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Correlation between the Kinetics of Activation and Inhibition of Adenosinetriphosphatase Activity by Divalent Metal Ions and the Binding of Manganese to Chloroplast Coupling Factor 1†

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ABSTRACT: Optimal activity of adenosinetriphosphatase (ATPase) in coupling factor 1 was obtained as a function of divalent metal ion–ATP complex rather than of either free metal or free ATP, indicating that the complex is the true substrate. High concentrations of either the complex, the free ATP, or the free metal ion were competitive inhibitors of ATPase activity. This suggests that the complex is attached through at least two points of attachment to the active site, one through the metal ion and another possibly through the base of the nucleotide. Divalent metal ions that were the best activators were found to be the strongest inhibitors in their free forms. This is seen when the order of V_{max} with the various complexes ($MnATP^{2-} > MgATP^{2-} > CaATP^{2-}$) is compared to the order of K_i for competitive inhibition by the free ions ($Ca^{2+} > Mg^{2+} > Mn^{2+}$). Direct binding studies indicated that CF_1 has one tight and five loose sites for Mn^{2+} binding. Various di- or triphosphonucleotides and pyrophosphate induce the appearance of a second tight site and the disappearance of one of the loose sites. It is suggested that the two tight sites are at the active site of the enzyme for the following reasons. (1) The K_i (5 μM) for free Mn^{2+} as a competitive inhibitor of ATPase activity is similar to the K_d ($\sim$1 μM) of Mn^{2+} binding. (2) All the effectors which tighten the binding of Mn^{2+} are either substrates or competitive inhibitors of ATPase activity. (3) A change from pH 8 to pH 6.5 similarly increases both the K_d for binding Mn^{2+} and its K_i as a competitive inhibitor.

Coupling factor 1 from chloroplasts (CF_1)[1] was found to be directly involved in the terminal step of photophosphorylation. It also catalyzes ATP hydrolysis which is probably a result of the reversal of ATP synthesis. CF_1 is composed of five different subunits, designated α, β, γ, δ, and ϵ (Nelson, 1976). The smallest subunit, ϵ, acts as an endogenous inhibitor of the ATPase activity of CF_1 (Nelson et al., 1972). The active site is probably located on the β subunit since 7-chloro-4-nitro-2,1,3-benzoxadiazole which inhibits ATPase activity was found to be covalently bound to a tyrosyl residue on the β subunit (Deters et al., 1975). Identification of the active site also was sought through the measurement of substrate binding. The membrane-bound enzyme contains two ATP and one ADP (Harris & Slater, 1975). However, after separation from the chloroplast, only one molecule of ADP remains on the enzyme (Roy & Moudrianakis, 1971). This single ADP molecule is located on either the α or β subunit (Magnusson & McCarty, 1976). In the presence of Mg^{2+} or Ca^{2+}, the isolated enzyme contains two high-affinity sites for ADP, ATP, or the ATP analogue AMPPNP, with a dissociation constant of less than 10 μM (Cantley & Hammes, 1975). Three weak binding sites were also found. A model was proposed in which the high-affinity sites act as allosteric conformational switches for ATPase activity. However, it was found that at high concentrations of Mg^{2+}, nucleotides of wide specificity bind to two sites of isolated CF_1 (Banai et al., 1978). The inert Co(III)–phenanthroline–ATP complex which competitively inhibits ATPase activity also binds at two sites to CF_1 (Hochman et al., 1979). It was suggested that these two sites might be the active sites of the enzyme. The uncertainty in the identification of the active site requires the use of reagents other than nucleotides for additional information. Divalent metal ions are activators of ATP synthesis and hydrolysis catalyzed by CF_1 (McCarty, 1979). In preliminary work (Hochman et al., 1976), we have found that divalent metal ions which also are competitive inhibitors of ATPase activity bind tightly to isolated CF_1. Metal ions were therefore used as probes of the active site. Conclusions were drawn from the correlation between binding of divalent metal ions and their effect on the kinetics of ATPase activity in isolated CF_1.

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[1] Abbreviations used: CF_1, chloroplast coupling factor 1 (ATPase); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; AMPPNP, adenosine imidophosphate.

