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Modulation by Divalent Metal Ions of the Autocatalytic Reactivity of Adenosinetriphosphatase from Chloroplasts†

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ABSTRACT: A nonlinear, pre-steady-state initial rate of ATP hydrolysis is obtained on the addition of a divalent metal ion–ATP complex to a heat-activated coupling factor 1 isolated from chloroplasts. The acceleration of the initial rate follows first-order kinetics. The observed first-order kinetic constant (k_{obsd}) changes with the concentration of the substrate, reaching half-maximal value at the K_m for ATP hydrolysis. Preincubation of the enzyme with divalent metal ions decreases the k_{obsd} from 1 to 0.04 s^{-1}. Saturation of the divalent metal ion effect was obtained at the micromolar range. It is suggested that the autocatalysis is a result of early stages in ATP hydrolysis which induce conformational changes in the enzyme. Binding of divalent metal ions in the absence of ATP slows down this change.

The CF_1[1] portion of the proton-translocating, reversible ATPase which catalyzes ATP synthesis in chloroplasts consists of five different subunits (Nelson, 1976). This membrane-bound enzyme is highly regulated, as seen from the fact that it does not catalyze ATP hydrolysis unless activated by an electrochemical potential of protons across the membrane in the presence of sulfhydryl reagents (Petrack et al., 1965). The state of activation is quenched by ADP and stabilized by phosphate (Carmeli & Lifshitz, 1972). The acquired reversibility is probably due to conformational changes induced by energization (Ryrie & Jagendorf, 1971). The effect of adenine nucleotides might also be conveyed through changes in conformation since they also increase the capacity of CF_1 to block proton leakage through the ATPase (McCarty et al., 1972).

The isolated enzyme is activated either by heat in the presence of sulfhydryl reagents (Farron & Racker, 1970) or by a mild tryptic digestion (Vambutas & Racker, 1965). Trypsin is suggested to cleave the sensitive ϵ subunit which was shown to act as ATPase inhibitor (Nelson, 1976). ADP acts as an allosteric inhibitor for ATPase activity (Nelson et al., 1972) while several other nucleotides prevent inactivation of the enzyme by cold (McCarty & Racker, 1966) and heat (Farron & Racker, 1970). It is possible that these regulative effects are due to conformational changes which affect the interaction among the various subunits in relation to the ϵ subunit. Subunit interactions are demonstrated by the fact that binding of subunit specific antibody (Nelson et al., 1973) and chemical modification in a single subunit (Deter et al., 1975; McCarty et al., 1972) caused conformational change in the enzyme. Slow changes in the K_i of Co^{3+}–phenanthroline–ATP complex (Hochman et al., 1979) and fluorescent changes on binding of ADP (Girault & Galmiche, 1977) are also indicators of conformational changes in the protein.

In preliminary reports (Carmeli et al., 1978, 1979), we showed that ATPase activity in isolated CF_1 goes through a transient state on addition of substrate. Similar results were obtained in ATPase from yeast mitochondria (Recktenwald & Hess, 1977). The data presented here indicate that the kinetic changes that take place during the early events in ATP hydrolysis could reflect conformational transformations of the enzyme which are expressed as various states of activity. It is suggested that one of these forms of the enzyme is a result of binding of divalent metal ions to the enzyme.

Experimental Procedures

Coupling factor 1 was prepared from lettuce (Romaine) chloroplasts according to the method used for spinach chloroplasts (Lien & Racker, 1971) and stored and prepared for use as indicated earlier (Carmeli et al., 1978). The protein concentration was determined according to the method of Hartree (1972). Following heat activation (Farron & Racker, 1970) the protein catalyzed ATP hydrolysis at a rate of 35 μmol mg^{-1} min^{-1}, indicating the high purity of the enzyme.

ATPase activity was measured spectrophotometrically by using the indicator cresol red for monitoring the acidification of the lightly buffered solution in Aminco DW-2 spectrophotometer equipped with a stopped-flow apparatus as described earlier (Carmeli et al., 1978).

