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Evidence for multiple effects in the methanol activation of chloroplast coupling factor 1

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Activation of the latent ATPase of soluble CF_1 by methanol is shown to involve several distinct effects. CaATPase activity of whole, but not epsilon-deficient or heat-activated CF_1, is stimulated by methanol. This suggests that one effect of methanol is to overcome inhibition by the epsilon subunit. In contrast, the MgATPase activities of both whole and epsilon-deficient CF_1 are further stimulated by methanol. This second activating effect can be traced in part to a greatly increased affinity of CF_1, due to methanol, for those anions which reverse the inhibitory effect of Mg^{2+}. Since the inhibition by free Ca^{2+} is much less severe than that caused by Mg^{2+}, anions have relatively little effect on CaATPase. Thus methanol has little or no effect when Ca^{2+} is the divalent cation, but stimulates the reaction when Mg^{2+} is used. Methanol also stimulates the MgATPase activity of ϵ-deficient CF_1 in the complete absence of activating anions. This additional effect is shown to arise from an increase in the V_{max} rather than from changes in either the K_m for MgATP or the K_i for free Mg^{2+}. Since this change in V_{max} occurs with the MgATPase but not the CaATPase, it can be inferred that different steps are rate-limiting in the two activities.

Introduction

Purified chloroplast coupling factor 1 (CF_1) is a latent ATPase. Expression of activity requires some activating treatment such as heat, proteolytic digestion or thiol reduction. The mechanisms involved in these activations are only partly understood. All lead to some change in the conformation of CF_1 although no general mechanism appears to be operating (for reviews, see Refs. 1 and 2).

Once activated, soluble CF_1 is capable of hydrolysing both Ca- and Mg-ATP but rates of hydrolysis with Ca^{2+} are usually much higher since free Mg^{2+} is more inhibitory than free Ca^{2+} [3,27]. High rates of MgATP hydrolysis are possible if certain oxyanions or organic acids are added [4,5]; the most effective anion has been shown to be SO_3^{2-} [6,7].

Incubation in organic solvents, particularly short-chain alcohols is an additional method of CF_1 ATPase activation. Both soluble [8] and thylakoid bound [9] CF_1 exhibit high rates of ATP hydrolysis if 20–35% methanol is added to the assay medium. A similar type of activation occurs if nonionic detergents such as octylglucoside are added [10].

In addition to simply activating CF_1, alcohols and detergents also affect the apparent divalent cation specificity. Alcohols, for example, appear to stimulate preferentially the MgATPase, especially if the enzyme has first been activated by heat or

Abbreviations: CF_1, chloroplast coupling factor one; Taps, 3-{[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid.

trypsin [9]. This enhancement of MgATPase activity is similar to the effect of anions and suggests some interrelationship may exist between the two.

Here we have examined the relationship between methanol and other forms of activation, and the possible interaction between alcohols and anions. In addition we have reexamined the reversibility of methanol activation of soluble CF_1 in view of our recent finding that methanol activation of thylakoid-bound CF_1 is not completely reversible [11].

Materials and Methods

Soluble CF_1 was prepared from spinach as described previously [12]; epsilon-deficient CF_1 was prepared as described in Ref. 13. Both preparations were stored at room temperature in a buffer containing: 25 mM Tris-Cl (pH 7.8)/1 mM ATP/2 mM EDTA/0.02% (w/v) NaN_3/20% (v/v) glycerol. Activity was completely stable for at least one month under these conditions.

For ATPase assays 2–5 μl (approx. 2–5 μg CF_1) of the stock CF_1 solutions were diluted into 0.5 ml of the assay medium. This assay medium contained 25 mM Taps-NaOH (pH 8.8), 5 mM ATP, 5 mM $MgCl_2$ or $CaCl_2$ and the indicated amount of methanol. For MgATPase assays the medium also contained Na_2SO_3 at the indicated concentrations. Reactions were started by the addition of the enzyme and proceeded for 2 min at 37°C. Liberated P_i was determined colorimetrically [14].

For methanol reversibility studies 34 μl CF_1 in storage buffer was mixed with 16 μl of either water or methanol (final methanol concentration, 32%) and incubated at room temperature. At the indicated times 5-μl aliquots were removed and diluted into 0.5 ml of an assay medium like that described above except lacking methanol. ATP hydrolysis was then allowed to proceed for 30 min at 37°C before liberated P_i was determined colorimetrically.

Dithioerythritol activation at room temperature was as in Ref. 15; heat activation without dithioerythritol as in Ref. 16. Protein concentrations were determined by a modified Lowry assay [17] with bovine serum albumin as a standard.

