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Bound Adenosine 5′-Triphosphate Formation, Bound Adenosine 5′-Diphosphate and Inorganic Phosphate Retention, and Inorganic Phosphate Oxygen Exchange by Chloroplast Adenosinetriphosphatase in the Presence of Ca^{2+} or Mg^{2+} †

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ABSTRACT: When the heat-activated chloroplast F_1 ATPase hydrolyzes [^{3}H,γ-^{32}P]ATP, followed by the removal of medium ATP, ADP, and P_i, the enzyme has labeled ATP, ADP, and P_i bound to it in about equal amounts. The total of the bound [^{3}H]ADP and [^{3}H]ATP approaches 1 mol/mol of enzyme. Over a 30-min period, most of the bound [^{32}P]P_i falls off, and the bound [^{3}H]ATP is converted to bound [^{3}H]ADP. Enzyme with such remaining tightly bound ADP will form bound ATP from relatively high concentrations of medium P_i with either Mg^{2+} or Ca^{2+} present. The tightly bound ADP is thus at a site that retains a catalytic capacity for slow single-site ATP hydrolysis (or synthesis) and is likely the site that participates in cooperative rapid net ATP hydrolysis. During hydrolysis of 50 μM [^{3}H]ATP in the presence of either Mg^{2+} or Ca^{2+}, the enzyme has a steady-state level of about one bound [^{3}H]ADP per mole of enzyme. Because bound [^{3}H]ATP is also present, the [^{3}H]ADP is regarded as being present on two cooperating catalytic sites. The formation and levels of bound ATP, ADP, and P_i show that reversal of bound ATP hydrolysis can occur with either Ca^{2+} or Mg^{2+} present. They do not reveal why no phosphate oxygen exchange accompanies cleavage of low ATP concentrations with Ca^{2+} in contrast to Mg^{2+} with the heat-activated enzyme. Phosphate oxygen exchange does occur with either Mg^{2+} or Ca^{2+} present when low ATP concentrations are hydrolyzed with the octyl glucoside activated ATPase. Ligand binding properties of Ca^{2+} at the catalytic site rather than lack of reversible cleavage of bound ATP may underlie lack of oxygen exchange under some conditions.

The CF_1 ATPase[1] component of the chloroplast ATP synthase retains a tightly bound ADP that has an apparent regulatory function [see review by Shavit (1980)] and appears to be at the catalytic sites on the β subunits of the enzyme [see Feldman & Boyer (1985) and references cited therein]. When chloroplasts are exposed to ^{14}C-labeled ATP, the isolated CF_1 ATPase retains a bound labeled ADP (Magnusson & McCarty, 1976). An adquate understanding of the factors

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[1] Abbreviations: CF_1 ATPase and MF_1 ATPase, ATPase portion of ATP synthase from spinach chloroplasts and beef heart mitochondria, respectively; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; EDTA, ethylenediaminetetraacetic acid.

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governing the appearance of this ADP, its relationship to any bound ATP and P_i, and its functional role are of obvious importance to the long-term goal of learning how ATP is made by ATP synthase.

This paper addresses several questions, in part stimulated by the observations of Kohlbrenner and Boyer (1983) that the chloroplast F_1 ATPase with Ca^{2+} activation, in contrast to Mg^{2+} activation, does not catalyze detectable oxygen exchange when low concentrations of ATP are hydrolyzed. This suggested that the catalytic behavior of the tightly bound ADP might differ in the presence of Mg^{2+} or Ca^{2+}. Questions that arise are as follows: Will Ca^{2+}, like Mg^{2+} (Feldman & Sigman, 1982), allow bound ATP formation from tightly bound ADP on the CF_1 ATPase and medium P_i? Can a steady-state level of bound [^{3}H]ADP formed from medium [^{3}H]ATP be detected on the CF_1 ATPase with either Ca^{2+} or Mg^{2+} present, analagous to that observed on the mitochondrial enzyme with Mg^{2+} activation (Gresser et al., 1982)? After hydrolysis of [γ-^{32}P,^{3}H]ATP followed by removal of medium ATP, ADP, and P_i, can tightly bound [γ-^{32}P,^{3}H]ATP, [^{3}H]ADP, and [^{32}P]P_i be detected with either Mg^{2+} or Ca^{2+}? If so, is the bound ATP subsequently converted to bound ADP and P_i, and does the P_i formed depart from the enzyme, leaving a tightly bound ADP? Can conditions be found where Ca^{2+} may catalyze an oxygen exchange analogous to that seen with Mg^{2+}?

The results obtained reveal additional characteristics of the formation, retention, and release of bound ATP, ADP, and P_i that are clearly relevant to understanding of the CF_1 ATPase mechanism and point to possible explanations for the differences in phosphate oxygen exchange in the presence of Ca^{2+} or Mg^{2+}.

Experimental Procedures

ATPase Preparation. CF_1 ATPase was prepared from market spinach essentially according to Binder et al. (1978). The crude ATPase was further purified on a Sepharose 6B column equilibrated and eluted with a buffer containing 40 mM Tricine–KOH, 1 mM ATP, and 2 mM EDTA at pH 7.8. The purified enzyme was precipitated by 50% saturated ammonium sulfate and stored at about 4 °C.