Results

Kinetics of Acceleration of ATPase Activity. The reaction was initiated by mixing the enzyme (plus EDTA) with an equal volume of CaATP containing 0.1 mM free Ca^{2+} and a pH indicator. The decrease in pH quantitatively measures ATPase activity. The earliest ATPase activity is nonlinear with time. As was shown by Schreiber et al. (1979), such curvature can be analyzed if the logarithm of change of ve-

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[1] Abbreviations used: CF_1, coupling factor 1 from chloroplasts (ATPase); Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; AMPPNP, adenyl-5′-yl imidodiphosphate; EDTA, ethylenediaminetetraacetate.

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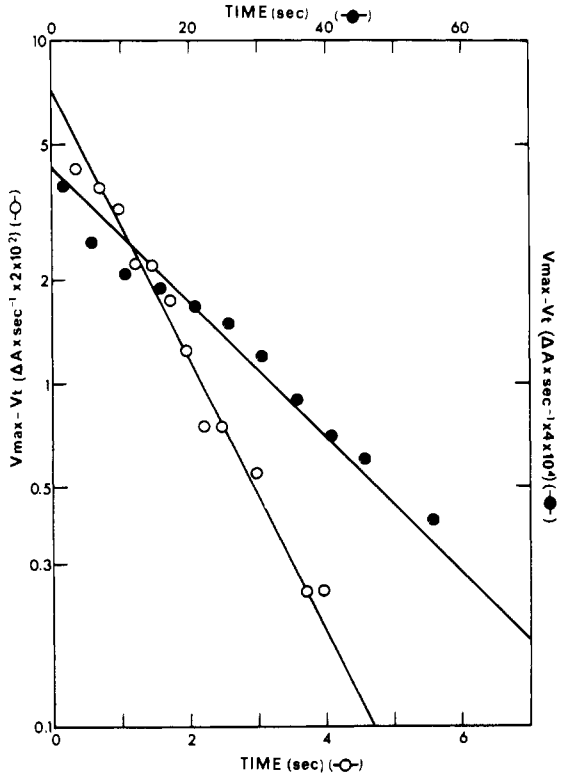

FIGURE 1: Determination of the observed rate constant for the pre-steady-state nonlinear initial rate of ATPase activity in CF_1. The change in the nonlinear initial rate of heat-activated ATPase was plotted semilogarithmically. ATPase activity was measured following preincubation either without (○) or with 0.1 mM $CaCl_2$ (●). The final reaction mixture contained 3 mM CaATP, 1 mM Tricine–NaOH (pH 8), 40 μg/mL CF_1 (heat activated), 50 μM EDTA, 25 μM cresol red, and 0.1 mM free Ca^{2+}.

locity is drawn as a function of time. The application of this treatment to the initial nonlinear kinetics of CF_1–ATPase is given in Figure 1. In this case, the acceleration had a k_{obsd} of 0.85 s^{-1}. If the enzyme was preequilibrated with the same concentration (0.1 mM) of free Ca^{2+} as was present in the assay mixture, the acceleration was much slower, and the k_{obsd} was 0.049 s^{-1} (note the difference in time scales of the two experiments).

As the enzyme preparation was purified and suspended in EDTA, it was free of Ca^{2+}. Thus it is concluded that the accelerated catalysis is an intrinsic property of the enzyme, while pretreatment with Ca^{2+} altered this property by slowing the rate of acceleration. Similar results were obtained (not shown) when the enzyme was preincubated with other divalent metal ions such as Ni^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+} and assayed in the presence of CaATP.

The effect of Ca^{2+} on the rate of acceleration was reversible (Table I). In this experiment, trypsin-activated CF_1 was suspended in a medium containing 50 μM EDTA. Ca^{2+} was added to a concentration of 100 μM and then back-titrated by EDTA. For each sample, the rate of acceleration was assayed. As seen, once the EDTA concentration was higher than the amount of free Ca^{2+}, the acceleration returned to the original constant.

