Biophys. Res. Commun. 63, 894-899.
Ohnishi, T., Blum, H., Sato, S., Nakazawa, K., Hon-nami, K., & Oshima, T. (1980) *J. Biol. Chem. 255*, 345-348.
Phillips, M. K., Hederstedt, L., Hasnain, S., Rutberg, L., & Guest, J. R. (1987) *J. Bacteriol. 169*, 864-873.
Prince, R. C., & Adams, M. W. W. (1987) *J. Biol. Chem. 262*, 5125-5128.
Rondeau, S. S., Hsu, P., & DeMoss, J. A. (1984) *J. Bacteriol. 159*, 159-166.
Rupp, H., Rao, K. K., Hall, D. O., & Cammack, R. (1978) *Biochim. Biophys. Acta 537*, 255-269.
Sambasivarao, D., Scraba, D. G., Trieber, C., & Weiner, J. H. (1990) *J. Bacteriol. 172*, 5938-5948.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Tartof, K. D., & Hobbs, C. A. (1987) *Bethesda Res. Lab. Focus 9*, 205.
Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8765-8785.
Weiner, J. H., Cammack, R., Cole, S. T., Condon, C., Honore, N., Lemire, B. D., & Shaw, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2056-2060.
Weiner, J. H., MacIsaac, D. P., Bishop, R. E., & Bilous, P. T. (1988) *J. Bacteriol. 170*, 1505-1510.
Werth, M. T., Cecchini, G., Manadori, A., Ackrell, B. A. C., Schroder, I., Gunsalus, R. P., & Johnson, M. K. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8965-8969.
Westenberg, D. J., Gunsalus, R. P., Ackrell, B. A. C., & Cecchini, G. (1990) *J. Biol. Chem. 265*, 19560-19567.
Wilson, G. S. (1978) *Methods Enzymol. 54*, 396-410.
Vincent, S. P. (1989) *Biochem. J. 177*, 757-759.

Characteristics of the Combination of Inhibitory Mg^{2+} and Azide with the F_1 ATPase from Chloroplasts†

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Received April 3, 1991; Revised Manuscript Received June 10, 1991

ABSTRACT: The interactions between ADP, Mg^{2+}, and azide that result in the inhibition of the chloroplast F_1 ATPase (CF_1) have been explored further. The binding of the inhibitory Mg^{2+} with low K_d is shown to occur only when tightly bound ADP is present at a catalytic site. Either the tightly bound ADP forms part of the Mg^{2+}-binding site or it induces conformational changes creating the high-affinity site for inhibitory Mg^{2+}. Kinetic studies show that CF_1 forms two catalytically inactive complexes with Mg^{2+}. The first complex results from Mg^{2+} binding with a K_d for Mg^{2+} dissociation of about 10–15 μM, followed by a slow conversion to a complex with a K_d of about 4 μM. The rate-limiting step of the CF_1 inactivation by Mg^{2+} is the initial Mg^{2+} binding. When medium Mg^{2+} is chelated with EDTA, the two complexes dissociate with half-times of about 1 and 7 min, respectively. Azide enhances the extent of Mg^{2+}-dependent inactivation by increasing the affinity of the enzyme for Mg^{2+} 3–4 times and prevents the reactivation of both complexes of CF_1 with ADP and Mg^{2+}. This results from decreasing the rate of Mg^{2+} release; neither the rate of Mg^{2+} binding to CF_1 nor the rate of isomerization of the first inactive complex to the more stable form is affected by azide. This suggests that the tight-binding site for the inhibitory azide requires prior binding of both ADP and Mg^{2+}.