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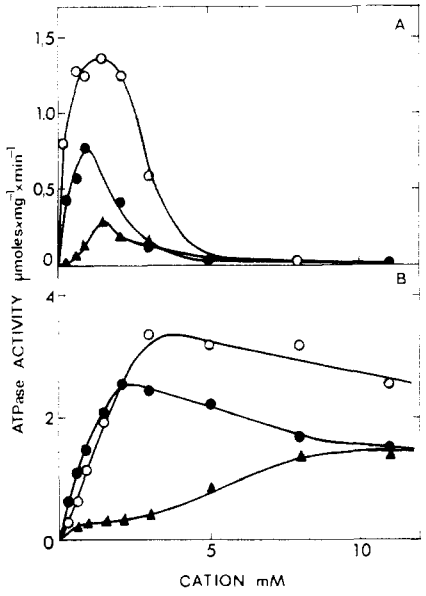

FIGURE 1: Effect of Ca^{2+} and Mn^{2+} on ATPase activity of CF_1. ATPase activity in heat-activated CF_1 was meausred in 40 mM Tris–Mes buffer at the various pH values as indicated in the presence of 3 mM ATP and of (A) various concentrations of $MnCl_2$ as indicated and (B) various concentrations of $CaCl_2$ as indicated at pH 6.5 (▲), 8 (○), and 9.5 (●).

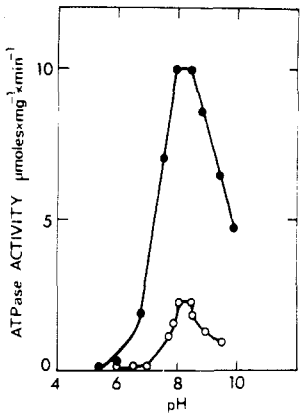

FIGURE 2: Effect of pH on Mn^{2+}-dependent and Ca^{2+}-dependent ATPase activity. ATPase activity of heat-activated CF_1 was measured in the presence of 5.4 mM $CaCl_2$ and 5.4 mM ATP (●) or 2.4 mM $MnCl_2$ and 14.4 mM ATP (○) at the various pHs as indicated. Experimental conditions were as described under Experimental Procedures except for the buffer which contained 40 mM Tris–Mes.

Experimental Procedures

Chemicals. AMPPNP, ATP, ADP, ADP-ribose, AMP, GDP, and CDP were purchased from Sigma Chemical Co., and phosphorylribose 1-pyrophosphate was from LP Chemicals. Highly labeled [^{32}P]ATP was prepared by phosphorylation of ADP as decribed (Avron, 1960). All other chemicals were the best available commercial grade, and all solutions were prepared with deionized double-distilled water.

CF_1 Preparation. CF_1 was prepared from lettuce leaves as described by Lien & Racker (1971) and was stored at 4 °C (Hochman et al., 1976). CF_1 was freed from $(NH_4)_2SO_4$ by passing on a Sephadex G-50 column (1 × 50 cm) with 40 mM Hepes–NaOH (pH 8) or with 40 mM Mes–NaOH (pH 6.5). CF_1 concentration was determined by the method of Lowry et al. (1951) assuming a molecular weight of 325 000 (Farron, 1970).

ATPase Activity. ATPase activity was measured following heat activation (Farron & Racker, 1970) in a reaction mixture containing 5 μg of CF_1, 60 μmol of Hepes–NaOH (pH 8) or 60 μmol of Mes–NaOH (pH 6.5), [^{32}P]ATP, and divalent metal ions as indicated in 1.5 mL at 37 °C as described (Hochman et al., 1976).

Mn^{2+} Binding. Binding of Mn^{2+} to soluble CF_1 was determined by electron paramagnetic resonance (EPR) method as described (Hochman et al., 1976). The assay solution contained 40–60 μM CF_1 in 40 mM Hepes–NaOH (pH 8) or 40 mM Mes-NaOH (pH 6.5) and various nucleotides as indicated. The binding constants of Mn^{2+} ions to CF_1 were determined by a Scatchard analysis, based on the EPR results (see eq 1). The data were fitted by a least-squares analysis to the equation by using the Maximum Likelihood principle (Rao, 1973) with the aid of a computer program MAXLIK (a subroutine package for Maximum Likelihood estimation by E. B. kaplan and R. C. Elston, University of North Carolina, June 1972) using a CDC 6600 computer.

Results

Heat-Treated CF_1-Catalyzed Divalent Metal Ion Dependent ATPase Activity. The activity increased with increasing concentrations of divalent ions when the concentration of ATP was kept at 3 mM (Figure 1). Both Ca^{2+} and Mn^{2+} inhibited ATPase activity beyond their optimal concentrations. Mn^{2+} was a stronger inhibitor of ATPase activity than Ca^{2+}. The V_{app} of ATPase activity was the highest at pH8. The concentration of the cation necessary for an optimal activity decreased with the increase in pH. This shift could be a result of stronger binding in higher pH. The pH dependence of ATPase activity is more clearly shown in Figure 2. Both Mn^{2+}- and Ca^{2+}-dependent ATPase activities had the same optimum at pH 8.2. The apparent low rates of the Mn^{2+}-dependent ATPase activity were due to the presence of relatively high concentrations of the free metal ions which are potent competitive inhibitors (Hochman et al., 1976). In other experiments where the concentrations of divalent cations were kept constant, high concentrations of free ATP also inhibited ATPase activity (not shown). The inhibition by free cations or by free ATP was similar to the inhibition previously observed in our study of ATPase activity in chromatophores from photosynthetic bacteria (Gepshtein et al., 1974). In analogy to the bacterial system, it is suggestd that the divalent cation–ATP complex is also the true substrate for ATPase in CF_1. For verification of this possibility and examination of the possible role of divalent cations, a system was set up in which the concentration of the complex, cation–ATP, was varied while the free ATP or free divalent cation concentrations were kept constant. The concentrations of the cation–ATP complex and its free components were calculated according to the stability constant determined for the various divalent cations (Tu & Heller, 1976).