These results are compatible with a mechanism assuming that in the presence of CaATP [which is the substrate (S)] the enzyme is converted from a state characterized by low catalytic (E′) activity to one with a high turnover (E″M) while Ca^{2+} acts as a ligand which converts the low turnover form to a third form with a very low activity (EM). Thus the conversion of EM to E″M is the rate-limiting process (see Scheme I).

Table I: Reversibility of the Effect of Ca^{2+} on the k_{obsd} of Acceleration of ATPase[a]

preincubation of CF_1		
first addition, 100 μM $CaCl_2$	second addition, EDTA (μM)	k_{obsd} (s^{-1})
none	none	0.27
+	none	0.025
+	10.0	0.037
+	43.3	0.25
+	76.6	0.26
+	110.0	0.26

[a] CF_1 (40 μg/mL) in 50 μM EDTA was first preincubated with 100 μM $CaCl_2$. Then EDTA at the indicated concentrations was added. Samples of the preincubated enzyme were assayed, and the k_{obsd} values were calculated as indicated in Figure 1.

Table II: Lack of AMPPNP Effect on the Rate of Acceleration of ATPase Activity in CF_1 Preincubated with $CaCl_2$[a]

preincubation of CF_1				
80 μM $CaCl_2$	AMPPNP (μM)	reaction [AMPPNP (μM)]	steady-state ATPase (μmol mg^{-1} min^{-1})	k_{obsd} acceleration (s^{-1})
none	none	none	5.5	0.38
+	none	none	5.46	0.039
+	50	25		0.034
+		50	3.5	0.037
+	100	50		0.039
+		100	2.1	0.026
+	200	100		0.026

[a] Heat activated CF_1 was preincubated with 50 μM EDTA and 80 μM $CaCl_2$ in the presence of AMPPNP as indicated and assayed for acceleration of ATPase activity as described under Figure 1 and for steady-state ATP hydrolysis in a pH meter under the same reaction conditions.

Effect of Substrate Analogues on the Rate of Acceleration. In order to check the above mechanism, we used the substrate analogue CaAMPPNP which was not hydrolyzed by the enzyme. This system of CF_1Ca^{2+} and AMPPNP was suitable to monitor the equilibration of CF_1 with the two ligands since it follows a strict equilibrium and was not upset by the rate of decomposition of E″MS to E″M and products. The enzyme (~70 μg/mL) was equilibrated with 80 μM Ca^{2+} and increasing AMPPNP concentrations (50–200 μM). After 10 min, the reaction was started by mixing the enzyme with an equal volume of CaATP. As a control, the enzyme in the absence of AMPPNP was mixed with CaATP and AMPPNP, giving the same final concentration of reactants as in the previous experiment. In the absence of Ca^{2+} in the preincubation mixture the apparent rate constant of acceleration was 0.39 s^{-1} (Table II) while in its presence it was 10 times slower. If AMPPNP were capable of shifting the equilibrium to the highest turnover form of the enzyme (E″), a marked increase in the rate of acceleration would have been expected. Furthermore, as AMPPNP is a competitive inhibitor, its interaction with the enzyme should have been expressed as a decrease in the maximal, steady-state rate of catalysis. The results summarized in Table II negate the possibility that AMPPNP could perturb (or shift) the equilibrium among the various forms of the enzyme. However, the decreased rate of catalysis as a function of the inhibitor concentration was evidence for the interaction of AMPPNP with the enzyme. However, a ligand concentration which inhibited the enzyme by more than 60% did not abolish or lower the ability of Ca^{2+} to affect the enzyme as estimated from the constant values

Table III: Effect of ADP on the Rate of Acceleration of ATPase Activity[a]

preincubation with CF_1		reaction (mM)			
$CaCl_2$	ADP	Ca^{2+} free	CaATP	CaADP	k_{obsd} (s^{-1})
none	none	0.25	3	none	0.430
0.2	1	0.35	3	0.50	0.041
0.2	none	0.35	3	0.50	0.037
none	none	0.35	3	0.50	0.090
0.2	0.1	0.35	3	0.05	0.047
none	none	0.35	3	0.05	0.500