One of the catalytic sites on the isolated F_1 ATPases from various sources binds ADP quite tightly (Futai et al., 1989; Harris, 1978; Strotmann, 1986). Such ADP together with Mg^{2+} participates in an unusual modulation of the catalytic activity (Carmeli et al., 1981; Drobinskaya et al., 1985; Feldman & Boyer, 1985; Milgrom & Boyer, 1990; Minkov et al., 1979, 1980; Zhou et al., 1988). When medium Mg^{2+} is present and ADP is bound at the catalytic site without bound P_i, a complex is formed that initially has little or no ATPase activity when Mg^{2+} and ATP are added (Drobinskaya et al., 1985; Feldman & Boyer, 1985; Milgrom & Boyer, 1990; Zhou et al., 1988). The ATPase activity can be restored by the chelation of medium Mg^{2+} with EDTA prior to assay of the enzyme with Mg^{2+} and ATP. If Mg^{2+} and ATP are added to the inactive complex, some ATPase activity slowly appears as the complex decomposes with release of the inhibitory ADP (Carmeli et al., 1981; Feldman & Boyer, 1985; Hackney, 1979; Vasilyeva et al., 1980, 1982).

Considerable uncertainty remains about the nature of the binding of the inhibitory Mg^{2+}. The apparent K_d for the binding to CF_1 is about 4 μM, and the inhibition may require the binding of only one Mg^{2+} per enzyme (Guerrero et al., 1990b). Where the inhibitory Mg^{2+} binds is not known. The CF_1 contains up to six binding sites for divalent Mn^{2+} or Mg^{2+} (Carmeli et al., 1979; Hiller & Carmeli, 1985, 1990), and three

† Supported by U.S. Public Health Service Grant GM-11094 and U.S. Department of Energy Grant P.A. FG03-88ER13845.

0006-2960/91/0430-8305$02.50/0

of these sites have dissociation constants for Mg^{2+} in the micromolar range. Limited experimental results suggested that the inhibitory Mg^{2+} could readily combine with CF_1 in the absence of catalytic site ADP (Guerrero et al., 1990b). One purpose of the present paper is to present more extensive data showing that the binding of inhibitory Mg^{2+} with a relatively low K_d occurs only when a catalytic site is occupied with ADP. In the course of these studies another interesting point emerged, namely, that the inhibition induced by Mg^{2+} has a biphasic time course. The initial combination with Mg^{2+} results in inhibition, but the initial complex isomerizes into another inhibited form with a lower K_d for Mg^{2+}.

An additional aspect of the studies reported here concerns the relationship of azide to the Mg^{2+}-induced inhibition. Azide is a potent inhibitor of the ATPase activity of the intact ATP synthase and the separated F_1 ATPase (Chernyak et al., 1988; Duggett et al., 1985; Ebel & Lardy, 1975; Harris, 1989; Milgrom et al., 1988; Minkov & Strotmann, 1989; Moyle & Mitchell, 1975; Vasilyeva et al., 1982; Wei et al., 1988; Yoshida et al., 1977). The interaction of azide with the mitochondrial enzyme has been widely studied (Chernyak et al., 1988; Duggett et al., 1985; Harris, 1989; Ebel & Lardy, 1975; Moyle & Mitchell, 1975; Vasilyeva et al., 1982) and has given convincing evidence that azide stabilizes the inactive Mg·F_1·ADP complex. With the recognition that the binding of the inhibitory Mg^{2+} is a relatively slow process (Guerrero et al., 1990b), it was of interest to find out if azide might increase the rate of combination of Mg^{2+} with the enzyme·ADP complex. Our results show that this is not the case. Also, with the recognition that binding of ATP at a noncatalytic site is necessary for the ATPase activity of CF_1 (Guerrero et al., 1990a; Milgrom et al., 1990, 1991; Xue & Boyer, 1989), the possibility that azide might inhibit by interfering with this activation needed checking. This somewhat unlikely possibility has been ruled out. In addition, the interaction of azide with the chloroplast enzyme has not been thoroughly studied, and some essential characteristics are reported here.

