Modulation by Bicarbonate, Phosphate, and Maleate of the Kinetics of Adenosinetriphosphatase Activity and of the Binding of Manganese Ions to Chloroplast Coupling Factor 1†

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ABSTRACT: Bicarbonate, maleate, and phosphate were shown to modulate adenosinetriphosphatase (ATPase) activity in coupling factor 1 from chloroplasts. Kinetic analysis of the changes in the ratio between the apparent K_m with and without effectors indicated that the stimulation of the activity by bicarbonate was a result of a decrease in the K_m for $MnATP^{2-}$. The inhibition by phosphate resulted from a decrease in the K_i for free ATP as a competitive inhibitor at pH 8. The effectors did not change V_{max} at this pH. However, at pH 6.5, both K_m and V_{max} of ATPase activity with $MnATP^{2-}$ were changed by maleate, yet the mode of inhibition by free ATP remained unaltered. In addition to decreasing the K_m, bicarbonate induced a 10-fold decrease in the K_d for binding of Mn^{2+} at the two tight binding sites in the presence of ATP at pH 8. At pH 6.5, maleate also decreased both the K_m for $MnATP^{2-}$ and the K_d for Mn^{2+} binding. A decrease in the K_m of a substrate induced by an effector is likely to be a result of a decrease in the binding constant of the substrate. Therefore, these results are in harmony with the suggested assignment of the two tight binding sites of Mn^{2+} at the active sites of the enzyme.

ATPase activity catalyzed by thylakoid membranes was found to require Mg^{2+} ions (Petrack & Lipman, 1961), which are also required for photophosphorylation. However, the isolated CF_1[1] seemed to be activated by Ca^{2+}, while Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, and Sr^{2+} gave only 3% of the activity (Vambutas & Racker, 1965). Yet, allosteric effectors such as maleate and bicarbonate modulated the soluble enzyme to catalyze appreciable rates of ATPase activity in the presence of Mg^{2+} (Nelson et al., 1972). The observed optimum curves for various divalent metal ions (Hochman et al., 1976; Posarski & Jagendorf, 1976) indicate that depending on the concentration, these ions are both activators and inhibitors of ATPase activity in CF_1. Thus, at low concentrations of ions, the V_{app} was in the following order: $Ca^{2+} > Mn^{2+} > Mg^{2+}$; the calculated V for the divalent metal ion–ATP complexes as substrates was in the following order: $MnATP^{2-} > MgATP^{2-} > CaATP^{2-}$. The fact that the K_i for free Mn^{2+} and Mg^{2+} ions was at the micromolar concentration range while that for free Ca^{2+} was at the millimolar concentration range (Hochman et al., 1976) explains why the former ions, which are better activators, gave lower apparent activities under experimental conditions where the free cations were in the millimolar concentration range. Similar to previous findings in *Chromatium* chromatophores (Gepshtein et al., 1974) and in F_1 isolated from these membranes (Gepshtein & Carmeli, 1977), both the free divalent metal ions and the free ATP were found to be competitive inhibitors while the divalent metal ion–ATP complex gave the highest activity of ATPase in CF_1 (Hochman et al., 1976). We therefore suggested a model in which the true substrate is the metal ion–ATP complex bound at least at two points to the active site. One point of attachment is through the metal in the complex. This is the site for the competitive inhibition by free metal ions. A second site of attachment is probably through the base in the nucleotide where the competition with the free nucleotides causes an inhibition of ATPase activity. Indeed, analogues of the substrate such as the inert Co(III)–phenanthroline–ATP were found to competitively inhibit ATPase activity in CF_1 (Hochman et al., 1979). The complex which binds at two sites to the enzyme also caused the release of bound Mn^{2+} from CF_1.

We have suggested that the two tight sites for Mn^{2+} binding to CF_1 could be at the active sites of the enzyme (Hochman & Carmeli, 1981). This suggestion was based on the observations that (1) the K_d for Mn^{2+} binding was similar to its K_i as competitive inhibitor of ATPase activity and (2) the specificity of triphosphonucleotides as substrates or of diphosphonucleotides as competitive inhibitors was similar to their specificity as agents which decreased the K_d of binding of Mn^{2+} to CF_1. Further support for this suggestion can be drawn from the data presented in this work, which show that allosteric effectors cause a similar change in the K_m of $MnATP^{2-}$ as substrate and in the K_d of the binding of Mn^{2+} to CF_1.