[a] Heat-activated (40 μg/mL) CF_1 in 100 μM EDTA was preincubated in the presence of various combinations of $CaCl_2$ and ADP as indicated and assayed for ATPase activity as indicated under Figure 1 except for changes in CaATP concentrations as indicated.

of k_{obsd}. The possibility that the acceleration represents the dissociation of AMPPNP from the enzyme was made unlikely by the fact that the same rate of acceleration was measured in the absence or in the presence of AMPPNP. It is interesting to note that preincubation with Ca^{2+} did not alter the steady-state rate of ATPase activity.

A similar experiment was repeated with ADP instead of AMPPNP. As seen from Table III, 200 μM Ca^{2+} slowed the rate of acceleration to 10%, and inclusion of 1 mM CaADP in the preequilibration mixture failed to counteract this effect of Ca^{2+}. This interaction of CaADP with the active site also failed to shift the equilibrium between various forms of the enzyme. CaADP which was added to the assay mixture of an enzyme which has not been preequilibrated with Ca^{2+} did, however, affect the k_{obsd}. This effect was dependent on ADP concentration and might indicate that though ADP could not counteract the effect of Ca^{2+}, it interacted with the enzyme as a competitive inhibitor and prevented CaATP from accelerating the enzymatic activity.

Correlation of Acceleration with Hydrolysis of ATP. As neither AMPPNP nor ADP could substitute for ATP in favoring the high turnover form of the enzyme, it was suspected that only a hydrolyzable substrate could achieve this transformation, namely that the catalytic events are coupled to the transformation. Once the enzyme was converted to its active form, it could proceed in the same catalytic reaction only at a high turnover capacity. If this were the case, any intervention with the actual velocity of steady-state catalysis would be reflected in the rate of acceleration. For investigation of this possibility, the preequilibration step was omitted, and a heat-activated enzyme (supplemented with EDTA to ensure absence of free Ca^{2+}) was mixed in the stopped-flow mixing chamber with various concentrations of CaADP (0–0.3 mM) and CaATP (1–8 mM) in order to obtain a wide range of steady-state catalytic velocities. For each mixture, the rate of acceleration was calculated. The correlation between these parameters is given in Figure 2. It is evident that the rate of acceleration is a function of the rate of steady-state catalytic activity of the enzyme.

Another method for correlating these two variables was to modulate the steady-state catalysis by lowering the substrate concentration. Analysis of the data was obtained from drawing a double-reciprocal plot of $1/k_{obsd}$ with respect to 1/CaATP (not shown). From the slope of the linear relationship, the CaATP concentration which gave a half-maximal rate of acceleration was obtained. It was similar to the apparent K_m for the hydrolysis of ATP under a similar concentration of free Ca^{2+} (Hochman et al., 1976). However, no effect of substrate concentration on k_{obsd} could be seen when the enzyme was preincubated with Ca^{2+} (not shown).

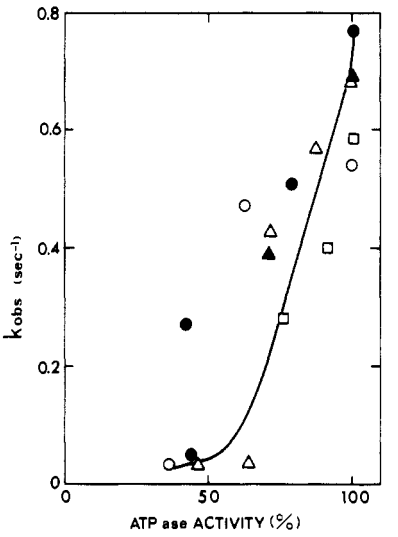

FIGURE 2: Correlation between k_{obsd} for acceleration and ATPase activity. For measurements of the rate of acceleration, ATPase activity was measured as described under Figure 1 while steady-state rates were measured under similar conditions by measuring changes in pH in a pH meter. The control rates of ATPase activity were taken as 100% in the presence of the following final concentrations of CaATP: 1 mM (○), 2 mM (●), 3 mM (△), 5 mM (▲), and 8 mM (□). The rates were varied by addition of 0–0.3 mM CaADP.

