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EFFECT OF METHANOL ON SPINACH THYLAKOID ATPase

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Methanol at 35% (v/v) overcomes the latency of spinach thylakoid ATPase. Activation is immediate and reversible involving changes in the V_{max}, not the K_m of the enzyme. MgATP is a much better substrate than CaATP; free Mg^{2+} noncompetitively inhibits activity. This inhibition can be overcome by the addition of Na_2SO_3. While both MgATP and MgGTP act as substrates, free ATP and GTP both inhibit activity. ADP and MgADP are also inhibitory. Insensitivity to certain inhibitors indicates that methanol neither induces the same conformational changes in CF_1 as illumination does, nor does it lead to coupling between H^+ movement through CF_0 and ATP hydrolysis. Methanol activation provides a much improved method for assaying thylakoid ATPase.

Introduction

The Mg^{2+}-dependent ATPase of thylakoid-bound coupling factor (CF_1) from spinach chloroplasts is latent, requiring activation either by prolonged incubation in dithiothreitol or by the combination of the high-energy state and dithiothreitol [1]. During ATP hydrolysis protons are translocated into the thylakoid lumen; this results in inhibition of the ATPase rates when the internal proton activity is too high [2]. On the other hand, the enzyme activity decays when the internal proton activity becomes too low. Hence, the observed activity depends on a delicate balance between proton entry and back-diffusion, is sensitive to the condition of the membrane and the presence of uncouplers, and depends on other components in the membrane besides CF_1. The rates obtained after activation by light are often lower and more variable than those seen with the solubilized CF_1.

As with soluble CF_1 trypsin also causes activation of ATPase [3] but in addition it causes most of the CF_1 to be detached from the membranes [4]. Thus, the trypsin-activated membranes show ATPase characteristics (for instance, in the use of Ca^{2+} as a divalent cation) similar to those of the solubilized enzyme. Octylglucoside at 40 mM activates ATPase of soluble CF_1 [5] and potentially could be used with thylakoid membranes. However, the high price of octylglucoside would make this impractical for use as a routine assay. Because of these limitations, we sought an improved assay for thylakoid ATPase activity.

Recently, several reports have appeared showing that organic solvents enhance the ATPase activity of soluble CF_1 [6–9]. In particular, methanol enhanced the ATPase activity of spinach CF_1 by as much as a factor of 50 [8]. We therefore examined the effect of methanol and report here that it activates the membrane ATPase instantaneously and reversibly. An analysis of the proper-

Abbreviations: Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Taps, 3-tris(hydroxymethyl)methylaminopropanesulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 4-morpholinepropanesulfonic acid; CF_1, chloroplast coupling factor 1; CF_0, chloroplast coupling factor 0; Chl, chlorophyll.

ties of this assay gives some insight into some of the differences between bound and soluble CF_1.

Materials and Methods

Spinach thylakoids were prepared essentially as described previously [10]. Market spinach was homogenized in a medium containing 0.3 M sucrose, 25 mM Tricine-NaOH (pH 7.6), 10 mM NaCl and 5 mM ascorbate. The brei was strained through four layers of cheesecloth and one of miracloth then centrifuged for 5 min at $4000 \times g$. The chloroplasts were resuspended and washed once in the grinding medium then twice with 50 mM NaCl and finally resuspended in the same. When market spinach was unavailable the washed thylakoids were stored under liquid nitrogen in the medium described by Cohen [11]. No difference in activity was noted between the fresh and frozen chloroplasts. Chlorophyll was determined by the method of Arnon [12].