ATPase activity was measured at various $MgATP^{2-}$ concentrations by keeping the concentration of free ATP constant at 13 and 20 mM (Figure 3). The highest apparent V_{max} was observed when ATPase activity was plotted as a function of the complex concentration rather than as a function of either divalent cations (Figure 1) or ATP concentration. As expected, free ATP was an inhibitor of the enzyme activity (Figure 3). Similar results were obtained when ATPase activity was measured at various $MnATP^{2-}$ or $CaATP^{2-}$ concentrations (not shown). For determination of the type of inhibition caused by free ATP and free cations, Lineweaver–Burk plots were used. The data were fitted to the rate equation by a least-squares analysis. The Lineweaver–Burk plot of the data indicated that free divalent cations and free ATP competitively inhibited the hydrolysis of the complex cation–ATP.

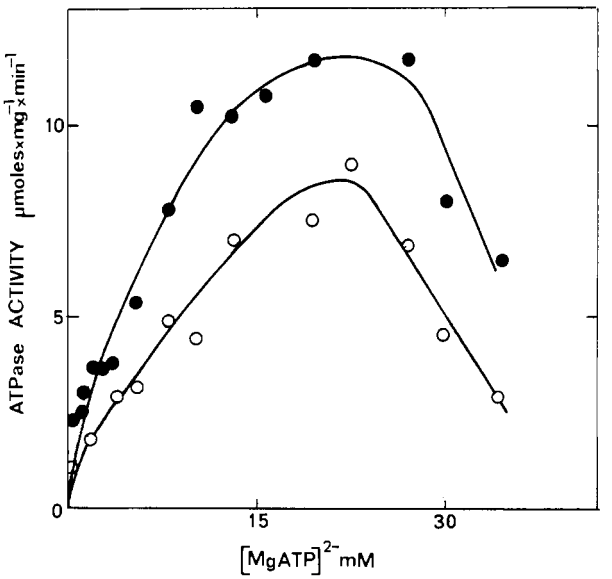

FIGURE 3: Dependence of the rate of ATPase activity on the concentration of $MgATP^{2-}$ complex. ATPase activity of heat-treated CF_1 was assayed at the indicated concentrations of $MgATP^{2-}$ while the concentrations of free ATP were 13 (●) and 20 mM (○). ATPase activity was measured at pH 8 as described under Experimental Procedures.

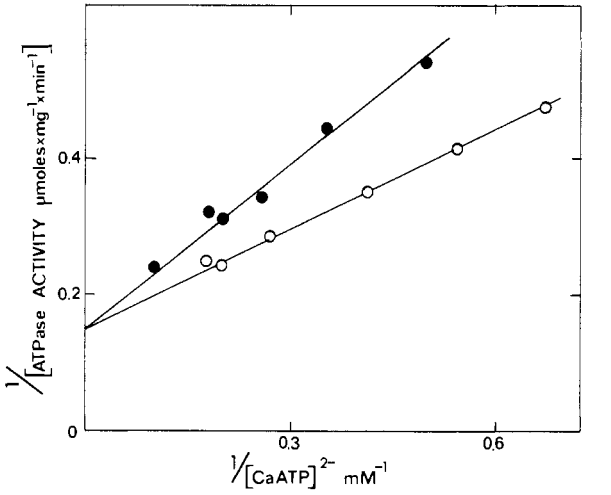

FIGURE 4: Dependence of the rate of ATPase activity on the concentration of $CaATP^{2-}$. ATPase activity in heat-activated CF_1 was measured at various concentrations of $CaATP^{2-}$ as indicated while the concentrations of free Ca^{2+} were 2 (○) and 7 mM (●). ATPase activity was measured at pH 8 as described under Experimental Procedures.

The competition by free Ca^{2+} is shown in Figure 4. Competition by other ions is not shown [but see Hochman et al. (1976)]. From the K_m apparent values obtained at two free ATP or free divalent cation concentrations, a K_m value for the complex, as substrate, and the K_i values for the inhibitors were determined.