Discussion

The apparent accelerated catalysis of ATPase activity by CF_1 is a sum of two independent and experimentally distinguishable processes. One is measured as an acceleration of the initial velocity following addition of saturating amounts of substrate to the metal (M) free enzyme. The second mechanism affecting the observed rate of acceleration results from the interaction of M with the substrate-free enzyme. This is most clearly seen as a marked (20-fold) decrease in the rate constant of acceleration in an enzyme preequilibrated with M. A minimal formulation which can explain these regulative events distinguishes between three interconvertible forms of the enzyme having increasing catalytic reactivity: EM < E′M < E″M, with E″M having the highest turnover number (see Scheme I, where substrate (S) represents ATP and product (P) ADP and P_i). For simplicity the enzyme in its various states is represented as the enzyme divalent metal ion form (EM) since such a complex is formed whenever substrate-containing free M is added to the enzyme. However, it is assumed that a reversible dissociation of all ligands from the enzyme takes place. The transition E′ → E″ is effected only by ATP. Thus even ligands with high affinity to the ATP binding site such as ADP or AMPPNP are not sufficient to change the E′/E″ ratio. Furthermore, there is an apparent correlation between the rate of E′ → E″ transformation and the rate of ATP hydrolysis. Conditions under which the catalytic activity is either limited by subsaturating substrate concentration or competitively inhibited by ADP are correlated with a slow rate of acceleration. All these points suggest that the transformation of the enzyme overlaps the hydrolytic reaction of the enzyme as presented in eq 1 and 2.

Scheme I

$$EM + MS \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EMS \xrightarrow{k_3} E''M + MP \qquad (1)$$

$$E'M + MS \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} E'MS \xrightarrow{k_3'} E''M + MP \qquad (2)$$

$$E''M + MS \underset{k_2''}{\overset{k_1''}{\rightleftharpoons}} E''MS \xrightarrow{k_3''} E''M + MP \qquad (3)$$

It is assumed that the rate constant of acceleration calculated at infinite substrate concentration gives a k_3' of 1 s^{-1} (eq 2) for the E′ ⇀ E″ conversion. This should be compared to the much faster rate constant for catalysis of the activated enzyme having $k_3'' = 150\ s^{-1}$ (eq 3).

When the enzyme is preincubated with divalent metal ions in the absence of substrate, it is converted to the EM form (eq 4). The rate constant for acceleration measured on addition

$$E' + M \rightleftharpoons E'M \rightleftharpoons EM \qquad (4)$$

of substrate to the EM enzyme gives k_3 ($\sim 0.05\ s^{-1}$) (eq 1) which is also the rate-limiting step of the total process. It is reasonable to assume that the rate constants for ligand association and dissociation from the various forms of the enzyme do not greatly differ. Under such conditions, the fact that k_3 is 20-fold slower than k_3' should be reflected in a K_m which is 20-fold smaller than K_m'. Indeed within the range of sensitivity of our experimental procedure, the conversion (EM ⇀ E″M) of the metal enzyme form to an active state (eq 1) was independent of substrate concentration.