Results

Previously alcohol activation of soluble spinach and algal CF_1 has been described as completely reversible [8,18]. However, we recently showed that this was not strictly true for thylakoid-bound CF_1 [11]. A reexamination of the CaATPase of soluble CF_1 also shows that methanol activation is not entirely reversible. When CF_1 was incubated in a 32% methanol solution for up to 5 min, then diluted into an assay medium lacking methanol, the ATPase rate more than doubled due to the prior exposure to methanol (Fig. 1). While this is a significant increase in activity, the absolute magnitude of the activity even after 5 min in methanol is still quite small, only 1.9 μmol/mg protein per min. This probably explains why it has been overlooked previously. If methanol was present during the assay a much larger increase in CaATPase activity occurred (Fig. 2), consistent with previous reports [8,19].

A recent report [13] showed that washing CF_1 bound to DEAE-cellulose with 20% ethanol results in an active ATPase due to the removal of the epsilon subunit. This suggests that the activating effect of alcohols may result from a change involv-

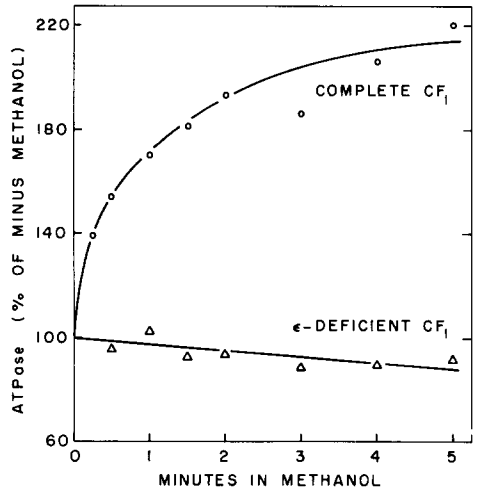

Fig. 1. Time-course for irreversible ATPase activation by a methanol pretreatment. Both complete and ε-deficient CF_1 were treated with methanol as described in Materials and Methods. CaATPase activity without any methanol treatment (=100% on graph) was 0.88 μmol/mg CF_1 per min for complete and 4.6 μmol/mg CF_1 per min for ε-deficient CF_1.

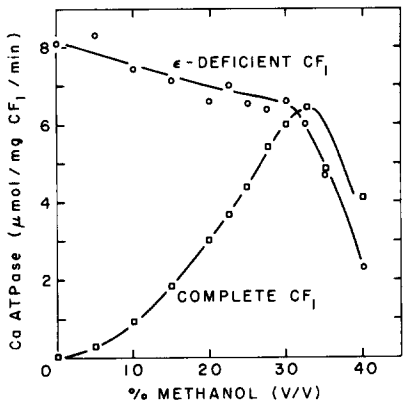

Fig. 2. Methanol stimulation of CaATPase. Both complete and ϵ-deficient CF_1 were assayed as described under 'Materials and Methods' in the presence of the indicated amount of methanol.

ing epsilon. To test this proposal the two types of CaATPase activation described above, reversible and irreversible, were measured using an epsilon-deficient CF_1 preparation. As shown in Figs. 1 and 2 neither type of activation occurred if an epsilon-deficient enzyme was used.

In contrast the MgATPase of both latent and epsilon-deficient CF_1 was activated by methanol and SO_3^{2-} (Fig. 3). This indicates that methanol must induce changes other than those involving epsilon. These other changes are apparently only

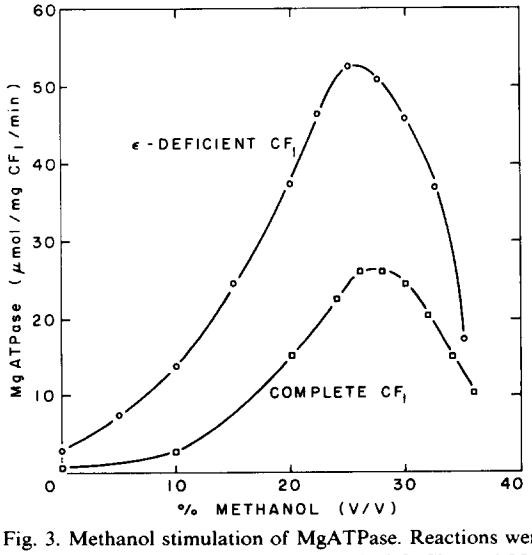

Fig. 3. Methanol stimulation of MgATPase. Reactions were as in Fig. 2 except $MgCl_2$ was used instead of $CaCl_2$, and 25 mM Na_2SO_3 was added.