Experimental Procedures

Preparation of CF_1. CF_1 was isolated and purified by the method of Binder et al. (1978) with the modifications of Shapiro and McCarty (1990). Heat activation in the presence of either ATP, ADP, or GDP was performed as published (Lien & Racker, 1971). Enzyme activated in the presence of different nucleotides is referred as (ATP-ha)CF_1, (ADP-ha)CF_1, or (GDP-ha)CF_1, respectively. For some experiments, 20–25 μM (GDP-ha)CF_1 was passed through two consecutive Sephadex centrifuge columns (Penefsky, 1977) equilibrated with a buffer containing, at pH 8.0, 50 mM Tricine, 50 mM tetrasodium pyrophosphate, and 2 mM EDTA and then dialyzed at room temperature against 500–1000 volumes of the same buffer. After dialysis, pyrophosphate was removed by passing the CF_1 through two Sephadex centrifuge columns (Penefsky, 1977) equilibrated with 50 mM Tricine-KOH, pH 8.0, buffer.

CF_1 Incubation and Mg-ATPase Assay. CF_1 at 0.2–1.0 μM was incubated at room temperature in a buffer containing 50 mM Tricine-KOH, pH 8.0; the time of incubation and concentrations of the Mg^{2+} and azide are indicated in the figure legends.

The Mg-ATPase activity of CF_1 was measured by P_i liberation in a medium containing 50 mM Tricine-KOH, 5 mM ATP, and 2 mM Mg^{2+}, at pH 8.0 and room temperature. After 10 s the reaction was stopped by the addition of a half-volume of a solution containing 2% sodium dodecyl sulfate and 10 mM EDTA. The P_i released was determined by a catalyzed phosphate assay (Ohnishi & Gall, 1978).

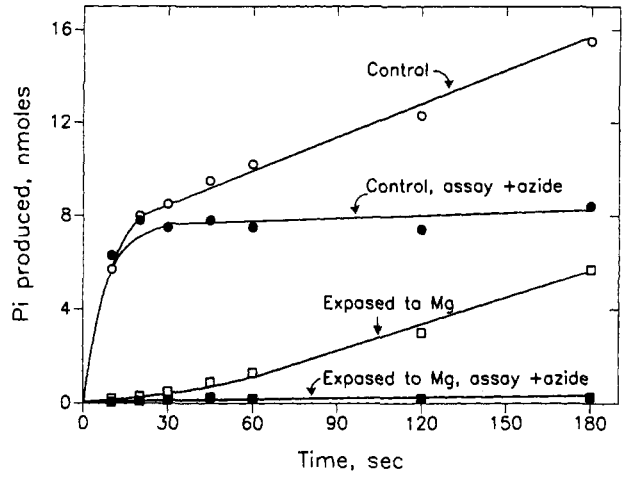

FIGURE 1: Time course of Mg-ATP hydrolysis by CF_1 with all noncatalytic sites filled with ATP. (ATP-ha)CF_1 was incubated in the presence of 1.0 mM $MgCl_2$, 20 μM ATP, 1 mg/mL pyruvate kinase, and 2.0 mM phosphoenolpyruvate for 20 min; then CF_1 was separated on a Sephadex centrifuge column equilibrated with 50 mM Tricine-KOH, pH 8.0, and 0.1 mM EDTA at pH 8.0. After a 30-min incubation with EDTA, the enzyme was passed through second centrifuge column equilibrated with 50 mM Tricine-KOH, pH 8.0. Kinetics of P_i formation (3.4 μg of CF_1/sample) were measured before (control) and after exposure of CF_1 to 1 mM Mg^{2+}. Where indicated, the assay medium contained 50 μM NaN_3.

Protein Assay. The concentration of the CF_1 protein was determined by the Lowry procedure (Lowry et al., 1951) using bovine serum albumin as a working comparative standard. The conversion factor was based on an $A_{227} = 0.483$ for 1 mg/mL CF_1 (Bruist & Hammes, 1981) and a molecular mass of 400 kDa (Moroney et al., 1983).