Assay conditions were based on those reported by Sakurai et al. [8] for soluble CF_1. The standard assay medium contained 25 mM Bicine or Taps buffer (pH 8.8 at 20°C), 25 mM NaCl, 35% (v/v) methanol, 5 mM ATP and 2.5 mM $MgCl_2$ in a volume of 0.5 ml. The effect of varying these assay conditions is described in Results. Chlorophyll concentration in the assay was between 5 and 25 μg/ml. Specific activity was not affected by the chlorophyll concentration when assays were run in this range. Reactions were started by the addition of the thylakoids and incubated at 37°C for 2 min or as indicated. Assays were stopped and the released P_i was determined colorimetrically by adding 0.5 ml of a solution based on the phosphate assay of LeBel et al. [13]. This solution contained: 2% (w/v) trichloroacetic acid, 2.4 M acetic acid, 0.4 M sodium acetate, 12 mM $CuSO_4 \cdot 5H_2O$, 1.0% (w/v) ammonium molybdate, 1.0% (w/v) Na_2SO_3, 0.4% (w/v) Elon, 1.0% (w/v) SDS. Color was allowed to develop for 10 min then 0.5 ml of a 5% sodium arsenate, 5% sodium citrate, 5% sodium acetate solution was added to prevent further color development [14].

Free Mg^{2+} concentrations were calculated according to the formulas of Adolfsen and Moudrianakis [15]. In experiments where Bicine was used as the buffer the apparent free Mg^{2+} concentrations were corrected for the weak complexation of Mg^{2+} by Bicine [16].

Results

The addition of methanol to the assay medium stimulated the rate of ATP hydrolysis by thylakoid membranes 40–50-fold (Fig. 1), with a sharp optimum between 30 and 35%. In 33% methanol the time course of ATP hydrolysis (Fig. 2) showed no apparent lag at the outset of the reaction and hydrolysis proceeded linearly for at least 2 min. After about 3 min the rate slowed down, probably as a result of denaturation of the enzyme. At methanol concentrations above 35% this denaturation apparently occurs more rapidly as the reaction proceeded for only a few seconds. The stimulation by methanol was not due to a change in the K_m for MgATP which remained at about 1 mM, but rather was due entirely to an increase in the V_{max} from about 15 μmol/mg Chl per h in the absence of methanol to over 600 μmol/mg Chl per h in 35% methanol (data not shown).

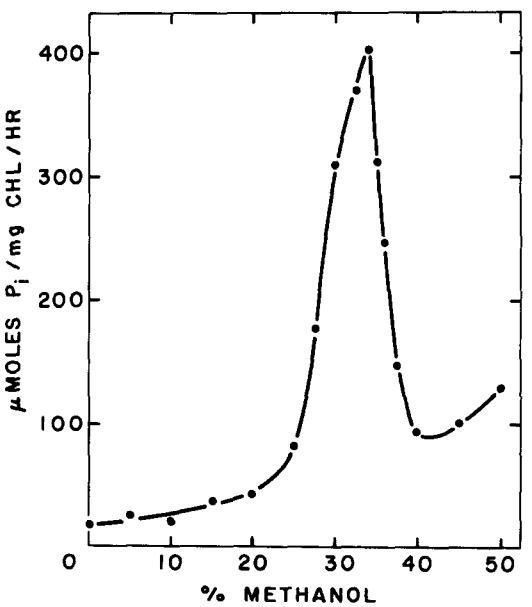

Fig. 1. Methanol stimulation of thylakoid ATPase. Reaction mixtures contained in 0.5 ml: 25 mM Bicine-NaOH (pH 8.8), 25 mM NaCl, 2.5 mM $MgCl_2$, 5 mM ATP, thylakoids at 10 μg Chl/ml, and methanol as indicated. Reactions were incubated for 2 min at 37°C and released P_i was determined as in Materials and Methods.