Ammonium sulfate precipitated CF_1 was desalted by passage through a Sephadex G-50 centrifuge column (Penefsky, 1979) equilibrated with 40 mM Tricine–KOH and 2 mM EDTA at pH 8.0 before being heat activated according to Lien and Racker (1971) in 40 mM Tricine–KOH, 4 mM EDTA, 20 mM ATP, and 7 mM dithiothreitol, pH 8.0, for 4 min at 63 °C. The reaction components were then removed by passage through another Sephadex centrifuge column equilibrated with 40 mM Tricine–KOH and 20 μM EDTA, pH 8.0.

Formation of Enzyme-Bound [γ-^{32}P]ATP from Enzyme-Bound [^{3}H]ADP and Medium [^{32}P]P_i. The heat-activated CF_1 was allowed to hydrolyze 200 μM [^{3}H]ATP (sp act. 10^{14} cpm/mol) in 40 mM Tricine and 200 μM $MgCl_2$ or $CaCl_2$ at pH 8.0 for 30 s. The reaction was quenched by the addition of sufficient EDTA to give a final concentration of 5 mM. The medium nucleotides and columns were equilibrated with 25 mM Tricine and 200 μM EDTA, pH 8.0. The enzyme was stored in this buffer at room temperature for 1–2 h before it was used for [γ-^{32}P,^{3}H]ATP synthesis. Under these conditions, approximately 0.7–0.8 mol of [^{3}H]ADP/mol of CF_1 remained tightly bound. The synthesis of tightly bound [γ-^{32}P,^{3}H]ATP from medium [^{32}P]P_i in the presence of Ca^{2+} was carried out under conditions similar to those described by Feldman and Sigman (1982) as outlined in the legend for Figure 1.

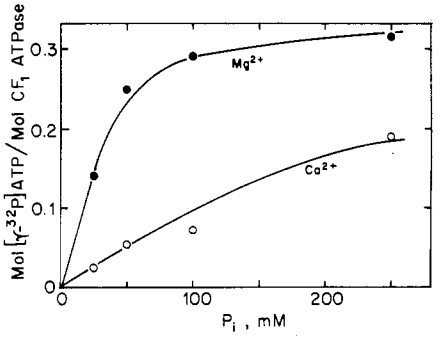

FIGURE 1: Dependence of synthesis of enzyme-bound ATP in the presence of Ca^{2+} or Mg^{2+} on phosphate concentration. Heat-activated CF_1 ATPase with tightly bound [^{3}H]ADP was prepared as described under Experimental Procedures. The ATPase was incubated at a final concentration of 5.6 μM for 30 min at room temperature and pH 6.0 with the indicated amounts of [^{32}P]P_i (sp act. 10^{12}–10^{13} cpm/mol) in 25 mM Tricine, 30 mM potassium acetate, and either 20 mM magnesium acetate or 20 mM calcium acetate, as indicated in the figure, in a final volume of 100 μL. The reaction was quenched with an equal volume of 1 M perchloric acid containing 2.5 μmol of unlabeled ATP, and the amount of [^{32}P]ATP synthesized was determined by addition of carrier ATP, separation of the nucleotides on a Dowex 1 column as described previously (O'Neal & Boyer, 1984), and measurement of ^{32}P and recovery of ATP in the ATP fraction.

Measurement of Bound ATP, ADP, and P_i. Reaction mixtures as given in the figure legends were quenched either with EDTA (final concentration 5 mM) or, when creatine kinase and pyruvate kinase were used, with NaOH (final concentration 50 mM). Unbound ATP, ADP, and P_i were removed by centrifuge Sephadex columns, and the proteins were precipitated by perchloric acid. After carrier additions, the labeled nucleotides and P_i were separated on Dowex AG 1X4 columns and measured as described previously (O'Neal & Boyer, 1984).

When high levels of creatine kinase and pyruvate kinase were used, a correction was made for the measured extent of binding of [^{3}H]ATP by the kinase. This was small for the pyruvate kinase but ranged up to a few percent of the CF_1 binding for the highest levels of creatine kinase.

Assays. Ca^{2+} ATPase activity was measured as P_i formation in an assay mixture at pH 8 and 30 °C containing 40 mM Tricine, varying concentrations of ATP (50 μM to 5 mM), and equivalent amounts of Ca^{2+}. Mg^{2+} ATPase activity was determined by monitoring the oxidation of NADH in a coupled assay system with phosphoenolpyruvate, NADH, lactate dehydrogenase, and pyruvate kinase.

Protein determinations were performed with Lowry reagent and bovine serum albumin as the standard. The CF_1 concentration was calculated with its M_r as 400 000 (Moroney et al., 1983) and corrected for the Lowry color equivalence of CF_1. The correction factor was based on the CF_1 extinction coefficient at 280 nm of 0.476 mL mg^{-1} cm^{-1} (Cantley & Hammes, 1975).

^{18}O Exchange Experiments. [γ-^{18}O]ATP (89.9% enriched) was synthesized as described (Stempel & Boyer, 1986). Experimental conditions are described in the legend to Table I. Controls were run without CF_1 ATPase but with added [^{18}O]P_i (98.5% enriched) or unenriched P_i. From the dilution of the PO_4 species in the [^{18}O]P_i, the amount of contaminating unenriched P_i was determined. The amount of nonenzymatic cleavage of [γ-^{18}O]ATP was determined from the control with added unenriched P_i.