Results

Some Azide Effects on CF_1 ATPase. The (ATP-ha)CF_1 has one noncatalytic site vacant, and the filling of this site with ATP can markedly promote GTPase or ATPase activity (Guerrero et al., 1990a; Milgrom et al., 1990, 1991; Xue & Boyer, 1989). The possibility that azide might prevent filling of this noncatalytic site was ruled out by the experiment reported in Figure 1. When all vacant noncatalytic sites on (ATP-ha)CF_1 were filled by prior exposure to Mg-ATP, azide still caused potent inhibition. In accord with results of Xue et al. (1988), azide strongly inhibited the steady-state Mg-ATPase activity of (ATP-ha)CF_1 but did not inhibit the initial rapid phase of Mg-ATP hydrolysis by CF_1.

CF_1 with ADP at a catalytic site is inactivated by prior exposure to Mg^{2+} and slowly reactivated upon exposure to MgATP (Feldman & Boyer, 1985). Figure 1 shows the partial reactivation with 5 mM ATP and 2 mM Mg^{2+} and that this reactivation is prevented by presence of 50 μM NaN_3. These results with CF_1 are quite similar to those found with mitochondrial F_1; azide has no effect on the initial rate of ATP hydrolysis, decreases the steady-state activity, and prevents the ATP-dependent reactivation that occurs with MF_1 inhibited by prior exposure to Mg^{2+} (Vasilyeva et al., 1982).

The Rate of Mg^{2+} Binding with and without Azide Present. As mentioned earlier, exposure of CF_1 with ADP at a catalytic site to Mg^{2+} gives an enzyme that is initially inactive upon addition of ATP. The opposite is not found; exposure of CF_1 to azide before the addition of Mg^{2+} and ATP gives no greater inhibition than when azide is added with Mg^{2+} and ATP (data not shown). Presence of medium Mg^{2+} is essential for azide inhibition. As noted with the mitochondrial enzyme (Vasilyeva et al., 1982), azide increases the extent of the Mg^{2+}-induced

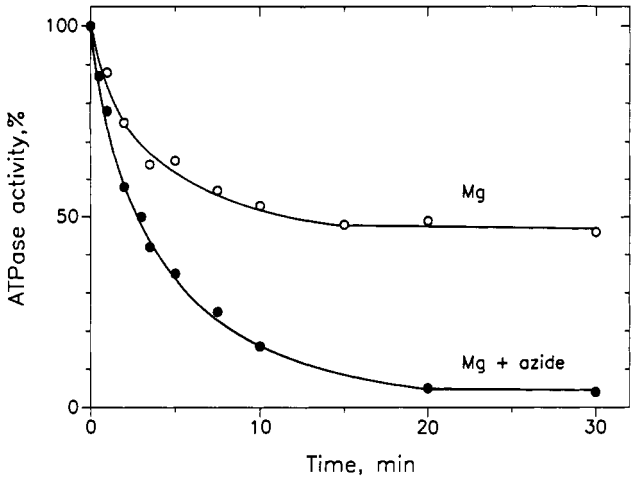

FIGURE 2: Effect of azide on the inactivation of (ATP-ha)CF_1 by 5 μM Mg^{2+}. CF_1 with noncatalytic sites filled with ATP was prepared as described in the legend to Figure 1. Then CF_1 was incubated in the presence of 5 μM Mg^{2+} or 5 μM Mg^{2+} and 50 μM NaN_3, and at the time indicated enzyme activity was measured. 100% activity corresponds to 9.8 μmol min^{-1} mg^{-1}.