Experimental Procedures

Materials. ATP, ADP, Hepes, and Mes were obtained from Sigma. [^{32}P]ATP was obtained by photophosphorylation of ADP in the presence of ^{32}P as described (Hochman et al., 1976). 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). Samples of CF_1 were stored at 4 °C as described (Hochman et al., 1976). Before use, CF_1 was desalted 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 Lowry et al. (1951) method, assuming a molecular weight of 325 000 (Farron, 1970).

ATPase Activity. ATPase activity was measured following heat activation (Farron & Racker, 1970) in the presence of

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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.

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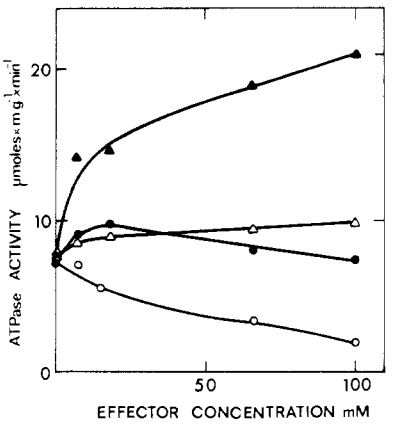

FIGURE 1: Effect of maleate and bicarbonate on ATPase activity in CF_1. ATPase activity of heat-treated CF_1 was measured in 40 mM Hepes–NaOH (pH 8) as described under Experimental Procedures. The reaction mixture contained (▲) 18 mM ATP and 6 mM $MnCl_2$, with bicarbonate as indicated, (△) 12.8 mM ATP and 0.8 mM $MnCl_2$, with maleate as indicated, (●) 8 mM ATP and 6 mM $CaCl_2$, with bicarbonate as indicated, and (○) 2.8 mM ATP and 0.8 mM $CaCl_2$, with maleate as indicated.

40 mM Hepes–NaOH (pH 8) or 40 mM Mes–NaOH by measuring the release of ^{32}Pi from [P^{32}]ATP as described (Hochman et al., 1976).

Binding of Mn^{2+} to soluble CF_1 was measured by the electron paramagnetic resonance (EPR) method in 40 mM Hepes–NaOH, pH 8, or in 40 mM Mes–NaOH, pH 6.5, as described (Hochman et al., 1976). Various nucleotides and effectors were added to the enzyme, and their effect on Mn^{2+} binding to CF_1 was studied. CF_1 concentration was 40–60 μM.

Results

The effect of maleate and bicarbonate at pH 8 on ATPase activity of CF_1 was dependent on the cation present in the reaction mixture. With Mn^{2+} (Figure 1), as with Mg^{2+} (not shown), bicarbonate accelerated ATPase activity. Maleate also activated ATPase with Mn^{2+}. In the presence of Ca^{2+}, bicarbonate was found to be a weak activator while maleate was an inhibitor of ATPase in CF_1. Phosphate was also found to be an inhibitor of ATPase in the presence of Mn^{2+} ions (see Figure 4). So that measurable rates of ATPase activity could be obtained, the concentrations of free Mn^{2+}, free Ca^{2+}, and ATP had to be varied (see legend to Figure 1) depending on the difference in the potency of the free cations as inhibitors and the variable effect of the effectors as activators of ATPase.