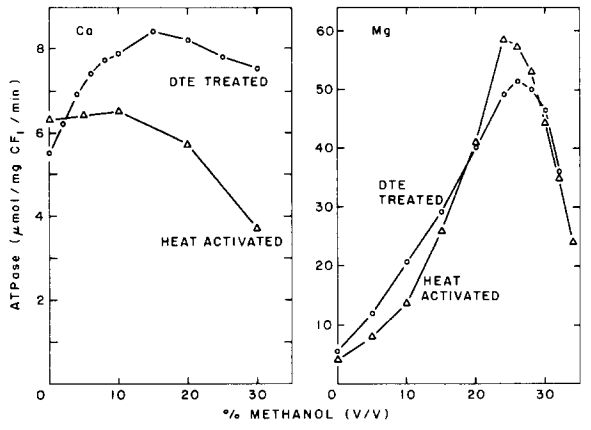

Fig. 4. Methanol effects on the Mg- and CaATPase activities of heat activated and dithioerythritol treated CF_1. The enzyme was either activated by exposure to 63°C without dithioerythritol, or by dithioerythritol at room temperature, and assayed for ATPase in the individual concentrations of methanol shown. 50 mM Na_2SO_3 was present in the MgATPase assays.

relevant to activation when Mg^{2+} is used as the divalent cation in the assay.

Recently, it has been shown that heat treatment and thiol reduction lead to distinctly different enzyme forms [15,20]. The effect of methanol on these two additional activated forms is shown in Fig. 4. The heat-activated enzyme responded to methanol in the same way as the epsilon-deficient form: the MgATPase was further activated, while the CaATPase was not. This suggests that heat treatment and epsilon removal lead to the same or similar activated state, and this is consistent with the proposal that heat activation is mediated via its effect on epsilon [4,21]. In contrast, both the Ca- and Mg-ATPase activities of the dithioerythritol-treated enzyme were further activated by methanol.

A major difference between Ca^{2+} and Mg^{2+}-dependent ATP hydrolysis is that free Mg^{2+} is highly inhibitory, while free Ca^{2+} is not. To overcome this Mg^{2+} inhibition, assays were performed in the presence of the activating anion SO_3^{2-}. The differential effect of methanol on the Ca vs. MgATPase suggests that the effect of methanol may be related to this divalent cation inhibition, or to anion activation. As shown in Fig. 5, the addition of 20% methanol had a dramatic effect on the apparent effectiveness of SO_3^{2-}. A double reciprocal plot of the data in Fig. 5 showed that the

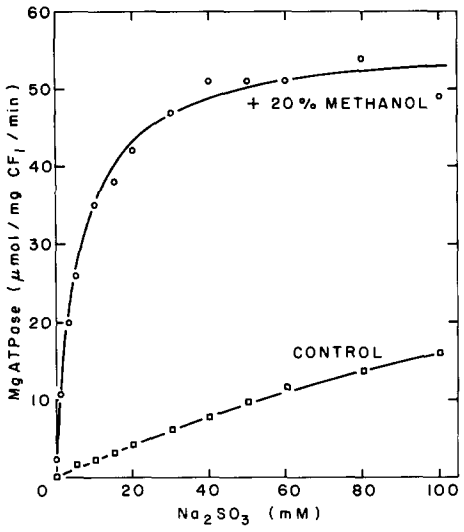

Fig. 5. Concentration dependence of the SO_3^{2-} stimulation of the MgATPase with and without methanol. Epsilon-deficient CF_1 was assayed as in Fig. 3 except that the amount of SO_3^{2-} was varied. Methanol, where added, was present at 20% (v/v).

addition of 20% methanol lowered the $K_{1/2}$ (the concentration for half maximal stimulation) of SO_3^{2-} from 205 to 6.5 mM. Similar results were obtained when HCO_3^{2-} was used instead of SO_3^{2-} (data not shown). Since the MgATPase assays in Figs. 3 and 4 were performed with 25 or 50 mM SO_3^{2-} present, the apparent stimulation of MgATPase activity by methanol can largely be accounted for by this change in the $K_{1/2}$ for SO_3^{2-}.

It is apparent, however, that methanol stimulates the MgATPase activity of ϵ-deficient CF_1 even in the complete absence of SO_3^{2-} (Fig. 5). This effect was also noted by Sakurai et al. [8] for both the trypsin and heat plus dithioerythritol activated enzymes. One possible explanation for this stimulation of the MgATPase activity of pre-activated CF_1 might be that methanol by itself reduces the inhibition caused by free Mg^{2+}. To test this possibility experiments were performed to see if the K_i for free Mg^{2+} was altered by methanol, with CF_1 previously activated by heat plus dithioerythritol. A double reciprocal plot of the initial rate of MgATP hydrolysis at several levels of free Mg^{2+} (Fig. 6b) showed that Mg^{2+} inhibition was of a mixed nature. This was also true with 20% methanol in the assay (Fig. 6a).