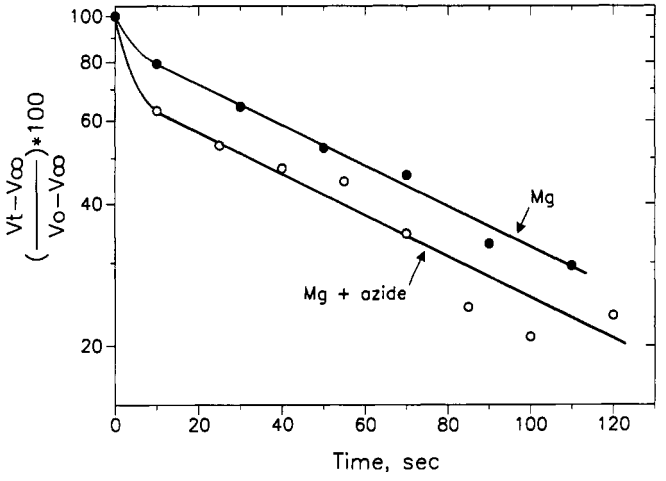

FIGURE 3: Semilogarithmic plot of (ATP-ha)CF_1 inactivation by Mg^{2+}. The enzyme was incubated in the presence of 10 μM Mg^{2+} or 10 μM Mg^{2+} and 100 μM azide. At the indicated time, aliquots were taken for ATPase assay. ATPase activity of CF_1 before Mg^{2+} addition was 10.4 μmol min^{-1} mg^{-1}.

inhibition of CF_1 (Figure 2). Other experiments showed that the affinity of CF_1 for the inhibitory Mg^{2+} in the absence of azide (K_d about 4 μM) is increased 4–5 times (K_d about 1 μM) in the presence of 50 μM azide (data not shown). This could result from promotion of the rate of Mg^{2+} binding or the inhibition of Mg^{2+} release or both. Figure 3 shows the semilogarithmic plot of CF_1 inactivation when exposed to 10 μM Mg^{2+} or to 10 μM Mg^{2+} plus 100 μM azide. The results demonstrate that azide does not affect the rate of binding of 10 μM Mg^{2+} to CF_1.

The effect of azide on the rate of Mg^{2+} binding was also checked at higher Mg^{2+} concentration. Results shown in Figure 4 demonstrate that the rate of inactivation by 2 mM Mg^{2+} is the same with or without 200 μM azide present. In the presence of 2 mM Mg^{2+}, the half-time for CF_1 inhibition is about 2 s, corresponding to a pseudo-first-order rate constant of about 0.4 s^{-1}. Taking into account the concentration of Mg^{2+}, this gives a second-order rate constant for the interaction of CF_1 and Mg^{2+} of about 10^3 M^{-1} s^{-1}, close to the value of 0.85×10^3 M^{-1} s^{-1} derived earlier from the inhibition of CF_1 at lower Mg^{2+} concentrations (Guerrero et al., 1990b). The rate-limiting step for the Mg^{2+}-induced inhibition of CF_1 is

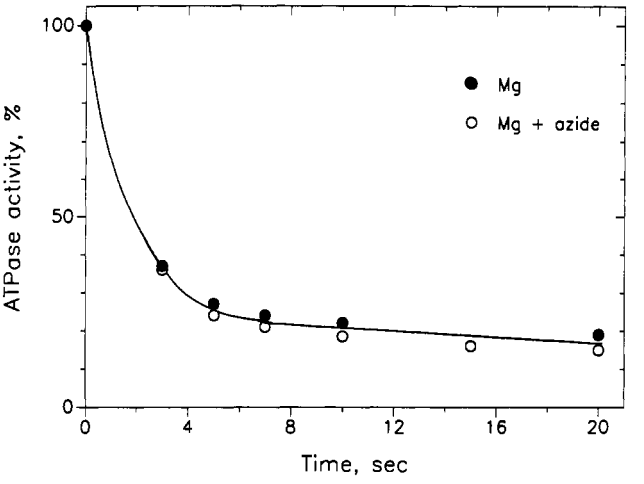

FIGURE 4: Kinetics of (ATP-ha)CF_1 inactivation by 2 mM Mg^{2+}. Incubations were carried out in the absence and presence of 200 μM NaN_3. The enzyme was prepared as described in the legend to Figure 1 and then was incubated in the presence of Mg^{2+} and azide, and at the indicated times aliquots were taken for the ATPase assay. 100% activity corresponds to 10.6 μmol min^{-1} mg^{-1}.