A better understanding of the stimulations and inhibitions was afforded by a detailed study of the changes in the kinetic parameters induced by the various effectors. Thus, the activity was measured with various concentrations of $CaATP^{2-}$ as substrate in the presence of maleate. A Lineweaver–Burk plot of the data indicated that maleate affected, at pH 8, the K_{app} of $CaATP^{2-}$ as substrate without changing the V_{max} (Figure 2). Increasing maleate concentration increased the K_{app} values for $CaATP^{2-}$. The same type of results was found when the effect of bicarbonate on ATPase activity, with $CaATP^{2-}$ as substrate, was studied. However, bicarbonate decreased the K_{app} values for $CaATP^{2-}$ without changing the V_{max} (Figure 4). In all cases, ATP remained a competitive inhibitor of ATPase activity. The effect of bicarbonate, phosphate, and maleate on the K_{app} of the substrates is shown in Figure 4. Increasing concentrations of the effectors changed the ratio between the K_{app} values, with or without effector, in a nonlinear fashion. High concentrations of the effectors saturated the system, as no further change in the ratio of the K_{app} values was observed. Even at saturating concentrations of the effectors, no change in V_{max} was observed. The interaction between the effectors and the enzyme was weak since half-saturation values between 10 and 30 mM were obtained depending on the effector used (Figure 4).

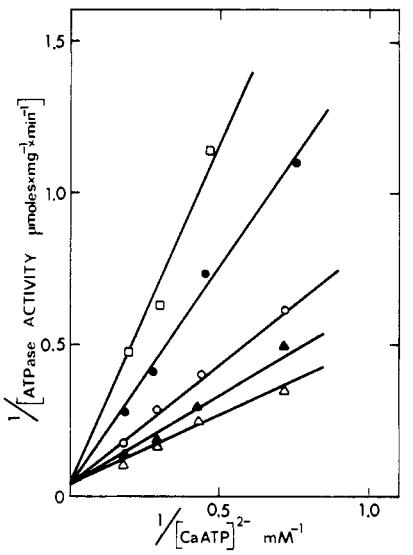

FIGURE 2: Effect of maleate on ATPase with $CaATP^{2-}$ as substrate. ATPase activity of heat-activated CF_1 was measured in 40 mM Hepes–NaOH (pH 8) as described under Experimental Procedures. The activity was determined with various concentrations of $CaATP^{2-}$, as indicated in the presence of 2 mM free ATP. The concentrations of maleate were (▲) 6.7 mM, (○) 16.7 mM, (●) 66.7 mM, (□) 100 mM and (△) without maleate. The concentration of the complex of ATP with Ca^{2+} and of free ATP was calculated from the total concentration of Ca^{2+}, ATP, and maleate on the basis of the association constants of ATP and of maleate with Ca^{2+} (Tu & Heller, 1976).

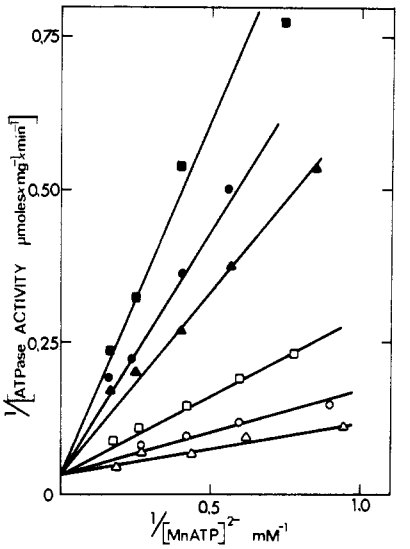

FIGURE 3: Effect of bicarbonate on ATPase activity with $MnATP^{2-}$ as substrate. ATPase activity of heat-activated CF_1 was measured in 40 mM Hepes–NaOH (pH 8) as described under Experimental Procedures. The activity was determined with various $MnATP^{2-}$ concentrations as indicated in the presence of (▲) 10 mM free ATP, (△) 10 mM free ATP and 100 mM HCO_3, (●) 15 mM free ATP, (○) 15 mM free ATP and 100 mM HCO_3, (■) 20 mM free ATP, and (□) 20 mM free ATP and 100 mM HCO_3. The concentration of the complex of ATP with Mn^{2+} and of free ATP was calculated from the total concentration of ATP, Mn^{2+}, and bicarbonate on the basis of the association constants of ATP and of bicarbonate with Mn^{2+} (Tu & Heller, 1976).

