RELATIONS BETWEEN DIVALENT CATION BINDING AND ATPase ACTIVITY IN COUPLING FACTOR FROM CHLOROPLAST

Y. HOCHMAN, A. LANIR and C. CARMELI

Department of Biochemistry, The George S. Wise Center for Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel

Received 28 November 1975

1. Introduction

Coupling factor from chloroplast (CF_1) was found to be directly involved in the terminal step of photophosphorylation. CF_1 also catalyzes ATP hydrolysis which is probably a result of the reversal of ATP synthesis. However, this enzymatic activity is latent whether the protein is bound to the chloroplast membrane or isolated in a purified soluble form. Several methods for the unmasking of ATPase activity were devised [1–4].

The catalysis of ATP hydrolysis by activated CF_1 was found to be dependent on divalent cations [2,5]. Based on kinetic analysis of ATPase activity in photosynthetic bacterial chromatophores we have suggested that the cation–ATP complex is the true substrate for ATPase activity [6]. This kinetic work is extended here in order to probe the mode of action of ATPase in purified CF_1. The kinetic data indicated that the cation–ATP complex was also substrate for ATPase activity in CF_1. Based on the analysis of inhibition it is suggested that the complex is attached at least at two points to the enzyme. One point of attachment is through the cation in the complex and the other through part of the ATP molecule. The measurements of cation binding were of great relevance to the understanding of the mode of action. Direct binding of Mn^{2+} ions as measured by the e.p.r. method indicated two tight and three loose cation binding sites. Based on the interpretation of the kinetic and binding data, suggestions were made as for the possible mode of substrate and inhibitors binding to the active site. The results were compared to measurements of nucleotide binding to CF_1 [7–9].

2. Materials and methods

Coupling factor from chloroplast (CF_1) was prepared from lettuce leaves as described by Lien and Racker [10] and was stored at 4°C in a solution containing 2 M $(NH_4)_2SO_4$, 2 mM EDTA, 1 mM ATP and 20 mM Tris–H_2SO_4 (pH 7.1). CF_1 was freed from $(NH_4)_2SO_4$ by passing it on a Sephadex G-50 column (1 × 25 cm) with 40 mM HEPES–NaOH (pH 8). CF_1 concentration was determined by the Lowry method [11], assuming a mol. wt. of 326 000 (12).

CF_1 was heat activated as described by Farron [12]. ATPase activity was assayed in a reaction mixture containing: 5 μg CF_1, 60 μmol HEPES–NaOH (pH 8), [^{32}P]ATP (containing 3 × 10^6 c.p.m.) and divalent cations as indicated in 1.5 ml at 37°C. The reaction was started by addition of the enzyme and was stopped after 10 min by the addition of cold TCA to a final concentration of 5%. The released [^{32}P]K_2HPO_4 was separated by the isobutanol-benzene procedure [13]. Highly labeled [^{32}P]ATP was prepared by phosphorylation of ADP according to the method of Avron [14].

Binding of Mn^{2+} ions to soluble CF_1 was determined by the electron paramagnetic resonance (e.p.r.) method. Concentration of free Mn^{2+} ions in samples containing the enzyme were determined from the amplitude of the e.p.r. spectrum of manganese, by comparison with the signal amplitude of $MnCl_2$ standard solutions. This was possible since the complex of Mn^{2+} with CF_1 was found to present extremely broad e.p.r. spectrum in the x band frequency region. The samples were contained in a 50 μl capillary tube maintained at 25°C. Spectra were obtained on a Varian E-3 spectrometer.

The binding constants of Mn^{2+} ions to CF_1 were determined by a Scatchard analysis based on the e.p.r. method.

3. Results

Heat activated CF_1 catalyzes a divalent cation dependent ATPase activity. As shown in fig.1 the activity of ATPase increased with increasing the concentration of divalent cations when the concentration of ATP was kept at 3 mM. Of all the tested cations the highest activity was measured in the presence of 4 mM $CaCl_2$. At low concentrations of divalent cations the rate of ATPase activity increased in the following order: $Mn^{2+} > Mg^{2+} > Ca^{2+} > Co^{2+}$ (fig.1). All divalent cations inhibited ATPase activity at high concentrations. In other experiments where the concentrations of divalent cations were kept constant, high concentration of free ATP also inhibited ATPase activity.