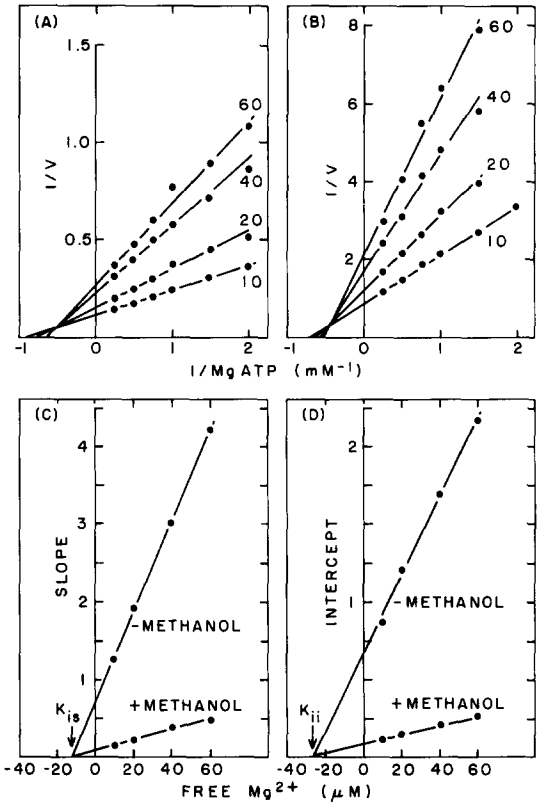

Fig. 6. Determination of K_i values for free Mg^{2+}. (A) CF_1 was activated by heat plus dithioerythritol, then assayed for MgATPase in 20% methanol as described in Materials and Methods, except that Na_2SO_3 was omitted. Variable amounts of $MgCl_2$ and ATP were added to give varying concentrations of MgATP and the four free Mg^{2+} concentrations indicated as μM values on the curves. The concentrations of free Mg^{2+} and of MgATP were calculated assuming a stability constant for MgATP of $8.3 \cdot 10^4$ as derived from the formula in Ref. 31. (B) As in (A) except methanol was omitted. (C) Replot of the values for the slopes of the lines in (A) and (B) vs. concentration of free Mg^{2+}. (C) Graph of the y-intercepts from (A) and (B) as a function of free Mg^{2+} concentration.

These results are in contrast to previous reports in which Mg^{2+} was said to act as either a purely competitive [3] or purely non-competitive [25] inhibitor. By replotting the slopes and y-intercepts from Fig. 6a and b the K_i slope (K_{is}) and K_i-intercepts (K_{ii}) values were determined (Fig. 6c and d). From these replots it is apparent that neither K_i value is significantly altered by the presence of methanol. Various kinetic parameters derived from Fig. 6 are listed in Table I. The only value which was changed by the methanol was that for V_{max}.

TABLE I

KINETIC PARAMETERS FOR MgATP HYDROLYSIS IN THE ABSENCE AND PRESENCE OF METHANOL

Values were determined from the plots in Fig. 6 according to the methods outlined in Ref. 32. K_{is} and K_{ii} refer to the K_i values for Mg^{2+}-derived from replots of the slopes and y-intercepts, respectively.

	Without methanol	With 20% methanol
K_m	1.07 mM	0.92 mM
V_{max}	1.53 μmol·mg^{-1}·min^{-1}	12.0 μmol·mg^{-1}·min^{-1}
K_{is}	12 μM	12 μM
K_{ii}	26 μM	28 μM

Thus its stimulating effect under these conditions cannot be ascribed to reversal of the inhibition by Mg^{2+}.

Discussion

The results presented here suggest that methanol activation of CF_1 involves multiple effects. While these effects are mostly reversible, some irreversible change also occurs. A marked difference also exists in the methanol effect depending on whether Ca^{2+} or Mg^{2+} is used as the divalent cation in the assay. Nevertheless, these results do suggest some general conclusions about the mechanisms of ATPase activation.

Both the reversible (Fig. 2) and irreversible (Fig. 1) methanol activation of the CaATPase require the presence of the epsilon subunit. This suggests that the principle effect of methanol in CaATPase activation is to displace epsilon or at least render it incapable of inhibiting ATPase activity. This change in epsilon can be reversed if the exposure to methanol is brief. Prolonged exposure leads to other irreversible changes, possibly the loss of epsilon, and permanent ATPase activation. The results of Richter et al. [13] where epsilon was removed by an ethanol wash of DEAE-cellulose-bound CF_1 would also indicate that epsilon is easily dislodged byalcohols.