Changes in the apparent rates of activity could result not only from alteration in the K_m for substrate but also from changes in the K_{app} resulting from effector-induced changes of the K_i values for free ATP. For verification of this pos-

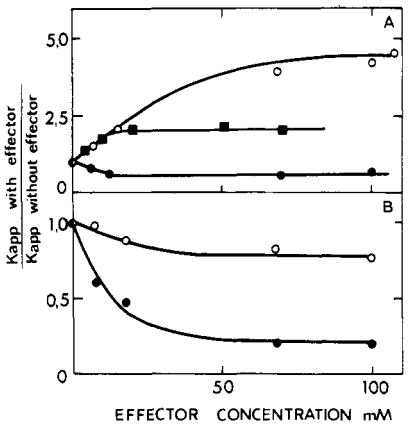

FIGURE 4: Effect of bicarbonate, phosphate, and maleate on the K_{app} values of $CaATP^{2-}$ and $MnATP^{2-}$ as substrates of CF_1. ATPase activity of heat-activated CF_1 was measured as described in Figure 3. The K_{app} ratio was calculated from the K_{app} values obtained with and without effector in the presence of the following: (A) (○) 2 mM free ATP and various concentrations of $CaATP^{2-}$, with maleate as indicated; (●) 2 mM free ATP and various concentrations of $CaATP^{2-}$, with bicarbonate as indicated; (■) 10 mM free ATP and various concentrations of $MnATP^{2-}$, with phosphate as indicated; (B) (○) 12 mM free ATP and various concentrations of $MnATP^{2-}$, with maleate as indicated; (●) 12 mM free ATP and various concentrations of $MnATP^{2-}$, with bicarbonate as indicated.

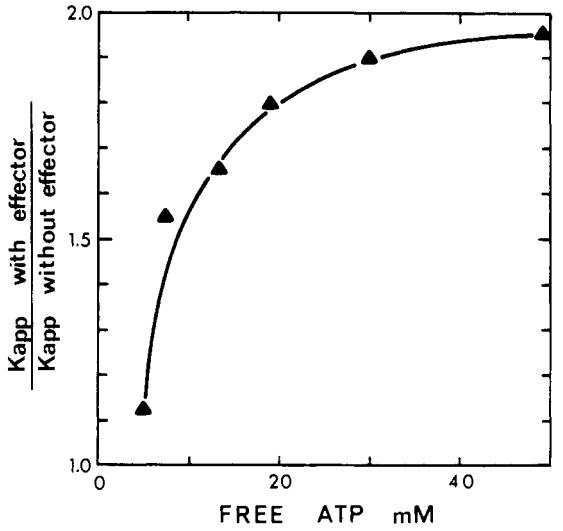

FIGURE 5: Effect of various concentrations of free ATP on the K_{app} for $MnATP^{2-}$ in the presence of bicarbonate. K_{app} values for $MnATP^{2-}$ were measured as described in Figure 3. The reaction mixture also contained as effector 30 mM phosphate (▲) with various concentrations of free ATP as indicated.

sibility, the influence of effectors on the K_{app} in the presence of varying concentrations of free ATP was studied. The dependence of the rate of ATPase activity on the concentration of $MnATP^{2-}$ in the presence or absence of a saturating concentration of bicarbonate (100 mM) indicated that the effector did not change the mode of inhibition by free ATP which remained competitive (Figure 3). The ratio between the K_{app} values with and without effectors also depended on the concentration of free ATP in a nonlinear fashion (Figure 5). There was a nonlinear increase in the K_{app} ratio which occurred in the presence of both bicarbonate, which is a positive effector, and phosphate, which is a negative effector (Figure 4).

Slightly different results were obtained when the effect of maleate on ATPase activity was studied at pH 6.5. At low pH, maleate caused an increase in the V_{max} but decreased the K_{app} for $MnATP^{2-}$ as substrate (Figure 6). However, as was found at pH 8, ATP remained a competitive inhibitor both