In the case of Mn^{2+} or Mg^{2+}, which are potent inhibitors, it was more convenient to obtain the K_i for the metal ions from sets of experiments in which ATPase activity was assayed at two constant concentrations of free ATP. Analysis was done by a variation of the equation suggested by Dixon & Webb (1964):

$$v = \frac{V_{max}}{1 + \frac{K_m}{MATP}\left(1 + \frac{M_f}{K_{iMf}} + \frac{ATP_f}{K_{iATPf}}\right)} \quad (1)$$

where MATP is the metal–ATP complex, and M_f, ATP_f, K_{iMf}, and K_{iATPf} are the free metal, free ATP, K_i for the free metal, and K_i for the free ATP, respectively. Substituting MATP by K_d/ATP_fM_f, the following term is obtained:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_dK_m}{V(K_{iMf})(ATP_f)} + \frac{K_dK_m}{V}\left(\frac{1}{ATP_f} + \frac{1}{K_{iATPf}}\right)\left(\frac{1}{M_f}\right) \quad (2)$$

At a constant ATP_f, there is a linear relation between $1/v$ and $1/M_f$. Since at constant ATP_f variation in the MATP results in changes of the M_f, the slope of the lines in a $1/v$ vs. $1/M_f$ plot of the data depends on these two variations. However, at the intercept of the two lines obtained at two constant ATP_f, K_{iMf} equals M_f (Figure 5). The kinetic parameters of ATPase activity (at pH 8) are shown in Table I. All three divalent cations were found to be good activators of ATPase. The order of the V_{max} values for the various complexes as substrates was $MnATP^{2-} > MgATP^{2-} > CaATP^{2-}$. The K_i values for free cations as competitive inhibitors were in the following order: $Ca^{2+} > Mg^{2+} > Mn^{2+}$. Ca^{2+} is a weak inhibitor (K_i = 5–7 mM) while Mg^{2+} and Mn^{2+} were found to be good inhibitors

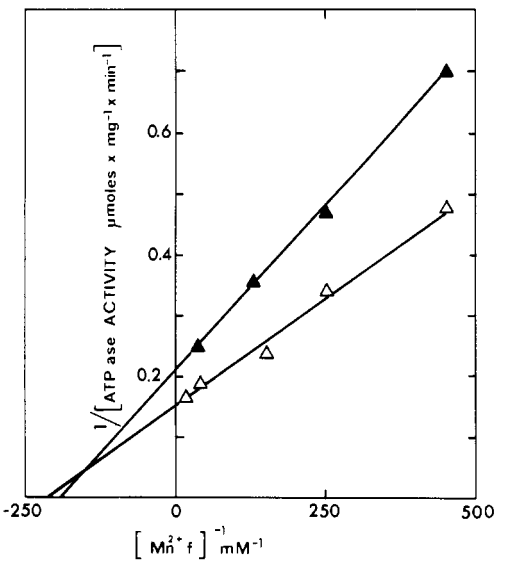

FIGURE 5: Determination of K_i for free Mn^{2+} from $1/v$ vs. $1/Mn^{2+}$ free plot. ATPase activity was measured in the presence of various concentrations of $MnATP^{2-}$ and in the presence of either 12 (△) or 26 mM (▲) free ATP. The concentration of free Mn^{2+} was calculated from the stability constants of the complex. The reaction was carried out at pH 8 as described under Experimental Procedures.

Table I: Kinetic Parameters of ATPase Activity of CF_1 at pH 8[a]

substrate	component in excess (mM)	K_m (mM)	K_i (ATP) free (mM)	K_i (cation) free (mM)	V_{max} [μmol (mg of protein)$^{-1}$ min^{-1}]
$CaATP^{2-}$	Ca^{2+}, 2 and 7	2.5 ± 0.2		7.0 ± 1.1	20 ± 1.8
$CaATP^{2-}$	ATP^{4-}, 2 and 7	2.3 ± 0.2	1.0 ± 0.2	5.0 ± 0.8	22 ± 1.6
$MgATP^{2-}$	ATP^{4-}, 13 and 20	2.0 ± 0.4	3.0 ± 0.4	0.020 ± 0.002	26 ± 2.9
$MnATP^{2-}$	ATP^{4-}, 12 and 26	2.0 ± 0.5	1.7 ± 0.4	0.005 ± 0.0004	36 ± 4.1

[a] CF_1 was activated as described under "Experimental Procedures". K_m, K_i, and V_{max} values were determined as described in the text from experiments similar to those shown in Figures 4 and 5 at pH 8.