Results

Formation of Enzyme-Bound ATP from P_i. A salient property of CF_1 ATPase is its ability to form a tightly bound

Table I: Phosphate Oxygen Exchange Accompanying [γ-^{18}O]ATP Hydrolysis with Octyl Glucoside Activated CF_1 ATPase and with either Mg^{2+} or Ca^{2+} Present[a]

cation addition	av no. of water oxygens in each P_i released	
	expt 1	expt 2
2 mM Mg^{2+}	1.42	1.47
15 mM Ca^{2+}	1.26	1.23

[a] Incubation mixtures with different CF_1 ATPase preparations at pH 8 and 37 °C contained 520 μM [γ-^{18}O]ATP (98.8% ^{18}O), 200 mM octyl glucoside, 50 mM Tricine, and either 2 mM Mg^{2+} and about 1.0 mg of CF_1 ATPase or 15 mM Ca^{2+} and 1.75 mg of CF_1 ATPase, in a total volume of 3.0 mL. Experimental Samples were incubated for about 1.5 h, and the P_i produced was isolated and analyzed for ^{18}O content as previously described (O'Neal & Boyer, 1984; Stempel & Boyer, 1986).

ATP from a tightly bound ADP in the presence of Mg^{2+} and high medium concentrations of P_i (Feldman & Sigman, 1982). This results from the reversal of hydrolysis of bound ATP to bound ADP and P_i with an equilibrium not far from unity. If reversal was slower with Ca^{2+} than with Mg^{2+}, shift of the equilibrium toward bound ADP and P_i could result. This would offer an explanation for the lack of intermediate $P_i \rightleftharpoons$ HOH exchange during Ca^{2+}-activated hydrolysis. To determine whether a shift of the equilibrium toward ADP and P_i occurs with Ca^{2+} and, if so, to what extent, the formation of bound ATP from medium P_i was measured. Results depicted in Figure 1 demonstrate that Ca^{2+} does promote the formation of bound ATP, although the concentration of medium P_i required is somewhat higher than that with Mg^{2+} as the activating cation. The principal difference between the two cations is that a higher concentration of P_i is required with Ca^{2+} than with Mg^{2+}. The equilibrium level of bound ATP attainable with Ca^{2+} is about half as great with Ca^{2+} compared to Mg^{2+}, indicating that the rate of reversal of hydrolysis of bound ATP is not far different with either cation present.

Detection of Oxygen Exchange during Ca^{2+}-Promoted Hydrolysis with Octyl Glucoside Activated ATPase. The ability of CF_1 ATPase to form tightly bound ATP from the bound ADP and medium P_i in the presence of Ca^{2+} suggested that further examination should be made of possible phosphate oxygen exchange accompanying hydrolysis of low concentrations of ATP in the presence of Ca^{2+}. The previous experiments in which no oxygen exchange was detected were done with heat-activated ATPase, which gives a considerably more rapid rate of hydrolysis with Ca^{2+} than with Mg^{2+}. We thus chose to examine oxygen exchange in the presence of Ca^{2+} using the octyl glucoside activated ATPase (Pick & Bassalian, 1983), which gives a slower net hydrolysis rate with Ca^{2+} than with Mg^{2+}, and the oxygen exchange observed with Mg^{2+} present is somewhat higher than that with the heat-activated enzyme (Kohlbrenner & Boyer, 1983).

Comparisons of the oxygen exchange observed with Mg^{2+} and Ca^{2+} activation during ATP hydrolysis by the octyl glucoside activated enzyme are shown in Table I. The ATP concentration chosen for these experiments was low enough to result in marked oxygen exchange with Mg^{2+} yet sufficient to allow formation of enough P_i for ^{18}O analyses. A higher Ca^{2+} than Mg^{2+} concentration was used because of the increased Ca^{2+} requirement with octyl glucoside activation (Pick & Bassalian, 1983). The results demonstrate that under the conditions used extensive incorporation of more than one water oxygen into each P_i formed occurs with both Ca^{2+} and Mg^{2+} activation. The octyl glucoside activated and the heat-activated enzymes thus show marked differences in oxygen exchange properties.

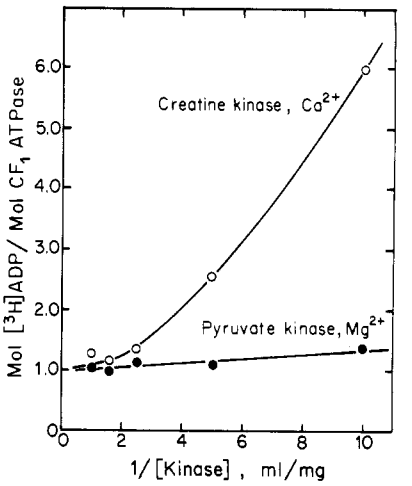

FIGURE 2: Measurement of creatine kinase or pyruvate kinase inaccessible ADP with Ca^{2+} or Mg^{2+} activation. Reaction mixtures of 0.5 mL containing 40 mM Tricine, 50 μM [^{3}H]ATP, 0.5 μM CF_1 ATPase (heat activated, then transferred to 40 mM Tricine and 20 mM EDTA at pH 8), and 50 μM $MgCl_2$ or $CaCl_2$ at pH 8.0 and 30 °C were incubated for 30 s with either a MgATP regenerating system (10 mM phosphoenolpyruvate and the indicated amounts of pyruvate kinase) or a CaATP regenerating system (15 mM phosphocreatine and the indicated amounts of creatine kinase) as indicated in the figure. The reaction was quenched with an equal volume of 100 mM NaOH, and the total amount [^{3}H]ADP was determined by addition of carrier ADP followed by adsorption and separation on an anion exchange column and measurement of the recovery of ADP and ^{3}H in the ADP fraction.