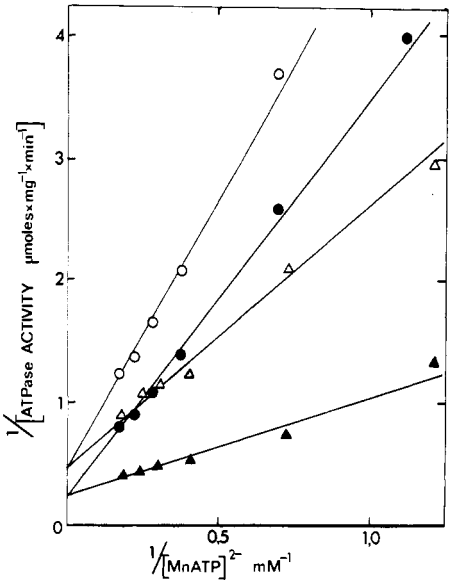

FIGURE 6: Effect of maleate on ATPase activity in CF_1 with $MnATP^{2-}$ at pH 6.5. ATPase activity of heat-activated CF_1 was measured in 40 mM Mes–NaOH (pH 6.5) as described under Experimental Procedures. The activity was determined with various concentrations of $MnATP^{2-}$ as indicated in the presence of (▲) 1 mM free $HATP^{3-}$ and 60 mM maleate, (△) 1 mM free $HATP^{3-}$, (●) 7 mM free $HATP^{3-}$ and 60 mM maleate, and (○) 7 mM free $HATP^{3-}$. Otherwise the reaction mixture was as in Figure 3.

in the presence and in the absence of the effector. It was previously observed (Nelson et al., 1972) that maleate induced changes in both the V_{max} and the K_m of ATPase activity measured in the presence of Mg^{2+} at pH 6. An attempt was made to determine the changes induced by the modulation of the K_{app} of substrates and of the K_i for competitive inhibition by free ATP and free divalent cations. For these determinations, results from experiments in which the changes in the ratio of the K_{app} with and without effectors caused by various concentrations of the inhibitors were used. The mathematical model was based on the formulation by Dixon & Webb (1964). It was assumed that bicarbonate, phosphate, and maleate were allosteric effectors which could change the affinity of both substrate and inhibitors to the active site. Without effector

$$K_{app} = K_m[1 + (I/K_i)] \quad (1)$$

and with effector

$$K_{app} = \alpha K_m[1 + (I/\beta K_i)] \quad (2)$$

where K_{app} is K_m apparent, I is the concentration of the competitive inhibitor (free ATP or free divalent cation), K_i is the inhibition constant of the competitive inhibitor, α is the change in K_m at saturating concentration of the effectors, and β is the change in K_i at saturating concentration of the effectors.

Analysis of the effect of various concentrations of free ATP on the K_{app} ratio (eq 3–5) at saturating concentration of the

$$A = \frac{K_{app}(\text{with effector})}{K_{app}(\text{without effector})} = \frac{\alpha K_m[1 + (I/\beta K_i)]}{K_m[1 + (I/K_i)]} = \alpha \frac{\beta K_i + I}{\beta(K_i + I)} \quad (3)$$

$$\lim_{I \to 0} A = \lim_{I \to 0} \alpha \frac{\beta K_i + I}{\beta(K_i + I)} = \alpha \quad (4)$$

$$\lim_{I \to \infty} A = \lim_{I \to \infty} \alpha \frac{(\beta K_i/I) + 1}{\beta[(K_i/I) + 1]} = \frac{\alpha}{\beta} \quad (5)$$

Table I: Effect of the Various Effectors on the Kinetic Parameters of CF_1 at pH 8[a]

effector	substrate	K_m (with effector)/ K_m (without effector)	inhibitor	K_i (with effector)/ K_i (without effector)
bicarbonate	$MnATP^{2-}$	0.10 ± 0.015	ATP^{4-}	0.52 ± 0.08
phosphate	$MnATP^{2-}$	1.10 ± 0.017	ATP^{4-}	0.58 ± 0.09

[a] Calculations of the K_m ratio and of the K_i ratio were made according to eq 1–5 from the data presented in Figures 4 and 5. The K_m and K_i values of the substrate and of free ATP or free divalent cations were calculated as described in a previous paper (Hochman & Carmeli, 1981).

effectors made it possible to distinguish between the effect of the various effectors on K_m and K_i values for the substrate and for the inhibitor, respectively.