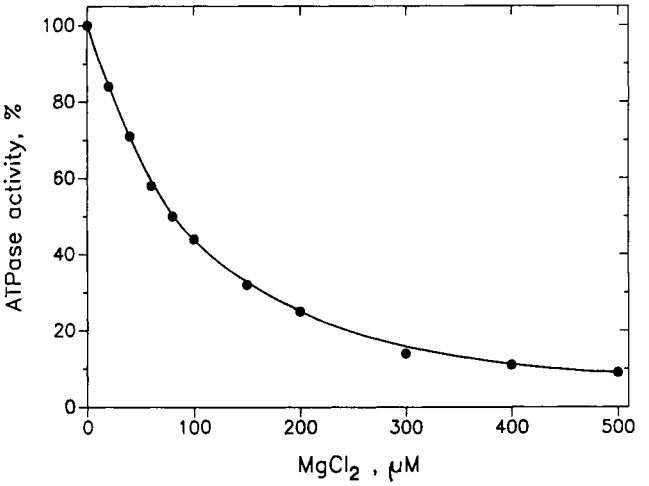

FIGURE 5: Dependence of (ATP-ha)CF_1 inactivation on the Mg^{2+} concentration. CF_1 was incubated in the presence of indicated concentrations of Mg^{2+} for 10 s, and then Mg-ATPase activity was measured in the presence of 1 mM NaN_3 as described under Experimental Procedures. 100% activity corresponds to 13.2 μmol min^{-1} mg^{-1}.

the binding of Mg^{2+}, i.e., after Mg^{2+} binding to CF_1 there is no step leading to the enzyme inhibition with a rate constant less than about 0.4 s^{-1}.

The Biphasicity of the Mg^{2+}-Induced Inhibition. Closer examination of the results given in Figures 3 and 4 shows that the inactivation of CF_1 by Mg^{2+} is not a monophasic process. An initial more rapid decrease in the activity is followed by further slower inactivation. Some exploration was made of the nature of the biphasicity by assessing the effect of various Mg^{2+} concentrations on the both phases of CF_1 inactivation.

Somewhat surprisingly, the concentration of Mg^{2+} has little or no effect on the slow phase of inactivation (data not shown). This suggests that the slow process of CF_1 inactivation results primarily from an isomerization of the first, more rapidly formed inactive complex of CF_1 with Mg^{2+}. The interaction of CF_1 with Mg^{2+} that leads to the loss of the enzyme activity appears to be a two-step process, although the initial binding of Mg^{2+} inactivates the enzyme. If this is so, higher Mg^{2+} concentrations should be capable of inducing nearly complete inactivation in the initial binding step. This was tested by measuring the rate of inhibition at increasing Mg^{2+} concen-

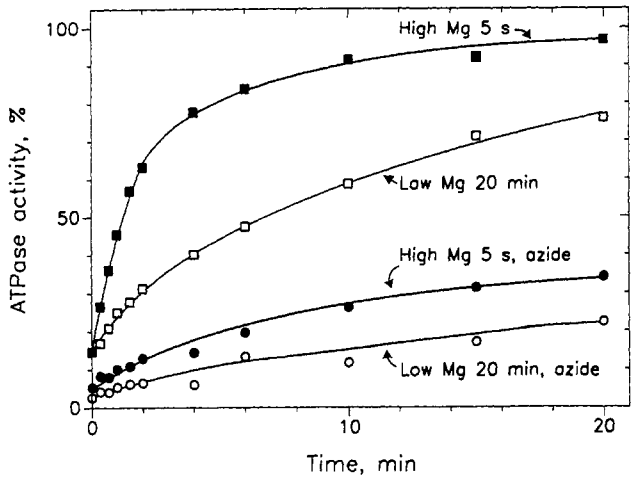

FIGURE 6: EDTA-dependent reactivation of CF_1. Before assay (ATP-ha)CF_1 was incubated in the presence of 2 mM Mg^{2+} for 5 s or in the presence of 30 μM Mg^{2+} for 20 min. Where indicated the reaction mixtures additionally contained 100 μM azide. Then EDTA to the final concentration 5 mM was added. At the time indicated after addition of EDTA, aliquots of the enzyme were taken for the Mg-ATPase assay. 100% activity corresponds to the activity of CF_1 before incubation with Mg^{2+} (10.4 μmol min^{-1} mg^{-1}).