Bound ADP Levels during Net ATP Hydrolysis. Previous attempts in our laboratory to demonstrate the retention of bound ADP at catalytic sites of CF_1 ATPase during steady state hydrolysis at low concentrations of ATP were not successful. Such retention would be expected if release of ADP and P_i from one catalytic site is slow unless ATP binds at another site. Indeed, the presence of bound catalytic ADP on MF_1 ATPase has been demonstrated during hydrolysis of [^{3}H]ATP by measurement of the [^{3}H]ADP that is inaccessible to pyruvate kinase (Gresser et al., 1982). We now recognize that the CF_1 ATPase can show a marked lag in the onset of ATP hydrolysis, particularly at low ATP concentrations if the enzyme has been exposed previously to Mg^{2+} or Ca^{2+} (Hochman & Carmeli, 1981; Feldman & Boyer, 1985), which is the case with the usual isolation procedures. This could explain why bound [^{3}H]ADP was not found shortly after addition of [^{3}H]ATP. As shown in Figure 2, by use of enzyme that had been stored in the presence of EDTA and assay conditions with somewhat lower Mg^{2+} and higher ATP concentrations than had been previously used, the formation of enzyme bound [^{3}H]ADP was readily demonstrated. Extrapolation to infinite pyruvate kinase concentration indicated that about one bound catalytic ADP per ATPase was present during the hydrolysis of 50 μM ATP.

To test for bound ADP levels with Ca^{2+} activation, creatine kinase was used because, unlike pyruvate kinase, it is readily activated by Ca^{2+}. The Ca^{2+}-activated ATP hydrolysis is more rapid, and relatively high levels of creatine kinase were required to remove most of the medium ADP as it is formed. As shown in Figure 2, an extrapolation to infinite creatine kinase concentration indicated the presence of about one bound catalytic ADP per ATPase during hydrolysis of 50 μM ATP. The similarity of bound ADP levels with either Mg^{2+} or Ca^{2+} activation indicates that during steady-state hydrolysis the equilibrium of bound ADP and P_i with bound ATP is about the same for both cations.

The ATP concentrations required for half-maximal velocity

Table II: ^3H-Labeled Nucleotides Bound to CF_1 ATPase after [^3H]ATP Hydrolysis[a]

conditions	enzyme-bound ^3H-labeled nucleotide (mol/mol of CF_1)		
	total ^3H-labeled nucleotide[b]	[^3H]ATP	[^3H]ADP
Mg^{2+}-activated hydrolysis			
pulsed with [^3H]ATP, then EDTA added (1)	0.78	0.35	0.41
control, as 1 but no CF_1 ATPase	0.03	0.00	0.02
control, as 1 but NaOH-inactivated ATPase	0.13	0.03	0.09
as 1, then Mg–ATP chase	0.08	0.03	0.08
Ca^{2+}-activated hydrolysis			
pulsed with [^3H]ATP, then EDTA added (1)	0.73	0.32	0.36
control, as 1 but no CF_1 ATPase	0.06	0.00	0.05
control, as 1 but NaOH-inactivated ATPase	0.14	0.02	0.09
as 1, then Ca–ATP chase	0.09	0.04	0.06

[a] A 50-μL volume of 2 μM heat-activated CF_1 ATPase was incubated for 1.0 min at pH 8.0 and 25 °C with 25 μL of a medium containing Tricine/NaOH buffer, pH 8.0, 40 μM ATP, and either 800 μM phosphoenolpyruvate, 20 μM $MgCl_2$, and pyruvate kinase (0.3 mg/mL) or 800 μM phosphocreatine, 20 μM $CaCl_2$, and creatine kinase (0.15 mg/mL). To this reaction was added 50 μL of a solution at pH 8.09 that gave final concentrations of 40 mM Tricine, 25 μM $MgCl_2$ or $CaCl_2$, 50 μM [^3H]ATP, 1 mM phosphoenolpyruvate or phosphocreatine, and pyruvate kinase (0.1 mg/mL) or creatine kinase (0.05 mg/mL). After 20 s, 25 μL of either water or a chase solution containing 30 mM ATP and 15 mM $MgCl_2$ or $CaCl_2$ was added followed by a quench of 10 μL of 200 mM EDTA. The CF_1 ATPase with tightly bound nucleotides was separated from the reaction mixture by passage through a Sephadex G-50 centrifuge column equilibrated with 40 mM Tricine and 2 mM EDTA at pH 8,.0, into an equal volume of M perchloric acid. The total bound nucleotides were measured by the ^3H in the perchloric acid extract. For independent measurements of bound [^3H]ATP and [^3H]ADP, the nucleotides were separated on a Dowex AG 1X4 column after addition of carrier ADP and ATP (O'Neal & Boyer, 1984). Values are averages of duplicate or triplicate analyses. [b] Separate measurement from [^3H]ATP and [^3H]-ADP measurements.

of ATP hydrolysis under the conditions used in Figure 2 were approximately 240 μM with Mg^{2+} and 200 μM with Ca^{2+}. Thus, with the 50 μM [^3H]ATP concentration used for the experiments of Figure 2, only about $^1/_5$ of the catalytic sites would be expected to be occupied if the enzyme had single noninteracting catalytic sites. The data of Figure 2 show that about one site per ATPase is occupied by [^3H]ADP. Some bound [^3H]ATP is very likely also present, as shown by experiments reported in the next section. Therefore, total catalytic site occupancy is probably greater than one per ATPase. During steady-state hydrolysis, considerable two-site catalysis is probably occurring, and both loosely and tightly bound ADP and ATP are likely present on different catalytic sites. It needs emphasis, however, that the present data do not allow us to draw conclusions about the amounts of bound ATP and ADP at catalytic sites during catalysis when excess ATP is present so that sites are saturated.