Table II: Kinetic Parameters of ATPase Activity of CF_1 at pH 6.5[a]

substrate	component in excess (mM)	K_m (mM)	K_i (ATP) free (mM)	K_i (cation) free (mM)	V_{max} [μmol (mg of protein)$^{-1}$ min^{-1}]
$CaATP^{2-}$	$HATP^{3-}$, 2 and 9	15.2 ± 2.8	17.0 ± 2.5	5.0 ± 0.7	3.6 ± 0.3
$MnATP^{2-}$	$HATP^{3-}$, 2 and 15	9.2 ± 2.0	14.0 ± 2.8	0.045 ± 0.009	6.2 ± 0.7

[a] CF_1 was activated, and K_m, K_i, and V_{max} values were determined as described under Table I but at pH 6.5.

Table III: Inhibition of ATPase by Various Nucleotides and by Pyrophosphate[a]

inhibitor	K_i (mM)
$CaADP^{1-}$	0.180 ± 0.027
$CaGDP^{1-}$	1.050 ± 0.17
$CaCDP^{1-}$	1.380 ± 0.19
$CaAMPPNP^{2-}$	0.018 ± 0.003
$CaPP_i^{2-}$	0.350 ± 0.052

[a] Heat-treated CF_1 which was preincubated for 30 min with 1 mM of either ADP, CDP, or GDP, 1.5 mM pyrophosphate, or 0.2 mM AMPPNP. ATPase activity was assayed in the presence of various concentrations of $CaATP^{2-}$ in the presence of the same concentrations of the inhibitors at pH 8 as described under Experimental Procedures. The K_i values were calculated from plots of the data similar to Figure 4.

(K_i = 20 μM and 5 μM, respectively). The K_m values for the substrate and the K_i values for free ATP were similar (1–3 mM).

The kinetics of ATPase activity were also determined at pH 6.5 (Table II). As at pH 8, the V_{max} values for the substrate were $MnATP^{2-}$ > $CaATP^{2-}$. The K_i values for free cations were in the following order: Ca^{2+} > Mn^{2+}. Ca^{2+} is a weak inhibitor (K_i = 5 mM). Although there was no change in the K_i for Ca^{2+}, a 9-fold increase in the K_i for free Mn^{2+} and an 8-fold increase in the K_i of free ATP resulted from the decrease of the pH to 6.5. The decreased pH caused also an approximately 6-fold increase in the *Km* values. Changing the pH from 8 to 6.5 changed the kinetic parameters of the enzyme as expected (Figure 1) but did not change the mode of action of the enzyme. At high as well as the low pH, the complex of divalent cations–ATP was found to be the true substrate of the enzyme. Free ATP and free cations were found to be competitive inhibitors of the enzyme. It was found that those cations which were the strongest inhibitors in their free form were the best activators when in complex with ATP. With Ca^{2+}, which is the weakest inhibitor of ATPase activity, the lowest values of V_{max} were obtained, while with Mn^{2+}, which is the strongest inhibitor, the highest value for V_{max} was determined (Tables I and II).

Not only the free components of the substrate complex but also divalent metal complexes of various analogues of the substrate were inhibitory. Nucleotides, such as CDP, GDP, ADP, AMPPNP, and pyrophosphate, were found to be competitive inhibitors of ATPase activity (Table III). It seems that adenine nucleotides are better inhibitors of the ATPase than other nucleotides. The K_i values were in the following order: $CaCDP^{1-}$ > $CaGDP^{1-}$ > $CaPP^{2-}$ > $CaADP^{1-}$. Even pyrophosphate interacted with the active site and inhibited ATPase activity. A wide nucleotide specificity was observed for both synthesis and hydrolysis of ATP (Bennun & Avron, 1965; Nelson, 1976). This observation supports the assumption