Bicarbonate, which activated ATPase, decreased the K_m for the substrate and the K_i for free ATP as competitive inhibitor (Table I). The principal change was the decrease in K_m which caused the acceleration of ATPase activity. Phosphate was an inhibitor of the ATPase in CF_1. This effector increased the K_m values for the substrate and decreased the K_i values for free ATP. The result was an inhibition of ATPase activity.

In a previous paper (Hochman & Carmeli, 1981), we showed that CF_1 can bind Mn^{2+}. The enzyme has two tight sites for Mn^{2+} binding, which are probably the active sites of the ATPase. The effects of the various effectors on Mn^{2+} binding to CF_1 are shown in Table II. Those effectors which inhibited ATPase activity also decreased the affinity of the tight sites for Mn^{2+}. Phosphate, which was an inhibitor of ATPase, increased the K_d values of Mn^{2+} binding to the tight site at pH 8. Phosphate was found to be most effective at a concentration of 30 mM. A lower concentration of phosphate had little effect on the binding of Mn^{2+} to the tight sites. Bicarbonate, which was an activator of ATPase, with $MnATP^{2-}$ as substrate, increased the affinity of Mn^{2+} to the tight sites. The K_d value of Mn^{2+} binding (0.98 μM) decreased to 0.07 μM in the presence of 50 mM bicarbonate. Maleate also increased Mn^{2+} binding at pH 6.5. At this pH, maleate was found to be an activator of ATPase, with $MnATP^{2-}$ as substrate. The various effectors had the same effect when binding was measured either with ADP or with ATP (Table II).

Table II: Effect of the Various Effectors on Binding of Mn^{2+} to CF_1[a]

reaction mixture (conditions)	tight sites		loose sites	
	n	K_d (μM)	n	K_d (μM)
no additions	1	3.80 ± 0.35	5	188 ± 12
2 equiv of ADP (pH 8)	2	0.83 ± 0.17	4	100 ± 14
2 equiv of ADP and 2 equiv of phosphate (pH 8)	2	1.43 ± 0.32	4	100 ± 28
2 equiv of ADP and 30 mM phosphate (pH 8)	2	1.20 ± 0.14	4	15 ± 1.8
2 equiv of ATP (pH 8)	2	0.98 ± 0.10	4	50 ± 10
2 equiv of ATP and 30 mM phosphate (pH 8)	2	2.00 ± 0.23	4	15 ± 1.6
2 equiv of ATP and 50 mM bicarbonate (pH 8)	2	0.07 ± 0.01	4	30 ± 3.0
1.8 equiv of ADP (pH 6.5)	2	42 ± 3.00	4	600 ± 60
2.0 equiv of ADP and 22 mM maleate (pH 6.5)	2	13 ± 1.10	4	125 ± 13
1.8 equiv of ATP (pH 6.5)	2	70 ± 5.00	4	500 ± 46
1.8 equiv of ADP and 22 mM maleate (pH 6.5)	2	39 ± 5.40	4	167 ± 23

[a] Mn^{2+} binding to 40–60 μM CF_1 was measured as described under Experimental Procedures. The number of binding sites (n) and the binding constants (K_d) were calculated as described (Hochman & Carmeli, 1981). Moles of added reagent per mole of CF_1 (equiv) were as indicated.