It is noteworthy that the CaATPase of heat activated CF_1 was not further activated by methanol (Fig. 4). Following heat treatment the epsilon subunit is readily removed from CF_1 by a variety of treatments [21,22]. This has been taken as an indication that the activating effect of heat is to dislodge or labilize epsilon. If the activating effect of methanol is also through its action on epsilon, then the lack of additivity between heat and methanol is not surprising. In contrast the enzyme activated by dithioerythritol at 20°C was further activated by methanol (Fig. 4). Additivity between the activation by dithioerythritol and by heat was shown previously [15,20]. The additivity of methanol and heat with dithioerythritol, but not with each other is consistent with the proposal that heat and alcohols have a similar basis (probably a change in the binding of the epsilon subunit), while thiol reduction leads to a distinctly different enzyme form.

While methanol further activates the CaATPase of both latent (Fig. 2, 'complete CF_1' curve) and thiol reduced (Fig. 4) CF_1, lower concentrations of methanol are effective with the thiol reduced than with the control enzyme. Thus half-maximal effect of methanol is achieved at 20% for the latent enzyme (Fig. 2), but at only 8% with the one pre-reduced by dithioerythritol (Fig. 4). Corresponding methanol concentrations for maximal activation are 32% for the control enzyme and 15% after its reduction by dithioerythritol. This suggests that the two effects are not entirely unrelated. It seems likely that the conformational change in the complex protein induced by reduction of the disulfide in the γ subunit renders the ϵ subunit more labile and easily displaced by methanol. Similarly, it was found earlier that the activation by trypsin is more rapid for enzyme reduced by dithioerythritol [23], and the reduction of the γ subunit disulfide is more rapid in CF_1 previously activated by heating [15].

The effects of methanol on the MgATPase are more complicated than those described for the CaATPase. The principle difference is that methanol stimulates the MgATPase activity irrespective of any prior activating treatment. The differential effect of alcohols on the MgATPase vs. the CaATPase has been noted by others [8,24], but no mechanism has been proposed to explain it. Under our experimental conditions the further-activating effect of methanol on the MgATPase can largely be accounted for by a change in the

apparent $K_{1/2}$ for the activating anion SO_3^{2-}. As has been shown for both thylakoid bound [9] and soluble CF_1 [7,25] as well as the soluble coupling factor from *Alcaligenes faecalis* [26], SO_3^{2-} activation results from a reversal of the inhibition by free divalent cations. Since free Ca^{2+} is only weakly inhibitory [3,27], SO_3^{2-} activation is negligible with the CaATPase; thus the increased affinity for SO_3^{2-} induced by methanol has little or no effect.

Since methanol stimulates, to a small extent, the MgATPase of ϵ-deficient CF_1 even in the complete absence of SO_3^{2-} (Fig. 5, points on the ordinate), it is apparent that a third methanol effect must exist besides those related to anion binding or to the epsilon subunit. The results shown in Fig. 6, using CF_1 activated by heat + dithioerythritol, indicate this effect in a sulfite-free medium is not caused by a change in the K_i for free Mg^{2+}. Rather, it resulted from an increase in V_{max}. It is not likely that this resulted from an increase in the total population of active ATPase molecules, since the preparation had been preactivated by other means. Therefore it is more likely to be due to an increased k_{cat} suggesting that methanol accelerates the rate-limiting step for MgATP hydrolysis. The fact that this increase does not occur with CaATPase suggests that different steps are rate limiting in Ca^{2+} than in Mg^{2+}-dependent ATP hydrolysis. A difference in the mechanism between the two cations is even more strongly supported by the differences in O exchange properties reported by Kohlbrenner and Boyer [28].

One well-known effect of alcohols is to lower the transition temperature for thermal denaturation of proteins [18,29]. It is possible that activation of CF_1 is in a sense a manifestation of the early stages of thermal denaturation. Heating CF_1 to 63°C in water or 37°C in 30% methanol would both be ways to put CF_1 in this partially denatured, activated, state. In support of this proposal we have found that glycerol, which is known to be antagonistic with methanol in lowering the transition temperature for thermal denaturation [30], is also antagonistic with methanol in ATPase activation. The addition of 25% glycerol reduced ATPase activity by 60% when assays were performed in 25% methanol. Since detergents such as octylglucoside also lower this transition temperature [10], the basis for their activating effect may be similar.

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