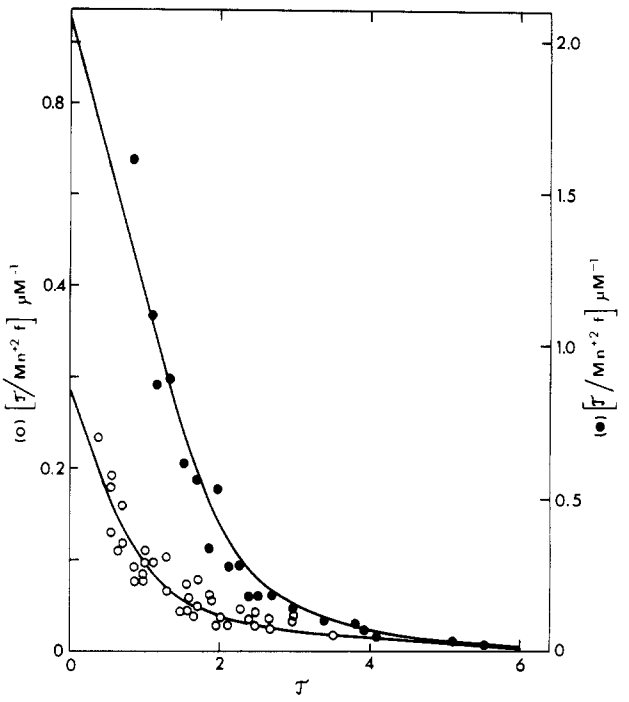

FIGURE 6: Binding of Mn^{2+} to CF_1. Binding of Mn^{2+} to CF_1 was measured in 40 mM NaHepes (pH 8) as described under Experimental Procedures. The number of moles of Mn^{2+} bound per mole of enzyme (τ) was plotted agianst the concentration of free Mn^{2+} (Mn^{2+}_f) divided by τ. Binding of Mn^{2+} to latent CF_1 was measured in the absence (○) and in the presence of 2 mol of ATP per mol of CF_1 (●). Theoretical binding curves were calculated by using eq 1 and the binding parameters shown in Table IV.

that the same site is involved in the catalysis of both reactions.

Mn^{2+} was the best activator of ATPase. It was also strongly bound to the enzyme since it had the lowest K_i as competitive inhibitor of ATPase activity when compared to other metal ions (Tables I and II). Therefore it could serve as a probe for ion binding to the active site of the enzyme. Indeed, in preliminary works, we have shown (Hochman et al., 1976, 1979) that Mn^{2+} binding to CF_1 can be measured by using EPR technique. Binding of Mn^{2+} ions was measured by titration, in the presence or absence of nucleotides, of a solution of 40–60 μM CF_1, which contained only one tightly bound ADP, with standard $MnCl_2$ solutions. The concentration of free Mn^{2+} ions was determined by using the amplitude of the EPR spectrum of Mn^{2+} ions. The binding constants of Mn^{2+} ions to CF_1 were determined by a Scatchard analysis based on the EPR results (Figure 6). These binding isotherms can be interpreted in terms of two mathematically equivalent, but conceptually different, methods: (a) interacting binding sites and (b) sets of independent noninteracting binding sites. The second model was arbitrarily utilized (Edsel & Wyman, 1958), and an attempt was made to verify if it fit the experimental data:

$$\frac{\tau}{[Mn^{2+}]} = \sum_{i=1}^{m} \frac{M_i}{K_{di} + [Mn^{2+}]} \qquad (3)$$

It was assumed that different sets of sites, with M_i sites in each set having a dissociation constant K_{di}, are related to the number of moles of Mn^{2+} bound per mole of CF_1 (τ) at a given concentration of free manganese ([Mn^{2+}]). The fit of the data to eq 1 by a least-squares analysis is shown in Figure 6. The values of M_i, the number of each type of site, were assumed to be integers. On the basis of this analysis, it was found that CF_1 has two sets of binding sites for Mn^{2+}, one set of tight binding sites and another set of loose binding sites. In the

Table IV: Parameters for Binding of Mn^{2+} to CF_1 at pH 8[a]

titration mixture (equiv of nucleotide or phosphate per mol of CF_1)	tight sites		loose sites	
	n	K_d (μM)	n	K_d (μM)
no additions	1	3.80 ± 0.35	5	188 ± 12
1 equiv of ATP	2	0.35 ± 0.036	4	52 ± 8
2–4 equiv of ATP	2	0.98 ± 0.10	4	50 ± 10
2 equiv of AMPPNP	2	0.98 ± 0.11	4	52 ± 9
2 equiv of AMP	1	2.96 ± 0.26	5	128 ± 28
2 equiv of AMP and 2 equiv of phosphate	1	6.06 ± 0.60	5	166 ± 24
1 equiv of ADP	2	0.30 ± 0.05	4	50 ± 10
2–5 equiv of ADP	2	0.83 ± 0.17	4	100 ± 14
2 equiv of ADP and 2 equiv of phosphate	2	1.43 ± 0.32	4	100 ± 28
2 equiv of ADP-ribose	2	1.20 ± 0.18	4	25 ± 8
2 equiv of GDP	2	2.00 ± 0.45	4	200 ± 20
2 equiv of CDP	2	2.20 ± 0.40	4	200 ± 20
2 equiv of pyrophosphate	2	2.50 ± 0.47	4	100 ± 28
2 equiv of phosphorylribose 1-pyrophosphate	2	0.63 ± 0.06	4	100 ± 15

[a] Mn^{2+} binding was measured as described in Figure 6. Each datum is a result of five titrations. Dissociation (K_d) constants were fit for the tight and loose sites to an integer number of sites (n) by a least-squares analysis as described under Experimental Procedures.

absence of added nucleotides, the enzyme has one tight and five loose binding sites. At pH 8, dissociation constants of 3.8 μM and 188 μM were fit for the tight and loose sites respectively (Table IV). Addition of 1 equiv of ATP per mol of CF_1 caused an appearance of a second tight site and a disappearance of one of the loose sites. Since the total number of sites was unchanged, it is possible that one of the loose sites underwent more than a 500-fold decrease in the K_d for binding of Mn^{2+} to be converted into a tight site. There was also a 10-fold decrease in the K_d for the loose sites. With 2 or more equiv, the number of each set of sites remained the same, but there was a 3-fold increase in the K_d of the tight sites compared to that obtained with only 1 equiv of ATP.