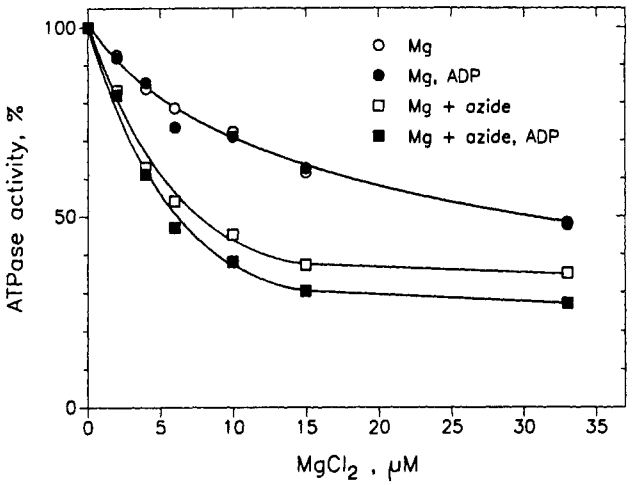

FIGURE 7: The effect of ADP on (GDP-ha)CF_1 inactivation by Mg^{2+}. Prior to the assay, CF_1 was incubated with the indicated concentrations of Mg^{2+} either in the absence or presence of 100 μM azide for 20 min and then, where indicated, ADP and EDTA to the final concentrations 250 μM and 100 μM, respectively, were added, and 10 s later the initial Mg-ATPase activity of CF_1 was measured. 100% activity corresponds to 10.5 μmol min^{-1} mg^{-1}.

trations. Figure 5 shows the dependence on the Mg^{2+} concentration of the extent of CF_1 inactivation after a 10-s incubation with Mg^{2+}. To prevent partial reactivation of CF_1 during the 10-s incubation with Mg-ATP (the time of the Mg-ATPase assay, see Figure 1), 1.0 mM NaN_3 was included in the assay mixture. Nearly complete inhibition of CF_1 is achieved by the short incubation with 500 μM of Mg^{2+}; even greater inhibition would be expected at higher Mg^{2+} concentrations. Fifty percent of activity is lost by a 10-s exposure to 80 μM Mg^{2+}. This corresponds to an apparent rate constant of Mg^{2+} binding near 0.1 s^{-1} and a bimolecular rate constant near 10^3 M^{-1} s^{-1}. Similar results were obtained in the absence of azide if correction was made for the about 15% reactivation that occurs during the assay (data not shown).

The Rate of Release of Inhibitory Mg^{2+} with and without Azide Present. The existence of two inactive complexes of CF_1 with Mg^{2+} is confirmed by the results of the following experiments. Figure 6 shows the effect of time of prior exposure to Mg^{2+} or to Mg^{2+} and azide on the EDTA-induced reactivation of Mg^{2+}-inhibited CF_1. The reactivation of the first, rapidly formed inactive complex of CF_1 (obtained during a 5-s incubation with 2 mM Mg^{2+}) has a half-time of about 1 min. The rate of reactivation is considerably slower if the exposure to Mg^{2+} is prolonged. The complex obtained from a 20-min incubation of the enzyme in the presence of 30 μM Mg^{2+} is reactivated more slowly ($t_{1/2}$ about 6–7 min, Figure 6).

Because azide does not increase the rate of Mg^{2+} binding to CF_1 (Figures 2–4), the cause of enhanced inhibition in the presence of azide is likely to be a decrease of the k_{off} for Mg^{2+}. The results in Figure 6 show that presence of azide effectively slows down the reactivation of both the rapidly and more slowly formed complexes with inhibitory Mg^{2+} as expected for a decreased rate of Mg^{2+} dissociation.