Levels of Tightly Bound P_i, ADP, and ATP at Catalytic Sites after Removal of Medium Reactants. When ATP hydrolysis is stopped by EDTA addition and the reaction mixture passed through a centrifuge Sephadex column (Penefsky, 1979), unbound medium nucleotides are removed. If the release of tightly bound ATP, ADP, and P_i from a catalytic

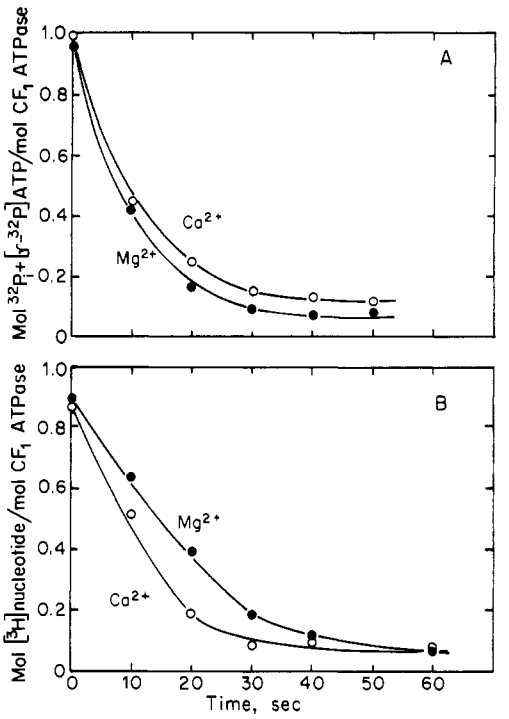

FIGURE 3: Presence and rate of replacement of ^3H- and ^{32}P-labeled nucleotides at catalytic sites following hydrolysis of [^3H,γ-^{32}P]ATP in the presence of Mg^{2+} or Ca^{2+}. Heat-activated ATPase, 2 μM, was incubated in medium containing 40 mM Tricine/NaOH, pH 8.0, 200 μM [^3H,γ-^{32}P]ATP, and 100 μM $MgCl_2$ or $CaCl_2$ in a 400–600 μL total volume for 20 s at 25 °C. Unbound components were removed by passing the enzyme through Sephadex centrifuge columns equilibrated with 40 mM Tricine and 2 μM EDTA, pH 8.0. Then the labeled enzyme was incubated in a 350-μL volume at pH 8 containing 40 mM Tricine, 20 μM ATP, and either 20 μM $MgCl_2$, 500 μM phosphoenolpyruvate, and 0.5 mg/mL pyruvate kinase or 20 μM $CaCl_2$, 500 μM phosphocreatine, and 0.15 mg/mL creatine kinase, as indicated in the figure. After exposure to unlabeled ATP for the times indicated, 100 μL of reaction mixture was removed and passed through a Sephadex centrifuge column equilibrated with 40 mM Tricine and 2 mM EDTA at pH 8.0. The total bound ^3H- and ^{32}P-labeled species remaining were determined by counting each label in the eluent. (A) Time course for the loss of ^{32}P label and (B) loss of ^3H label from CF_1.

site is very slow unless another catalytic site binds nucleotides, as in the binding change mechanism, after passage through the column, the ATPase would be expected to retain an equilibrium mixture of ATP, ADP, and P_i at the catalytic site. Table II gives measurements of the total bound ^3H-labeled nucleotides and separate measurements of bound [^3H]ATP and [^3H]ADP. About 0.3 mol each of bound [^3H]ATP and [^3H]ADP was present after steady-state cleavage of [^3H]ATP with either Mg^{2+} or Ca^{2+} activation. As shown in Table II, all the bound ^3H-labeled nucleotides were effectively removed if the enzyme was allowed to turnover with excess unlabeled ATP before column separation. These results are consistent with tightly bound P_i being present together with ADP and ATP at a catalytic site.

To provide a more direct assessment of the presence of a bound P_i at a catalytic site, measurements were made with ATP labeled with both ^3H and ^{32}P. These experiments included separation of the ATPase with bound ^3H- and ^{32}P-labeled nucleotides and measurement of their rate of replacement during subsequent hydrolysis of only 20 μM unlabeled ATP. Results given in Figure 3 show that with either Ca^{2+} or Mg^{2+} present each mole of ATPase initially retained 0.8–1.0 mol of ^3H- and ^{32}P-labeled species. From the data of Table II, the ^3H present represented about equal parts of ATP and ADP. Thus, the total amount of ^{32}P-labeled species present was about

