Stempel, K. E., & Boyer, P. D. (1986) *Methods Enzymol. 126*, 618–639.
Vinkler, C., Rosen, G., & Boyer, P. D. (1978) *J. Biol. Chem. 253*, 2507–2510.
Yoshida, M. (1983) *Biochem. Biophys. Res. Commun. 114*, 907–912.