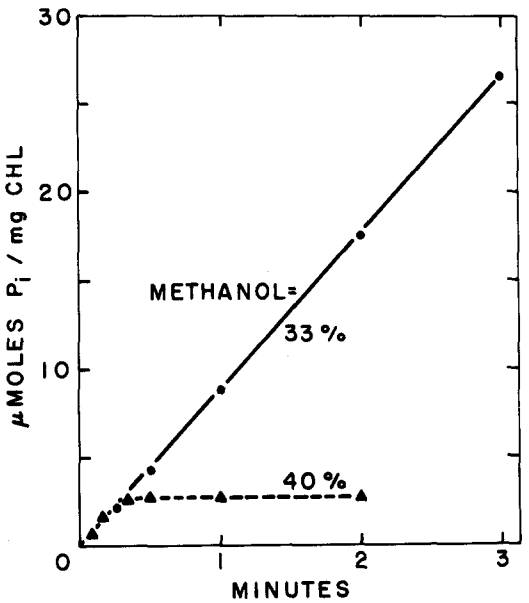

Fig. 2. Time course for ATP hydrolysis. Reaction conditions were as described for Fig. 1 except in a volume of 5 ml and at the two methanol concentrations indicated. At the indicated times 0.5-ml aliquots were assayed for P_i.

The activation by methanol was completely reversible. This was demonstrated by first incubating the thylakoids in 35% methanol for 1 min, then diluting the methanol and collecting the chloroplasts by centrifugation. The pelleted chloroplasts were resuspended, and assayed either in the presence or absence of added methanol. Both control and methanol-pretreated chloroplasts had low rates (less than 15 μmol/mg Chl per h) without methanol in the final assay, and high rates (greater than 500 μmol/mg Chl per h) when methanol was included. Thus, the methanol pretreatment did not cause permanent activation or inactivation of the thylakoid-bound CF_1, nor did it cause solubilization and loss of the enzyme during the washing step. Similar results were obtained if the chloroplasts were preincubated in methanol, then diluted into the assay mixture without an intervening centrifugation step: the activity observed depended entirely on the concentration of methanol present during ATP hydrolysis. Methanol-induced ATPase was strongly inhibited by anti-CF_1 antiserum confirming that the activity was in fact due to the coupling factor enzyme (not shown).

The divalent cation requirement for methanol-activated ATPase is satisfied by Mg^{2+} or Mn^{2+}, with Co^{2+} and Cd^{2+} giving somewhat lower activity and Ca^{2+} being nearly ineffective (Table I). The response to Mg^{2+} concentration varied with the amount of ATP added (Fig. 3). The optimum

TABLE I

CATION REQUIREMENT FOR METHANOL-ACTIVATED THYLAKOIDS

Reaction mixtures contained in 0.5 ml: 25 mM Taps-NaOH (pH 8.8), 25 mM NaCl, 35% (v/v) methanol, 5 mM ATP, thylakoids at 10 μg Chl/ml and the indicated divalent cations at 3 or 10 mM. All divalent cations were added as the chloride salt. Reactions were incubated at 37°C for 2 min and released P_i was determined as described in Materials and Methods. Essentially no ATP hydrolysis was seen in the absence of any divalent cation (not shown).

Cation	ATPase (μmol P_i/mg Chl per h)	
	3 mM	10 mM
Mg^{2+}	404	100
Mn^{2+}	360	92
Cd^{2+}	110	116
Co^{2+}	305	58
Ca^{2+}	56	78

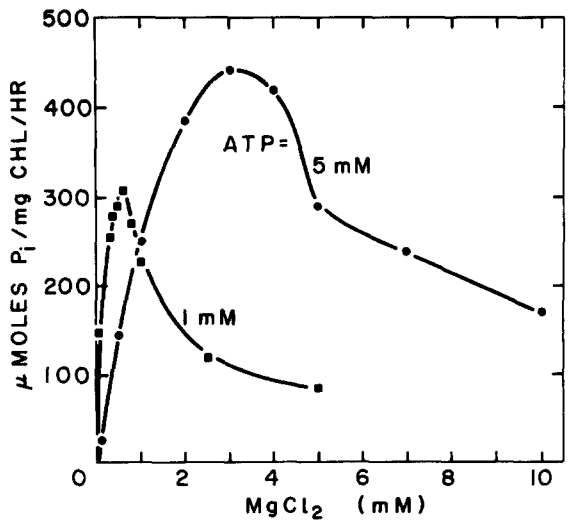

Fig. 3. $MgCl_2$ curves for the methanol-dependent thylakoid ATPase. Reactions contained in 0.5 ml: 25 mM Taps-NaOH (pH 8.8), 25 mM NaCl, 35% (v/v) methanol, 1 or 5 mM ATP, thylakoids at 10 μg Chl/ml, and $MgCl_2$ as indicated. Reactions were incubated for 2 min at 37°C and released P_i was determined as in Materials and Methods.