The inhibitions by free cations or by free ATP were similar to those previously observed in our study of ATPase activity in chromatophores from photosynthetic bacteria [6]. In analogy to the bacterial system it was suggested that the cation–ATP complex is also the true substrate for ATPase in CF_1. In order to verify this possibility and to examine the possible role of divalent cations, a system was set up in which the concentration of the complex, cation–ATP was varied keeping free ATP or free cation at constant excess. Concentrations of the cation-ATP complex and its free components were calculated according to the stability constant-determined by Wallas [15].

Highest apparent V (table 1) were observed when ATPase activity was plotted as a function of the complex concentration rather than a function of either divalent cations (fig.1) or ATP concentrations. In order to determine the type of inhibition caused by free ATP. ATPase activity was measured at various concentrations of $(Mn–ATP)^{2-}$ keeping the free

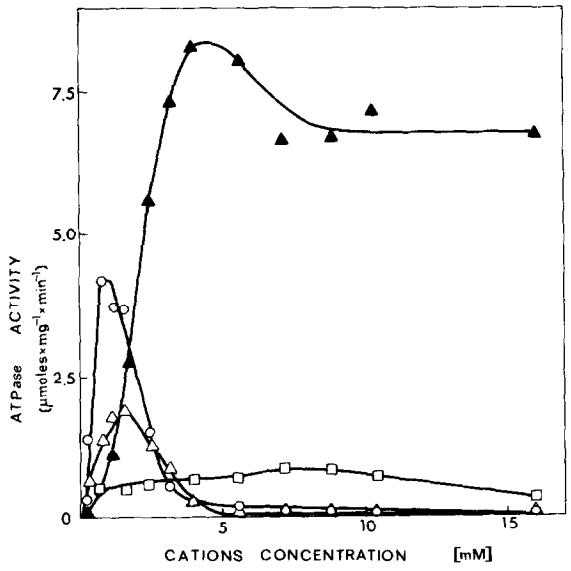

Fig.1. Effects of divalent cations on ATPase activity of CF_1. CF_1 was heat activated as described by Farron [12]. The reaction mixture and assay condition were as described under Materials and methods. The concentration of ATP was 3 mM with the indicated concentration of $MgCl_2$ (△); $CaCl_2$ (▲); $MnCl_2$ (○); $CoCl_2$ (□).

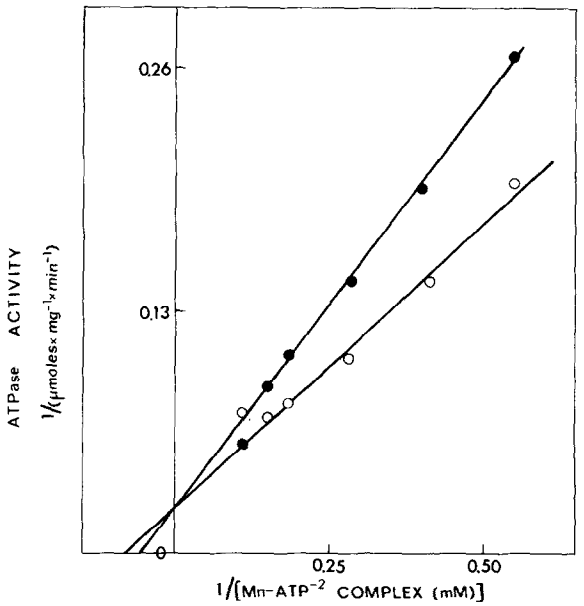

Fig.2. Dependence of the rate of ATPase activity on the concentration of $Mn–ATP^{2-}$ complex. CF_1 was heat activated as described by Farron [12]. Concentration of the $Mn–ATP^{2-}$ was varied as indicated while the concentrations of free ATP were: 15 mM (○) and 25 mM (●).