Essentially, the effect of conversion of the enzyme from a form that has one tight binding site to a form with two tight binding sites has a wide nucleotide specificity. Not only ATP but also AMPPNP, ADP, GDP, CDP, and ADP-ribose are effective. However, the base in the nucleotide was not enough to cause the change since AMP was not effective. On the other hand, a polyphosphate chain with at least two phosphates was essential since even pyrophosphate or 5-phosphorylribose 1-pyrophosphate was active in strengthening the binding of Mn^{2+} to CF_1 while phosphate had little effect. Kinetic evidence for the interaction of all these effectors with the enzyme can be drawn from the fact that all of them were competitive inhibitors of ATPase activity (Table III). ATP and ADP were more effective than GDP and CDP in inducing the relatively small changes of 2–3-fold decrease in the K_d of the four loose sites while pyrophosphate had an intermediate effect. It should be noted that a K_d of $\sim$1 μM for the two tight sites in the presence of ATP is not dissimilar to a K_i of 5 μM for competitive inhibition of ATPase activity by free Mn^{2+} ions.

The increase in the concentration of divalent metal ions required for optimal activity (Figure 1) and the increase in the K_m for the metal–ATP complex or the K_i of the free metal as a function of the decrease of the pH of the medium (Tables I and II) were also reflected as a change in Mn^{2+} binding. There was almost a 10-fold decrease in the K_d for Mn^{2+} binding of both the tight and the loose sites as a result of the change from pH 8 to 6.5 (Table V). Yet the enzyme contained five loose sites and one tight site at pH 6.5. ADP, ATP, and pyrophosphate caused an appearance of a second tight site and a disappearance of one of the loose sites. However, at pH 6.5, pyrophosphate was slightly more effective than the adenine nucleotides in decreasing the K_d for both the tight and the loose sites of Mn^{2+} binding. A striking similarity is seen between the K_d (70 μM) for Mn^{2+} binding to the two tight sites in the presence of ATP and the K_i (45 μM) of free Mn^{2+} as competitive inhibitor of ATPase activity at pH 6.5.

Table V: Parameters for Binding of Mn^{2+} to CF_1 at pH 6.5[a]

titration mixture (equiv of nucleotide per mol of CF_1)	tight sites		loose sites	
	n	K_d (μM)	n	K_d (μM)
no additions	1	44 ± 3.6	5	1300 ± 240
1.8 equiv of ADP	2	42 ± 3.0	4	600 ± 60
2.2 equiv of pyrophosphate	2	26 ± 3.2	4	143 ± 27
1.8 equiv of ATP	2	70 ± 5	4	500 ± 46

[a] Mn^{2+} binding was measured at 40 mM Mes–NaOH, pH 6.5, as described in Table IV.

Discussion

It was previously shown that ATPase activity is catalyzed by the same enzyme which takes part in ATP formation. Understanding of the mechanisms of ATPase activity could therefore contribute to the understanding of ATP synthesis. Although there were variations in the kinetic parameters of ATPase activity in pH 8 and 6.5 and with Mn^{2+}, Mg^{2+}, and Ca^{2+}, the data suggested that the same mechanism was involved with all cations at the various pH values. The complexes of Mn^{2+}, Mg^{2+}, and Ca^{2+} with ATP were the true substrates for ATP hydrolysis. Free ATP or free cations were competitive inhibitors of the enzyme. Free Mn^{2+} or Mg^{2+} were strong inhibitors of ATPase activity while Ca^{2+} was a poor inhibitor. At a relatively high concentration of free metal ion, CF_1 catalyzes an apparent Ca^{2+} ATPase only because this ion was a poorer inhibitor than free Mn^{2+} and Mg^{2+}. It was found that those cations which were the strongest inhibitors in their free forms were best activators when in complex with ATP. Indeed Mn^{2+} and Mg^{2+} are also best activators of ATP synthesis in the membrane system (Nelson, 1976).