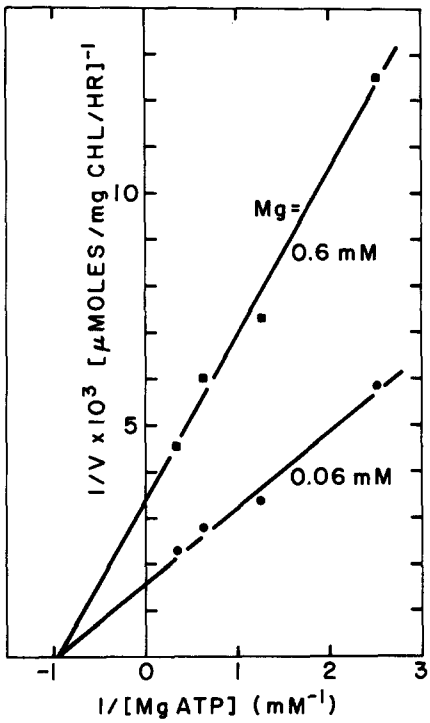

Fig. 4. A double-reciprocal plot of ATP hydrolysis in 35% (v/v) methanol at two free Mg^{2+} concentrations. Reaction conditions were as described for Fig. 1 except that the amounts of $MgCl_2$ and ATP added were varied to give the indicated Mg^{2+} and MgATP concentrations, calculated as described in Materials and Methods.

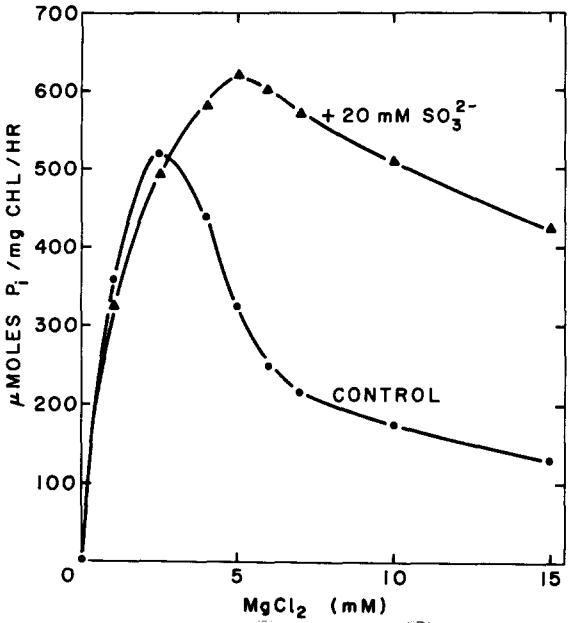

Fig. 5. Effect of SO_3^{2-} on methanol-dependent thylakoid ATPase. Conditions were as described for Fig. 3 with an ATP concentration of 5 mM and Na_2SO_3 as indicated.

for Mg^{2+} occurred at about half the ATP concentration indicating that free Mg^{2+} is highly inhibitory. The nature of the inhibition by free Mg^{2+} was analyzed by measuring the ATP dependency of the initial velocity of ATP hydrolysis at two free Mg^{2+} concentrations (Fig. 4). These Lineweaver-Burk plots show that Mg^{2+} acts as a noncompetitive inhibitor. The apparent K_m for MgATP of about 1 mM is unchanged by excess Mg^{2+}. From the intercepts measured at the two free Mg^{2+} concentrations a K_i for Mg^{2+} of 0.29 mM and a V_{max} of 698 μmol/mg Chl per h were calculated.