The saturation curve obtained for the dependence of k_{osbd} on the concentration of M during preincubation of the enzyme in the absence of ATP (saturation reached at $\sim 15\ \mu M\ CaCl_2$; Carmeli et al., 1979) also supports the assumed existence of an EM form of the enzyme (eq 1). Such a form should exist if the binding and dissociation constants of the E′M are relatively faster than the conversion of E′M to EM. These are reasonable assumptions since the E′M ⇀ EM transformation should involve conformational changes in the enzyme which can be slower than dissociation of ligands. If this were the case, the concentration of half-saturation is not equivalent to the dissociation constant of the E′M form. This could explain why even 10 mM free M in the presence of saturating amounts of substrate did not slow down the rate of acceleration beyond the effect of M as competitive inhibitor of ATPase activity. Indeed, the dissociation constant of Ca^{2+} from the active enzyme (measured as the K_i for competitive inhibition of ATPase activity by free Ca^{2+}) was 3 mM (Hochman et al., 1976) while $\sim 15\ \mu M\ CaCl_2$ sufficed to slow down the acceleration by formation of EM.

It should be noted that the added substrate (M-ATP) contains free M at a concentration (0.1–2 mM) which is saturating for the formation of EM (saturation concentration $\sim 15\ \mu M$). When both the substrate and free M are at saturation, the highest rate constant for acceleration is observed, thus indicating that $k_3 < k_3'$. It seems, however, that at subsaturation of the substrate there is an increase in the rate of EM formation which results in a decreased k_{obsd} for acceleration. Quantitatively, there is a linear relation between ATPase activity which is a function of the degree of saturation and the k_{obsd} for acceleration down to about ~50% activity. This is shown in experiments where the activity is controlled by the competitive inhibitor ADP. Below ~50% saturation, the rate of EM formation becomes faster than the rate of E″M formation since k_{obsd} for acceleration equals k_3 and becomes independent of substrate concentration. This line of reasoning is supported by the observed dependence of the k_{obsd} of acceleration on substrate concentration. Here, too, variations were observed between k_{obsd} of 1 s^{-1} at infinite substrate concentrations and a low k_{obsd} at low substrate concentrations which approaches the k_{obsd} obtained with an enzyme which was preincubated with divalent metal ions.

When ADP is used to modify the rate of activity, there is a good correlation between k_{obsd} and the rate of activity, only down to 50% activity. It is possible that under conditions where ADP/ATP ratio is high (resulting in low activity) ADP acts as an allosteric effector of the enzyme, reducing the k_{obsd} beyond its effect on accessibility of substrate to the active site. Indeed we have previously shown that the action of ADP as a negative effector of ATPase activity is counteracted by ATP with a 4-fold higher K_i value (Carmeli & Lifshitz, 1972). This finding is also in agreement with the observed increase in the lag of ATPase activity caused by addition of ADP (Recktenwald & Hess, 1979; Roveri et al., 1980).

Alternatively it can be suggested that binding of the divalent metal ion–triphosphonucleotide complex to the enzyme causes a conformation change which activates the enzyme. The wide nucleotide specificity and the similarity in the concentration dependence to the K_m for catalysis could indicate that binding to the active site of the enzyme causes a change in reactivity through a cooperativity effect. However, the lack of an effect by the analogue AMPPNP on the k_{obsd} of acceleration would have been difficult to explain.

The effect of divalent metal ions in decreasing the k_{obsd} for acceleration could be another expression of the observed acceleration of the decay of the light-activated state of ATPase by Mg^{2+} ions in situ (Bakker-Grunwald & Van Dam, 1974). The nature of the effects of divalent cations and ATP and their effective concentrations are within the limits found in the intact chloroplast. Furthermore, it is well-known (Krause, 1977) that upon light–dark transition the stroma's Mg^{2+} concentration varies as a result of ion transport through the thylakoid membrane. Thus it seems possible that these variations in divalent metal ions and metabolites might alter the concentration of the high turnover form of CF_1. It was found (Inue et al., 1978) that light activates ATPase in intact chloroplasts even in the absence of any added SH reagent. It is possible that on turning off the light, Mg^{2+} ions will slow the process of acceleration of the potentially activated enzyme to steady-state catalysis while the ADP formed will increase the decay of the activated state, thus stopping the hydrolysis of the ATP formed in the light.

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