Table 1
Kinetic parameters of ATPase activity of CF_1

Substrate	Component in excess (mM)	K_M (mM)	K_i ATP (mM)	K_i cation (mM)	V_{app} (μmole X mg^{-1} X min^{-1})	V (μmole X mg^{-1} X min^{-1})
$(Mn-ATP)^{2-}$	ATP^{4-}; 15 and 25	4	13	0.004	17	37
$(Mg-ATP)^{2-}$	ATP^{4-}; 13 and 20	2	–	0.020	16	18
$(Ca-ATP)^{2-}$	ATP^{4-}; 2 and 6	2.7	2	–	9	12
$(Ca-ATP)^{2-}$	Ca^{2+}, 2 and 7	2.5	–	7	9	10.5

CF_1 was activated as described in Materials and methods. K_M, K_i and V values were determined as described under Fig.2.

ATP concentrations constant at 12 and 25 mM. The Lineweaver and Burk plot (fig.2) of the data indicated that free ATP competitively inhibited the hydrolysis of $(Mn-ATP)^{2-}$. From the K_M apparent values obtained at two inhibitor concentrations a K_M value of 4 mM was determined for $(Mn-ATP)^{2-}$ complex and a K_i of 13 mM for free ATP. Free Mn^{2+} ions also competitively inhibited ATPase activity with K_i value of 4 μM. In a similar kinetic analysis the same type of competitions between other cation–ATP complexes and the free cations or the free ATP were found (table 1).

K_i values for Mg^{2+} and Ca^{2+} ions were found to be 20 μM and 7 mM respectively. The lack of inhibition of ATPase activity by excess of Ca^{2+} ions (fig.1) is explained by the relative high K_i value obtained for this ion. The V values for the hydrolysis of cation–ATP complexes by ATPase was: $Mn-ATP^{2-} > Mg-ATP^{2-} > Ca-ATP^{2-}$. The same order was observed for the rate of activity at low cation concentration (fig.1). The K_M values for all cation–ATP complexes as well as the K_i values for inhibition by ATP, were found to be at the milimolar concentration range.

A possible high affinity of cations to the catalytic site was inferred from the low K_i values obtained. Therefore, direct measurements of bindings of cation to CF_1 were performed. Binding of Mn^{2+} ions was measured by titration, in the presence of absence of nucleotides, of a solution of 50 μM CF_1 with standard $MnCl_2$ solution. The concentration of free Mn^{2+} ions was determined using the amplitude of e.p.r. spectrum of Mn^{2+} ions.

Two moles of nucleotides per mole of CF_1 were used in these experiments in order to prevent excess of free nucleotides in the assay solution. This ratio was used since it was found [9] that two nucleotides were tightly bound in the presence of divalent cations. Scatchard plots of the binding data (fig.3) show that CF_1 binds Mn^{2+} ions tightly at 2 sites and weakly at 3 sites (table 2). Addition of ATP or ADP increased the affinity of the tight sites without changing the number of binding sites. Heat activation of CF_1 did not significantly change the binding of Mn^{2+} ions to the enzyme. It should be noted that the tight binding constants of Mn^{2+} to ATPase measured in the presence of nucleotides by the e.p.r. method were similar to those obtained by inhibition measurements. Mg^{2+} and Ca^{2+} ions were found to compete with Mn^{2+} for binding to CF_1 (fig.4). As expected from the kinetic data, Mg^{2+} competed better than Ca^{2+} for the Mn^{2+} ions binding sites.

Table 2
Binding of Mn^{2+} to CF_1

Nucleotide	Number of Binding Sites	K diss. (μM)
–	(1,1)2 ± 0.25	9,20
	3 ± 0.5	53
ATP	2 ± 0.25	5
	3 ± 0.5	20
ADP	2 ± 0.25	1
	3 ± 0.5	58

The binding was measured by the e.p.r. method under the condition indicated in fig.3.