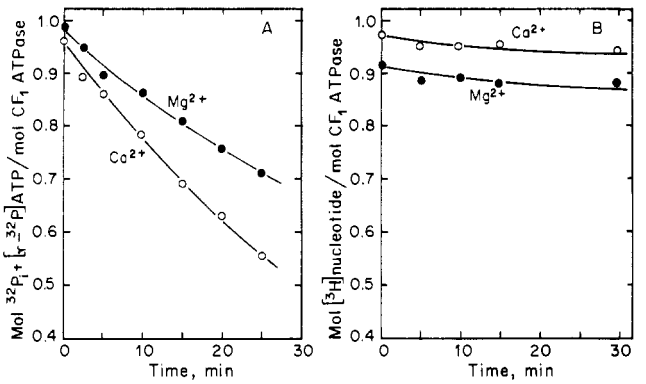

FIGURE 4: Measurement of dissociation of ^3H and ^{32}P labels from CF_1 ATPase in the presence of EDTA. Reaction conditions were essentially the same as for Figure 3 except that after the enzyme was labeled it was incubated in 40 mM Tricine and 2 mM EDTA at pH 8.0 and 30 °C instead of the ATP regenerating systems. Aliquots were removed and treated as described in the legend for Figure 3. (A) Loss of ^{32}P label from CF_1 and (B) loss of ^3H label.

twice as large as that accounted for by the bound [γ-^{32}P]ATP present. This shows that about an equivalent amount of bound [^{32}P]P_i was also present. The presence of such bound [^{32}P]P_i was also confirmed by separate [^{32}P]P_i analyses.

As noted in Figure 3, with initiation of hydrolysis of unlabeled ATP, both the ^{32}P and ^3H labels were replaced. The replacement rates as reported in this figure are considerably slower than the maximum enzyme turnover rates, reflecting the relatively low (20 μM) ATP concentration used and the probable occurrence of some Mg^{2+}- or Ca^{2+}-induced inhibition (Feldman & Boyer, 1985). The bound ^{32}P- and ^3H-labeled species were removed at about the same rate, as would be expected if a conformational change resulted in a change of the binding of both ADP and P_i from tight to loose.

From the reported properties of the membrane-bound enzyme and isolated ATPase (Harris & Slater, 1975; Magnussen & McCarty, 1976; Carlier & Hammes, 1979; Smith et al., 1983), even without an unlabeled ATP chase, a continued slow conversion of the bound ATP to bound ADP and P_i seems likely, driven by a preferential loss of the bound P_i. Measurements demonstrating that this is indeed the case are shown in Figure 4. The labeling and initial separation of the ATPase with tightly bound reactants was done as described with Figure 3, but the enzyme was kept for the indicated periods of time in the EDTA buffer mixture before separation and measurement of the tightly bound reactants. The results demonstrate a considerable loss of the bound ^{32}P over a 25-min period (Figure 4A), accompanied by only a slight loss of the bound ^3H label (Figure 4B). After more prolonged incubation, only bound [^{3}H]ADP is found on the enzyme after column separation. No [γ-^{32}P]ATP is released to the medium, and thus, the loss of the bound ^{32}P species does not occur by ATP dissociation but reflects conversion of the bound ATP to bound ADP and P_i and the slow loss of bound [^{32}P]P_i.

Discussion

Our results add to the accumulating evidence consistent with the binding change mechanism proposed for catalysis by the ATP synthase and F_1 ATPases. They also clarify the comparative roles of Mg^{2+} and Ca^{2+} as activators of hydrolysis, but they do not reveal why, under some conditions, reversal of bound ATP hydrolysis is observed with Mg^{2+} but not with Ca^{2+} activation. These and other facets of the results are considered here.

For our interpretation, we consider that the tightly bound [^{3}H]ADP found on the enzyme after [^{3}H]ATP hydrolysis is at catalytic sites. A recent paper from this laboratory (Feldman & Boyer, 1985) presents data supporting this view and summarizes earlier evidence. Although this view now seems adequately justified, the alternative possibility that the ADP is at a regulatory site that has weak catalytic activity but does not participate in rapid net ATP hydrolysis has not been conclusively eliminated and is still favored by some investigators.

Formation and Release of Bound ATP, ADP, and P_i. In our experiments following exposure of the CF_1 ATPase to [γ-^{32}P,^{3}H]ATP and separation of the enzyme from medium nucleotides and P_i, we found radioactive ATP, ADP, and P_i bound to the enzyme. This is consistent with the formation of an equilibrium mixture of bound reactants at a catalytic site. Similar levels and rates of release were observed with either Ca^{2+} or Mg^{2+}. Over a period of minutes, the P_i drops off, and the bound ATP is converted to bound ADP, which is then is slowly released. A similar behavior is found with the *Escherichia coli* enzyme (Wise & Senior, 1984) and is akin to the slow single-site catalysis observed with mitochondrial F_1 ATPase (Cross et al., 1982). Our results could be regarded as not in agreement with earlier experiments of Carlier and Hammes (1979). They exposed CF_1 ATPase to [γ-^{32}P,^{3}H]-ATP for 30 s and found about one [^{3}H]ADP but little or no ATP or P_i on their separated ATPase. However, they used column chromatography on Sephadex G-25 for their subsequent enzyme isolation with an unspecified time period. Under these conditions, there was likely time for most of the bound ATP to form bound P_i and for the P_i to dissociate. Thus, their results are not in conflict with our findings.