A wide variety of inorganic anions are known to stimulate the MgATPase activity of soluble CF_1. Of these SO_3^{2-} has been shown to be the most effective [17]. We found SO_3^{2-} stimulated the methanol-dependent ATPase, with an optimum at 20–30 mM, but only when the Mg^{2+} concentration was above its optimum (Fig. 5). Thus, the apparent SO_3^{2-} stimulation is in fact a reduction in the extent of Mg^{2+} inhibition. Mg^{2+} and SO_3^{2-} do combine to form a complex; adding SO_3^{2-} would thus reduce the true free Mg^{2+} concentration which in turn would reduce the extent of Mg^{2+} inhibition. However, this does not appear to be the primary mechanism for SO_3^{2-} stimulation, since the association constant for the $MgSO_3$ complex is weak [18] and CO_3^{2-}, which forms a similar

TABLE II

EFFECT OF SO_3^{2-} ON Mg^{2+} INHIBITION

Kinetic parameters were determined from the experiment shown in Fig. 4 and from the same experiment except that it was performed in the presence of 20 mM SO_3^{2-}; when SO_3^{2-} was present the apparent free Mg^{2+} concentrations were corrected for the weak complexation of Mg^{2+} by SO_3^{2-} assuming an association constant of 100 [19].

	K_m (mM)	V_{max} (μmol P_i/mg Chl per h)	K_i Mg^{2+} (mM)
Control	1.04	699	0.29
+20 mM SO_3^{2-}	1.03	660	3.26

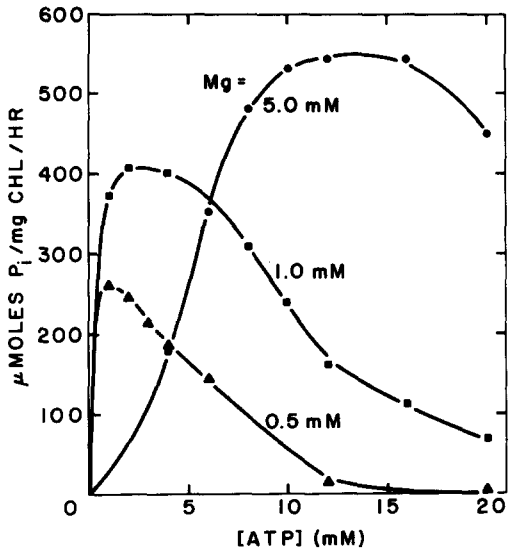

Fig. 6. ATP concentration curves for methanol-dependent thylakoid ATPase. Conditions were as in Fig. 3 except that $MgCl_2$ was present at 0.5, 1.0 or 5.0 mM and the ATP concentration was varied as indicated.

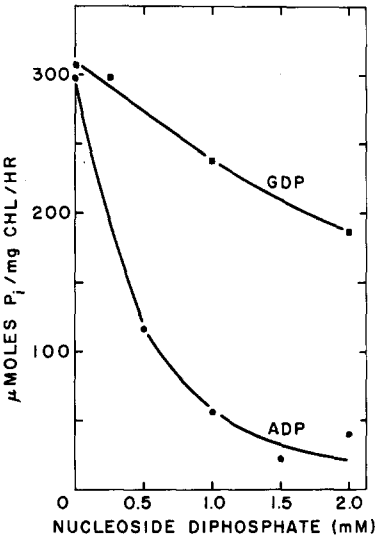

Fig. 7. Inhibition of methanol-dependent thylakoid ATPase by ADP and GDP. Conditions were the same as for Fig. 3 except that the ATP concentration was 1.0 mM and the $MgCl_2$ was 0.5 mM. ADP or GDP were added at the indicated concentrations.

complex, stimulates activity only slightly (not shown). It is more likely that SO_3^{2-} interacts directly with CF_1 to prevent inhibitory Mg^{2+} binding. With 20 mM SO_3^{2-} present, the K_i of free Mg^{2+} was raised by a factor of 10 (Table II). No changes were observed in the other kinetic parameters.