Discussion

It was assumed in this work that bicarbonate, maleate, and phosphate are allosteric effectors of ATPase activity in CF_1. This assumption was based on the observation that depending on the conditions, these agents either stimulated or inhibited the activity. The stimulation and inhibition of the activity depended on the pH, on the type of effector, and on the cation used for activation. Thus maleate stimulated ATPase activity with $MnATP^{2-}$ but inhibited the activity when $CaATP^{2-}$ was used as substrate, while bicarbonate stimulated the activity in the presence of both cations. Except for the increase in V_{max} of ATPase activity induced at pH 6.5 by maleate when $MnATP^{2-}$ was used as substrate, no other effector caused a change in K_{cat} under the conditions tested in this work. In the presence as well as in the absence of the effectors, the divalent metal ion–ATP complex was the true substrate of the enzyme while both free ATP and the free cation were competitive inhibitors of ATPase activity. It was assumed therefore that the effectors caused changes in the K_m for substrate or in the K_i for free ATP and free divalent metal ions. The kinetic analysis (eq 1–5) of the ratio between the K_{app} with and without effectors showed which of the constants was changed. Thus bicarbonate mainly caused a decrease in the K_m of $MnATP^{2-}$ while phosphate changed mainly the K_i for free ATP at pH 8. In the mathematical analysis, it was assumed that the effector changed the K_m and K_i by binding to an allosteric site on the enzyme. This assumption was partially verified by the observation that the changes in the K_m and in the K_i calculated from these equations (eq 1–5) matched quantitatively the observed changes in activity. Bicarbonate caused a 10-fold decrease in the K_m of $MnATP^{2-}$ and only a 2-fold decrease in the K_i for free ATP, thus inducing a stimulation in ATPase activity. Phosphate inhibited the activity partially because of a 2-fold decrease in the K_i for free ATP. Although it is not very likely, it is still possible that the same effectors would have two different modes of action which change alternatively, depending on the conditions. For example, maleate could act as an allosteric effector in stimulating ATPase activity with $MnATP^{2-}$ but as a competitive inhibitor in the presence of $CaATP^{2-}$ at pH 8. Indeed, from the double-reciprocal plot of the data, it seemed that maleate competitively inhibited the activity in the presence of $CaATP^{2-}$. If that were the case, the system could have been analyzed as if it responded to the two types of competitive inhibitors (Dixon & Webb, 1964), the free ATP or the free cation and the effector. When K_{i1} and K_{i2} are the inhibition constants of I_1 and I_2 as competitive inhibitors of ATPase, the K_{app} ratio is given by

$$A = \frac{K_{app}(\text{with effector})}{K_{app}(\text{without effector})} = 1 + \frac{K_{i1}}{(K_{i1} + I_1)K_{i2}} I_2 \quad (6)$$

When the concentration of I_1 is constant, B is constant:

$$B = \frac{K_{i1}}{(K_{i1} + I_1)K_{i2}} \quad (7)$$

Under these conditions, a linear relation is expected between A (the K_{app} ratio) and the concentration of I_2 because

$$A = 1 + BI_2 \quad (8)$$

However, nonlinear relations were obtained when the ratios between the K_{app} values with effectors and the K_{app} values without effectors were plotted as a function of the effector concentration. Similarly nonlinear relations were obtained when the ratios between the K_{app} in the presence of a saturating concentration of effector and the K_{app} without effector were plotted against variable concentrations of one of the competitive inhibitors. These results indicated that bicarbonate, phosphate, and maleate acted as allosteric effectors under all the conditions tested.

Since divalent metal ions interact with the active site both as part of the substrate complex and as competitive inhibitors, it is expected that this binding might be modulated by effectors which alter the K_m and the K_i values. In correlation with the kinetic experiments, it was found that those effectors which accelerated ATPase activity increased Mn^{2+} binding to the tight sites while those which inhibited ATPase decreased the binding of Mn^{2+} to the tight sites. High concentrations of the various effectors also increased the binding of Mn^{2+} to four loose sites of CF_1. However, this effect on the loose sites was nonspecific. Since maleate and bicarbonate, which accelerated ATPase activity, or phosphate, which inhibited ATPase, had the same effect on Mn^{2+} binding to the loose sites of CF_1, the influence of the effectors on Mn^{2+} binding might be due to a general nonspecific anionic effect.

Analysis of the kinetic changes induced by the various effectors required the use of saturating concentrations in these experiments. However, a large extent of their effect was obtained even at a concentration of 10 mM (see Figure 1). This is certainly within the concentration range of carbonate, phosphate, and organic acids in the chloroplasts. Jagendorf & Uribe (1966) showed that organic acids accelerated ATP formation in chloroplasts. It is possible that they play a role in the activation of CF_1. Bata & Jagendorf (1965) found that bicarbonate had also affected ATP synthesis in chloroplasts. Phosphate was also shown to be an effector of membranal ATPase activity (Carmeli & Lifshitz, 1972). The regulation of ATPase activity by various intermediates of the carbon cycle in chloroplasts may be relevant to its function in vivo.

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