Our demonstration that the tightly bound ATP is converted to bound ADP points to the tight ADP binding site as a catalytic site. Support for this interpretation also comes from the demonstration (Figure 1) that this tightly bound ADP will form bound ADP when exposed to high medium P_i concentrations. Thus, the tightly bound [^{3}H]ADP formed by cleavage of medium [^{3}H]ATP has the same properties as the tightly bound [^{3}H]ADP obtained by prolonged exposure to medium [^{3}H]ADP in the experiments of Feldman and Sigman (1982) and Bruist and Hammes (1982).

Our interpretation that the tightly bound ADP is at a catalytic site differs from that of Bruist and Hammes (1982) and Hammes (1983), who favored the view that the tightly bound ADP is at a noncatalytic regulatory site. Our interpretation is in harmony with other recent findings. The labeling experiments of Abbott et al. (1984) give evidence that the tightly bound 2-azido-ADP or 2-azido-ATP labels the same site on the β subunit of the chloroplast ATP synthase. Other results (Hochman & Carmeli, 1981; Feldman & Boyer, 1985), showing that exposure to Ca^{2+} or Mg^{2+} can give rise to sluggish forms of the enzyme that can account for a slow release of ADP from catalytic sites, have eliminated the principal basis for the objections of Bruist and Hammes (1982) to the bound ADP being at catalytic sites. Bruist and Hammes (1982) and Hammes (1983) did recognize that their results could also be explained by participation of alternating catalytic sites. Subsequent results as presented and discussed here show that such recognition was appropriate.

The form of the enzyme with bound ADP present but with no bound ATP or P_i is likely not an intermediate in net catalysis with sufficient ATP present for positive catalytic cooperativity to occur. Under these circumstances, conformational changes accompanying ATP binding to another catalytic site promote formation of ADP and P_i and their release.

Differences in Oxygen Exchange with Ca^{2+} or Mg^{2+} Activation. Extensive oxygen exchange is observed during net ATP hydrolysis by heat- or trypsin-activated CF_1 ATPase at low ATP concentrations with Mg^{2+} but not with Ca^{2+} activation (Kohlbrenner & Boyer, 1983). A similar behavior is found with MF_1 ATPase; when the ATP concentration is reduced to below 1 μM with MF_1, over 400 reversals of bound ATP hydrolysis occur before the P_i formed is released (O'Neal & Boyer, 1984). Our experiments address a number of possible reasons for this lack of detectable exchange with CF_1 ATPase in the presence of Ca^{2+}.

One possible explanation for the lack of oxygen exchange with Ca^{2+} activation of the heat-activated ATPase is that once the P_i is formed at the catalytic site it is rapidly released. Our results show that this is not the case. Both P_i and ADP are found tightly bound at the catalytic site after net ATP hydrolysis with Ca^{2+} activation; the hydrolysis of bound ATP can be reversed, as shown by the formation of bound ATP from bound ADP at high concentrations of medium P_i in the presence of Ca^{2+}. Another explanation warranting consideration is that although an equilibrium of bound ATP with bound ADP and P_i is reached, the rates of the forward and reverse reaction are much slower with Ca^{2+} than with Mg^{2+}. However, this does not appear to be the case, as the concentration of bound ADP that is present at catalytic sites is nearly the same during steady-state hydrolysis with either Ca^{2+} or Mg^{2+} and similar levels of tightly bound ADP and ATP are found on the enzyme when net catalysis is stopped by removing medium nucleotides. The conversion of bound ATP to bound ADP during steady-state net hydrolysis must be at least as rapid as the net catalytic rate. If the reversal of this reaction were not about equally rapid, a higher steady-state level of bound ADP would be expected with Ca^{2+}. Also, when medium ATP is removed, little or no bound ATP remaining at catalytic sites would be anticipated. The presence of about equal amounts of bound ADP and ATP suggests that there is no marked difference in the rates of formation and cleavage of bound ATP in the presence of either Ca^{2+} or Mg^{2+}.

Under certain conditions, the phosphate oxygen exchange can accompany ATP hydrolysis in the presence of Ca^{2+}, as shown by our data with the octyl glucoside activated enzyme. This behavior may be related to the observation that the rate of ATP cleavage with the octyl glucoside activated form of the CF_1 ATPase is less with Ca^{2+} than with Mg^{2+} (Pick & Bassalian, 1983), in contrast to properties of the heat- or trypsin-activated enzyme with which the hydrolysis rate with Ca^{2+} is considerably higher than that with Mg^{2+}. Subtle but important conformational differences accompanying the two modes of enzyme activation could alter the catalytic properties of the bound cations. Some possibilities for how reversal of bound ATP cleavage may occur without a phosphate oxygen exchange are considered in the next section.

Possible Explanations for Lack of Phosphate Oxygen Exchange. Several possibilities warrant consideration for how reversible cleavage of bound ATP could continue without oxygen exchange when low concentrations of ATP are hydrolyzed in the presence of Ca^{2+} by the heat-activated enzyme. One way this could be accomplished would be if the oxygen displaced from P_i when ATP is formed does not mix with medium water. This would mean that the same oxygen that is incorporated into P_i when ATP is hydrolyzed would be somehow retained at the active site when ATP is re-formed. With continued reversal of bound-ATP cleavage, only one of the four oxygens of the bound P_i would be derived from water. Such events would occur if, during ATP hydrolysis, the water oxygen attacking the P atom of the γ-phosphoryl group is associated with Ca^{2+} and this same oxygen when present in the P_i remains bound to Ca^{2+} in a structurally restrained complex that does not allow exchange of the Ca^{2+}-liganded oxygen with water oxygen. This explanation is not attractive because Ca^{2+} is known to replace its oxygen ligands much more rapidly than is Mg^{2+}.