Excess ATP was also inhibitory, in experiments varying the ATP concentrations at fixed Mg^{2+} levels (Fig. 6). The optimum ATP level depended on the Mg^{2+} concentration, so the inhibition is apparently due to the excess free ATP and not high levels of the substrate MgATP. Similar results were obtained when GTP was used in place of ATP (not shown).

ADP inhibits the ATPase of both soluble CF_1 and light-activated thylakoids [20,21]. We found that this was also the case with methanol-activated thylakoids (Fig. 7). Unlike the inhibition due to excess nucleotide triphosphates (above), the inhibition by diphosphates is more specific for adenine nucleotides. Furthermore, the inhibition by ADP is independent of the amount of Mg^{2+} added suggesting that both ADP and MgADP are effective in causing this inhibition (not shown).

When the coupling factor is activated by light, thiol reagents, or both, ATP hydrolysis is coupled to H^+ transport across the thylakoids [2]; compounds such as DCCD, oligomycin and triphenyltin which interact with CF_0 [10,21,22] inhibit ATPase activity. These inhibitors had essentially no effect in the methanol-dependent ATPase (not shown) and thus it appears that a functional CF_0 is

TABLE III

EFFECT OF A DCCD PRETREATMENT ON SUBSEQUENT METHANOL-ACTIVATED ATPase

Thylakoids were prepared as described in Materials and Methods except that the final resuspension was in 25 mM Mops (pH 7.0), 25 mM NaCl, and 1 mM EDTA at 1 mg Chl/ml. DCCD, dissolved in ethanol, was added to give a final concentration of 0.4 mM; controls received an equal amount of ethanol (4%, v/v, final concentration). Samples were then incubated at 35°C for 30 min. Aliquots were removed and diluted 100-fold into the ATPase assay medium and activity was determined at variable Mg^{2+} concentrations as described for Table II.

Mg^{2+} concentration in assay (mM)	ATPase (μmol P_i/mg Chl per h)	
	Control	DCCD pretreated
2.5	436	423
5.0	233	308
10.0	120	244

not required for ATP hydrolysis in this case.

Recently, DCCD has been shown to interact directly with soluble CF_1 when incubations are carried out for longer periods of time and at higher concentrations than those used to inhibit CF_0 [23]. The effect of a 30 min preincubation in 400 μmM DCCD on subsequent methanol ATPase activity is shown in Table III. Unlike the case with the soluble heat-activated enzyme, DCCD preincubation had no inhibitory effect. On the contrary, when the assay was carried out in excess Mg^{2+} a noticeable stimulation occurred. While not as large, this effect is very similar to the SO_3^{2-} effect described above. DCCD thus appears to interact with a regulatory Mg^{2+} site but not with the catalytic site when CF_1 is membrane bound.

N-Ethylmaleimide modifies and inhibits membrane-bound CF_1 only when the thylakoids are in the high-energy state [26]. Sensitivity to *N*-ethylmaleimide can therefore be used as an indicator that CF_1 is in the light-induced 'active' conformation. We therefore tested the ability of added methanol to induce *N*-ethylmaleimide inhibition. While illumination of thylakoids in the presence of *N*-ethylmaleimide reduced subsequent methanol-induced ATPase by about 50%, a methanol plus *N*-ethylmaleimide pretreatment had no effect (data not shown).