On the basis of the analysis of the mode of inhibition of ATPase by free ATP or free cations, it was suggested that the substrate, a complex of ATP and dilvalent cations, was attached at least at two points to the active site. One point of attachment was through the cation in the complex and the other through part of the ATP molecule. Binding of the substrate through the two points was essential for the catalytic activity of CF_1 as an ATPase. Free ATP or free cations could bind directly to the active site and inhibit ATPase activity. The binding of Mn^{2+} or Mg^{2+} to the active site was strong while binding of free Ca^{2+} and ATP was weaker. The inhibition of ATPase activity at a high substrate concentration was also in harmony with this model. Binding of two complex molecules to the active site, at high substrate concentration, prevented the binding of the substrate through the two essential points of attachment and inhibited the ATPase activity of CF_1. ADP, GDP, CDP, AMPPNP, and even pyrophosphate, which can form complexes with divalent cations, were found to be competitive inhibitors of ATPase activity. Since the common denominator of all these compounds is a polyphosphate cation

complex, it was probably their interaction with the cations' binding site which inhibited the enzyme activity. That does not exclude the possibility that some of these compounds inhibit also by interacting through other parts of the complex. There are many indications that the base of adenosine and of some other nucleosides also interacts with the enzyme. Binding of free ATP to the active site is weak, as seen from the K_i values for free ATP as competitive inhibitor, which are of the same magnitude as the K_m values for the substrate.

Manganese ions were good possible probes of binding to the active sites of the enzyme since they were the best activators of ATPase activity; yet the K_i for competitive inhibition by free Mn^{2+} was the lowest among the tested divalent metal ions. CF_1 used for the binding studies had only one firmly bound ADP which could be removed only by partial irreversible denaturation of the protein. Since this enzyme had one tight and five loose sites for Mn^{2+} binding, it is tempting to suggest that the tight site was located at the bound ADP. Indeed addition of 1 or more equiv of ADP or ATP caused an appearance of a second tight site. However, there was no evidence that the Mn^{2+} binding site was at the bound ADP since the depleted enzyme was denatured. Furthermore, it was very difficult to relate the sites of Mn^{2+} binding to the multiple tight binding sites of nucleotides (Roy & Moudrianakis, 1971; Cantly & Hammes, 1975; Girault & Galmiche, 1977; Higashida & Mukohata, 1976; Carlier & Hammes, 1979). Indeed a variety of diphosphonucleosides and pyrophosphate, which induced tight binding of Mn^{2+}, were shown to be competitive inhibitors of the ATPase activity. Therefore, it is possible that if a formation of a tertiary complex between nucleotide, Mn^{2+}, and the enzyme causes the tight binding, these sites might be at the active sites of the enzyme. In other work, it was shown that the active site of ATPase is relatively not specific for nucleotides (Bennum & Avron, 1965) while a variety of phosphonucleosides (Banai et al., 1978) and pyrophosphate (Girault et al., 1973) were shown to bind to CF_1 in the presence of Mg^{2+}. The similarity between the K_d (~ 1 μM) for the two tight binding sites and the K_i (5 μM) for free Mn^{2+} ions as competitive inhibitors of ATPase activity gave further support for the suggestion that these sites might be at the active site of the enzyme. This correlation was supported by the fact that a change from pH 8 to 6.5 caused similar increases in both the K_d for binding of the tight sites and in the K_i for inhibition by free Mn^{2+} ions. The suggestion that these active sites were located at the two β subunits was in harmony with the finding of two tight sites for Mn^{2+} binding. It seems that a pyrophosphate group was an essential moiety of the various effectors since all di- and triphosphonucleosides tested, pyrophosphate, and ribose pyrophosphate were good effectors while inorganic phosphate and AMP did not change the binding constants. Therefore, binding of a pyrophosphate moiety to the Mn^{2+} in the divalent metal ion binding site might be sufficient to cause the major change which results in a conversion of one of the loose sites to a tight binding site.

In the presence of ADP, ATP, and pyrophosphate, up to a 3-fold decrease in the K_d of the loose binding was observed while the K_d for the tight sites was lower than that in the presence of GDP and CDP, which did not affect the loose sites. These differences could not be considered to be the result of the properties of ADP and ATP as allosteric effectors (McCarty et al., 1971; Nelson et al., 1972; Carmeli & Lifshitz, 1972) unless it is shown that pyrophosphate is also an allosteric effector. Yet the fact that 1 equiv of the effector per mol of CF_1 caused a change in two or more binding sites could indicate cooperativity interaction.

Both ATPase activity and phosphorylation catalyzed by the membrane bound enzyme show stricter specificity to divalent metal ions, a lesser degree of inhibition by the free components of the metal–nucleotide complex, and indications that this complex serves as the true substrate. It seems that these features become more pronounced as a result of the conformational change that occurs in the isolated protein. Yet it is not too far reaching to speculate that the catalysis in the chloroplast involves two active sites at which the complex is attached at least at two points, one through the metal and the other to the base in the nucleotide.

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