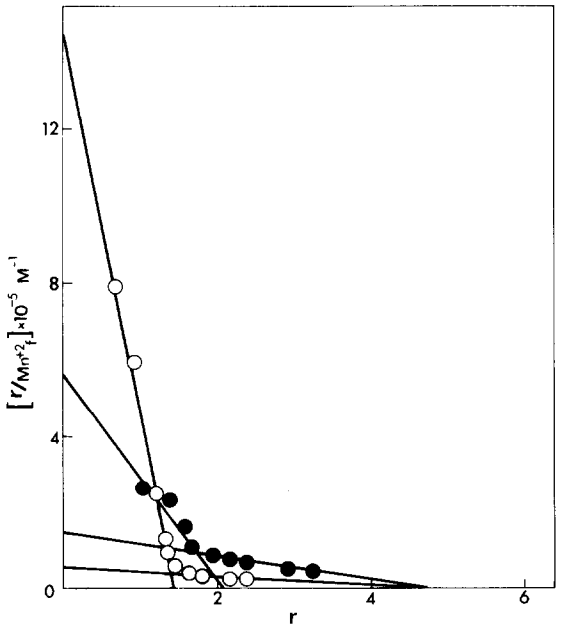

Fig.3. Scatchard plot of Mn^{2+} ions binding to CF_1. τ is the number of moles of Mn^{2+} binding per mole of enzyme. Binding was determined from the changes in the e.p.r. signal as described under Materials and methods. The assay solutions contained 50 μM CF_1, 40 mM HEPES (pH 8) and (•) 100 μM ATP or (○) 100 μM ADP.

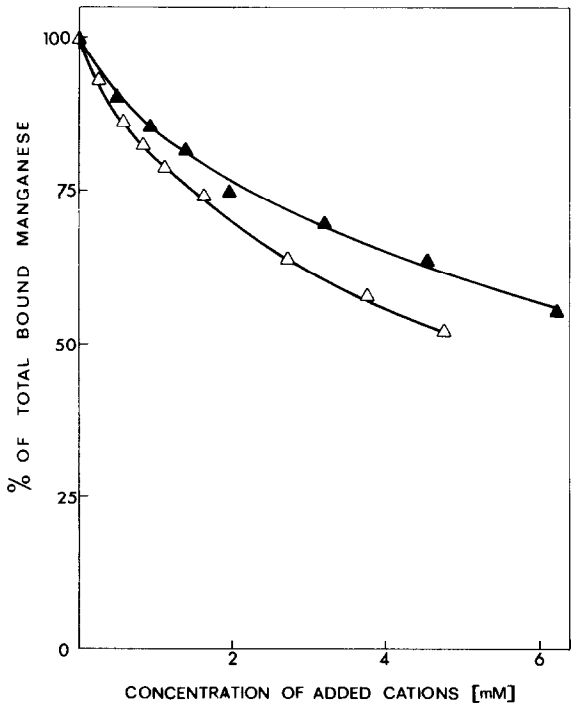

Fig.4. Effect of divalent cations on the release of Mn^{2+} ions from CF_1. A solution containing 90 μM $MnCl_2$, and 50 μM CF_1 was titrated with $MgCl_2$ (△) or $CaCl_2$ (▲).

4. Discussion

It was previously shown that ATPase activity is catalyzed by the same enzyme which takes part in ATP formation. Since ATP hydrolysis is probably the reversal of ATP formation, understanding of the mechanism of ATPase activity could contribute to the understanding of ATP synthesis. As was shown, CF_1 catalyzed ATPase activity in the presence of several divalent cations. For Mn^{2+}, Mg^{2+} and Ca^{2+} ions it was shown that the complex cation–ATP was the substrate for ATP hydrolysis and free ATP or free cations were competitive inhibitors for the complex. The similarity in the kinetic behaviour of ATPase activity with Mn^{2+}, Mg^{2+} and Ca^{2+} ions suggested that one protein was involved with all cations. The competitive inhibition by free cations suggests that the free divalent cations and the cation in the complex bind to the same site on CF_1. The proposed binding of divalent cation to CF_1 was verified by direct measurements of Mn^{2+} ions as determined by the changes in its e.p.r. signal.