Discussion

The ability of methanol to enhance the MgATPase activity of thylakoid membranes by a factor of 40 or more is in accord with the reported stimulation of soluble spinach and *Chlamydomonas* CF_1 by alcohols and other organic solvents [6–9]. A variety of enzymes including mitochondrial F_1 are also stimulated by organic solvents [25–27] but generally to a much smaller extent (a factor of three or less). Additionally, methanol stimulation of F_1 occurs only with the soluble enzyme and not with submitochondrial particles [27]. We found that methanol changed the V_{max} but not the K_m for ATP indicating that the stimulation is due to a greatly increased rate of enzyme turnover and not a change in substrate binding. Changes primarily in V_{max} rather than K_m have been reported in the solvent stimulation of soluble CF_1 [8] and other enzymes [25]. It is of interest that this is apparently not the case with mitochondrial F_1 as it has been reported that methanol lowers the K_m but does not raise the V_{max} [26].

Activation of CF_1-ATPase by methanol obviously does not require the thylakoid membranes to be in the high-energy state, nor is the active ATPase obligately coupled to proton movement through CF_0. This is shown by the failure of methanol to make CF_1 sensitive to *N*-ethylmaleimide (an indicator for the high-energy state) and by the inability of DCCD, oligomycin or triphenyltin to inhibit methanol-activated ATPase. This insensitivity to CF_0 inhibitors is similar to the reported effect [28] of butanol on submitochondrial particles. The ready reversibility of the activation by diluting out the methanol indicates activation is not likely to result from loss of an inhibitory protein subunit [29] or of tightly bound nucleotides [19] as has been suggested for the light-dependent activation. The latter point is under further investigation, however. At the moment it seems possible that ATP hydrolysis induced by methanol could be due to a greatly enhanced turnover rate of the 'latent' form of the enzyme rather than a conversion to its previously described active forms.

In any event, the methanol-activated membrane-bound spinach CF_1 retains the specificity for Mg^{2+} as divalent cation, and inability to use Ca^{2+}, that is seen with the light- and dithiothreitol-activated enzyme [1]. In this respect it differs strongly from the soluble enzyme, whether activated by heat, trypsin, or organic solvents [1,3,6]. Solubilization must lead to a change in conformation at the active site such that CaATP can bind more readily. It is interesting to note a species difference in this respect: *Chlamydomonas* F_1 ATPase has low activity with Ca^{2+} whether membrane-bound or soluble [7].

The inhibition of soluble CF_1-ATPase by free Mg^{2+} was reported earlier, with K_i values between 20 and 56 μM [9,30,31]. Free Mg^{2+} also inhibits the membrane-bound, methanol-activated ATPase but with a K_i value of 290 μM (Fig. 6). It is possible that this higher K_i is due to the presence of the methanol as it has been reported that ethanol reduces the extent of Mg^{2+} inhibition with soluble spinach and *Chlamydomonas* CF_1 [9,31]. However, thylakoids assayed in the absence of organic

solvents are also only weakly inhibited by free Mg^{2+} (Ref. 34 and unpublished results). Thus, the reduced extent of Mg^{2+} inhibition is likely to be an indicator of membrane-bound CF_1 rather than an effect of the methanol.

Since experiments with soluble CF_1 showed competitive kinetics, inhibition by free Mg^{2+} was proposed to result from competition between Mg^{2+} and MgATP for the catalytic site [30]. However, we found that Mg^{2+} inhibition was noncompetitive in nature (Fig. 6). Workers using the CF_1 from pea [31] and *Chlamydomonas* [9], as well as bacterial F_1 [17] have also reported noncompetitive inhibition. Why Mg^{2+} inhibition is sometimes observed to be competitive and other times noncompetitive is not clear. The apparent nature of the inhibition may be affected by the specific assay conditions. With *Chlamydomonas* CF_1 the kinetics of inhibition are changed from noncompetitive to apparently competitive by the addition of ethanol. However, given that noncompetitive inhibition does occur under some conditions, an allosteric Mg^{2+}-binding site most likely exists. Inhibition of methanol-activated thylakoids presumably occurs via this regulatory site.