A second way that the oxygen exchange could be prevented would be if the bound P_i was restricted from rotating or tumbling at the active site so that oxygens of P_i are not randomized. Randomization of oxygens in a bound M^{2+}–P_i could occur if M–O bound in a bidentate complex opened randomly and then the resulting $-OPO_3$ bound in monodentate form rotated more rapidly than re-formation of the bidentate complex. There would then be one chance out of three that the bidentate would re-form with a different oxygen than that previously coordinated with the metal. If Ca^{2+} with its larger ionic radius formed a stable bidendate complex, oxygen exchange could be prevented. However, Ca^{2+} might not give a more stable bidentate complex because, as mentioned previously, it exchanges ligands with oxygen more readily than does Mg^{2+}. For example, Mg^{2+} makes and breaks its bonds to oxygen ligands as in ATP only about 10^3 times per second; in contrast, Ca^{2+} releases and re-forms its bond to oxygen at greater than 3×10^5 times per second (Baslo & Pearson, 1967; Eigen & Hammes, 1960; Vasavada et al., 1984).

We tend to favor another explanation for the differences in oxygen exchange in the presence of Ca^{2+} or Mg^{2+}. The dissociation constants of Mg^{2+} and Ca^{2+} for phosphates are about the same (Davies & Hoyle, 1953; Clarke et al., 1954). The more rapid M–O bond rupture with Ca^{2+} must thus be accompanied by more rapid bond formation. Consequently, the P_i bound with Mg^{2+} will spend longer periods of time in the unliganded state than P_i bound with Ca^{2+}. This longer time period, with only one or no ligands to the Mg^{2+}, could give adequate time for rotation and tumbling of the P_i, whereas with Ca^{2+} the rebinding after each release could be sufficiently rapid so as to prevent rotation or tumbling and thus oxygen exchange. With the octyl glucoside activated enzyme, the slower net hydrolysis rate may increase the probability of phosphate oxygen randomization, or the characteristics of the active site differ in a way that favors randomization.

Steady-State Level of Bound ADP during Net ATP Hydrolysis. During net [^{3}H]ATP hydrolysis in the presence of either Mg^{2+} or Ca^{2+}, a steady-state level of about one bound [^{3}H]ADP per mole of enzyme was found. This was demonstrated by inaccessibility of the nucleotide to an appropriate kinase. Such a relatively high level of bound nucleotide could result if during steady state the equilibrium of bound reactants is shifted considerably toward bound ADP at one occupied catalytic site. However, other data make this unlikely. When catalysis is stopped by rapid Sephadex column separation of medium [^{3}H]ATP from the enzyme, considerable bound [^{3}H]ATP is initially found on the enzyme (Table II, Figures 3 and 4). It is probable that such bound [^{3}H]ATP is present during the steady-state hydrolysis. If so, then to account for both the [^{3}H]ADP and [^{3}H]ATP binding, more than one catalytic site must be occupied during steady-state hydrolysis. That more than one catalytic site actively participates during net ATP formation by the ATP synthase was shown by Rosen et al. (1979). Sequential participation of at least two and probably three catalytic sites during net ATP formation by the intact synthase or during net ATP hydrolysis by the isolated CF_1 ATPase offers an attractive explanation for present findings.

Positive Cooperativity of Catalytic Sites with Ca^{2+} Activation. The occurrence of intermediate $P_i \rightleftharpoons HOH$ exchange when low concentrations of ATP are hydrolyzed was one of the early findings that suggested sequential participation of catalytic sites such that ADP and P_i release from one site was promoted by ATP binding at another site. The occurrence of such positive cooperativity of catalysis accompanied by negative cooperativity of substrate binding is well supported experimentally for the Mg^{2+}-activated mitochondrial and chloroplast ATPases in the presence of Mg^{2+} (Cross, 1981). With the octyl glucoside activated enzyme, positive catalytic cooperativity is readily evident with either Ca^{2+} or Mg^{2+}. Data in this paper support the occurrence of positive catalytic cooperativity with the heat-activated enzyme with Ca^{2+} present even though oxygen exchange is not found when low concentrations of ATP are hydrolyzed. The results best explained by such positive cooperativity are (a) the retention of bound ADP at the catalytic site during steady-state hydrolysis at ATP concentrations considerably below that for half-maximal velocity, (b) the retention of bound ATP, ADP, and P_i at catalytic sites for a short time period when medium nucleotides are removed, and (c) the accelerated release of such bound ADP and P_i when ATP and Ca^{2+} are added so that net ATP hydrolysis again occurs.

Thus, in the presence of either Ca^{2+} or Mg^{2+}, we believe that catalytic site cooperativity is occurring. However, under some conditions this cooperativity is not expressed by ATP modulation of oxygen exchange in the presence of Ca^{2+}. It is interesting to speculate that if no oxygen exchange accompanied the Mg^{2+}-catalyzed ATP synthesis or hydrolysis, the recognition of the binding change mechanism might have been considerably delayed.

Registry No. P_i, 14265-44-2; ATP, 56-65-5; ADP, 58-64-0; ATPase, 9000-83-3; O_2, 7782-44-7; Mg, 7439-95-4; Ca, 7440-70-2.

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