It was shown that other divalent cations such as Mg^{2+} and Ca^{2+} compete with Mn^{2+} for the same site in CF_1. Since the activity of ATPase with cation–ATP complex was competitively inhibited by either free cations of free ATP and since it was shown that CF_1 has binding sites for divalent cations it is suggested that catalysis of ATP hydrolysis by CF_1 require attachment of the complex cation–ATP at least at two points to the enzyme. One of the sites is attached to the cation in the complex while the other site is attached to the ATP. Binding of free ATP or free cation to one of these sites inhibited competitively the activity. Using the e.p.r. method it was found that two Mn^{2+} ions were tightly bound to CF_1 with a binding constant similar to the K_i of the free Mn^{2+} ions as determined from the kinetic analysis of ATPase activity. Three additional loose binding sites of Mn^{2+} ions to CF_1 were also observed. The binding

sites of divalent cations were probably the same sites for ATP binding in the presence of divalent cations since measurements of nucleotide binding in the presence of Mg^{2+} and Ca^{2+} determined by equilibrium dialysis [9], indicated two high affinity and three low affinity sites with binding constants similar to those obtained for Mn^{2+} ions in the present work. The difference between the K_M values which were in the milimolar-range and tight binding sites which were in the micromolar range prompted the suggestion that the tight sites are regulating rather than catalytic binding sites [9]. The new information presented in this work induced us to suggest that the two tight binding sites were probably the catalytic site. This suggestion is based on the fact that the K_i values for the competitive inhibition between free cations and the complex cation–ATP was similar to both the dissociation constant of the Mn^{2+} ions as measured by the e.p.r. method and to the binding of nucleotides in the presence of divalent cations [9]. It is likely that the free divalent cations inhibited by preventing the cation–ATP complex from binding through the cation binding site which is proposed to be one of at least two binding sites of the complex required for catalysis. The other site is probably a lower affinity site which binds free ATP having a K_i in at the millimolar range. The K_M for catalysis is also at the millimolar range since it is determined by the low affinity site.

Since at least two subunits of the CF_1 protein are involved in catalysis [16] it is possible that binding to one of the sites will effect the binding to the second site in an allosteric manner. It is possible that the observed allosteric effects induced by nucleotides [5,17,18] could be affected through binding of a cation–nucleotide complex to the catalytic site. These might not require two points of attachment of the complex and therefore have low K_M values similar to those of the cation binding.

References

[1] Vambutas, V. K. and Racker, E. (1965) J. Biol. Chem. 240, 2660–2667.
[2] Farron, F. and Racker, E. (1970) Biochem. 9, 3829–3836.
[3] McCarty, R. E. and Racker, E. (1968) J. Biol. Chem. 243, 129–137.
[4] Petrack, B. and Lipman, F. (1961) Light and Life (McElroy, W. D. and Glass, H. B., eds.) pp. 621–630 Johns Hopkins Press.
[5] Nelson, N., Nelson, H. and Racker, E. (1972) J. Biol. Chem. 247, 6506–6510.
[6] Gepshtein, A., Hochman, Y. and Carmeli, C. (1974) Proceeding of the 3rd International Congress on Photosynthesis (Avron, M., ed.) pp. 1189–1197. Elsevier Scientific Publishing Company, Amsterdam.
[7] Roy, H. and Mandrianakis, E. N. (1971) Roc. Nat. Acd. Sci. 68, 2720–2724.
[8] Harris, D. A. and Slater, E. C. (1975) Biochem. Biophys. Acta 387, 335–348.
[9] Cantley, L. C. and Hammes, G. G. (1975) Biochem. 14, 2968–2975.
[10] Lien, S. and Racker, E. (1971) in: Methods in Enzymology (San Pietro, ed.) Vol. 13 pp. 547–555. Academic Press, New York and London.
[11] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[12] Farron, J. (1970) Biochem. 9, 3823–3828.
[13] Avron, M. (1960) Biochem. Biophys. Acta 40, 257–271.
[14] Avron, M. (1961) Anal. Biochem. 2, 535–543.
[15] Walaas, E. (1958) Acta. Chemica Scandinavica 12, 528–536.
[16] Deters, D. W., Racker, E., Nelson, N. and Nelson, H. (1975) J. Biol. Chem. 250, 1041–1047.
[17] Carmeli, C. and Lifshitz, Y. (1972) Biochem. Biophys. Acta 267, 86–95.