Stimulation of coupling factor Mg^{2+}-dependent ATPase by various anions has been reported for soluble CF_1 [17,20], mitochondrial F_1 [33] and bacterial F_1 [18] among others. The stimulation of methanol-dependent ATPase by SO_3^{2-} is shown here (Fig. 5) to be entirely a matter of reversing the inhibition by excess Mg^{2+}; the K_m for ATP is unaffected. This was shown previously to be the case for soluble CF_1 [31] and for the soluble coupling factor from *Alcaligenes faecalis* [18]. Indeed, the correspondence between results with the latter enzyme, and the membrane-bound, methanol-activated CF_1 (noncompetitive inhibition by free Mg^{2+}, and raising the K_i for free Mg^{2+} by a factor of 10 when SO_3^{2-} is added) is striking, and suggests that the effects we report here are general and not just some peculiarity of methanol activation. Additionally, the ability of SO_3^{2-} to raise the K_i for Mg^{2+} without affecting the K_m for MgATP is a further argument that the catalytic site is not the site for Mg^{+2} inhibition.

Inhibition by high levels of ATP has been widely observed with CF_1 and other coupling factors [18,30,34]. This effect has been ascribed to either 'substrate' inhibition, implying the existence of a second noncatalytic substrate-binding site [34], or to a direct competition between free ATP and the ATP-metal ion complex for the catalytic site [30]. Since the inhibition by ATP depends on the amount of Mg^{2+} present, the latter proposal seems more likely. The fact that GTP gives qualitatively similar results to ATP also supports this proposal.

With light-activated thylakoids ADP is a potent inhibitor having a K_i of about 2 μM [19]. We also found that ADP inhibited but only at millimolar concentrations. It is thus unlikely that the inhibition we observe involves the tight binding site for ADP. Two distinctly different forms of ADP inhibition have been observed with heat-activated soluble CF_1: one half-saturated at 10 μM [34], the other at 0.5 mM ADP [20]. The latter weak ADP effect is not a simple competitive inhibition [20] and is more specific for adenine nucleotides than is activity [1]. The inhibition we observe is also half-maximal at 0.5 mM ADP and shows the same adenine nucleotide specificity. Thus, while regulation by tightly bound ADP, which occurs in light activation, does not occur with the methanol assay, ADP still affects activity in a manner similar to that observed with the soluble enzyme. This is very different from that which has been reported for alcohol-stimulated *Chlamydomonas* CF_1 where ethanol eliminated inhibition by ADP [9].

It is of interest that the higher levels (400 μM) of DCCD that inhibit soluble coupling factor by itself, have no inhibitory effect on the methanol-activated ATPase. This does not represent a reversal of existing DCCD inhibition by methanol, since we found that the methanol-activated soluble ATPase is indeed inhibited by a DCCD pretreatment (data not shown). It does not represent a total failure of DCCD to react with the thylakoid-bound enzyme either because after DCCD treatment the extent of inhibition by free Mg^{2+} is diminished (Table IV). Thus DCCD must react with or at least affect the regulatory binding site for free Mg^{2+}. A similar change in the extent of inhibition by free Mg^{2+} following modification of DCCD or its relative *N*-cyclohexyl-*N'*-β-(4-methylmorpholinoethyl)carbodiimide (CMCD) was noted earlier for soluble CF_1 [35].

Finally, we should emphasize the practical aspects of this assay. Previously, the only available

methods for measuring thylakoid ATPase activity were light or trypsin activation, both of which require a pretreatment prior to the actual assay. Such two-step procedures are time-consuming and also increase the amount of variability. We have found that the methanol-induced activity is highly reproducible and gives rates of ATP hydrolysis comparable to the highest rates obtained with trypsin activation. Additionally, CF_1 remains membrane bound and thus retains properties of the bound enzyme. Unlike activation by light, the methanol-dependent ATPase does not require other thylakoid components for activity. It is thus well suited for monitoring residual activity in chemical modification studies of membrane-bound CF_1. We are currently using this assay routinely to measure activity in such chemical modification experiments.

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