Effect of Catalytic Site ADP on Inhibitory Mg^{2+} Binding. Results obtained in the course of these investigations suggested that the binding of inhibitory Mg^{2+} to CF_1 showed a more pronounced dependence on the prior binding of ADP at the catalytic site than indicated by earlier studies (Guerrero et al., 1990b). Data presented here show that the binding of ADP at CF_1 catalytic site considerably increases the affinity for inhibitory Mg^{2+}.

Heat activation of CF_1 in the presence of GDP gives an enzyme with less than one catalytic site ADP per mole of enzyme. Such (GDP-ha)CF_1 can only be partially inactivated by the addition of saturating Mg^{2+} and azide but is all rapidly inactivated if ADP is added to complete the filling of a catalytic site (Guerrero et al., 1990b). Trials on the rate of (GDP-ha)CF_1 inhibition with prior exposure of (GDP-ha)CF_1 to 2 μM ADP or 25 μM Mg^{2+} indicated that the same rate of enzyme inactivation occurs as with the simultaneous addition of Mg^{2+} and ADP or Mg^{2+} alone. This is as expected if the inhibitory Mg^{2+} does not bind if catalytic site ADP is not present.

For a better assessment of binding of inhibitory Mg^{2+} an enzyme with less catalytic site ADP was desirable. We observed that the level of catalytic site ADP in the (GDP-ha)CF_1 could be reduced by dialysis against 50 mM PP_i. Dialysis of 20–25 μM (GDP-ha)CF_1 for 4 h at room temperature against 500–1000 volumes of 50 mM PP_i at pH 8.0 gave preparations that could be inactivated by only 30–35% by 100 μM Mg^{2+} and 100 μM azide. If CF_1 without tightly bound ADP at catalytic site is able to bind inhibitory Mg^{2+}, exposure to Mg^{2+} in the presence or absence of azide should produce a complex that could be inactivated readily by the short incubation of CF_1 with ADP because the rate of ADP binding to the catalytic site is high (Guerrero et al., 1990b). To reduce the possibility of inactivation of CF_1 by some binding of Mg^{2+} after addition of ADP 100 μM EDTA (final concentration) was included in the medium. The same behavior was noted, however, in the absence of the EDTA addition as expected if little or no Mg^{2+} inhibition occurred in the 5-s incubation. The data obtained are presented in Figure 7. Incubation of CF_1 with various concentrations of Mg^{2+} for 20 min in the absence or presence of 100 μM azide gave only partial inhibition of the enzyme activity (curves 1 and 2). This inhibition likely reflects the amount of enzyme that still has a catalytic site ADP present. After the incubation with Mg^{2+}, a short exposure to 250 μM ADP and 100 μM EDTA before the Mg-ATPase assay does not change the pattern of Mg^{2+}-induced inhibition in the absence as well in the presence of 100 μM azide (curves 3 and 4). The data show that in the absence of ADP bound at the catalytic site of CF_1, Mg^{2+} up to 35 μM concentration does not form a complex with CF_1 that can be

Table I: Values of Rate Constants for the Interaction of CF_1 with Inhibitory Mg^{2+} [a]

rate or equilibrium constant	present work	from Milgrom and Murataliev (1989)
k_1 (M^{-1} s^{-1})	1×10^3	
k_{-1} (s^{-1})	1×10^{-2}	1×10^{-2}
K_1 (M)	1×10^{-5}	
k_2 (s^{-1})	7×10^{-3}	7×10^{-3}
k_{-2} (s^{-1})	1.8×10^{-3}	0.4×10^{-4}
K_2	0.26	0.08
$K_1 \times K_2$ (M)	2.6×10^{-6}	

[a] See Scheme I.

rapidly inactivated upon ADP binding.

Discussion

Our results show that the interactions of CF_1 with azide are quite similar to those of the mitochondrial enzyme. With the enzyme that has not been inhibited by prior exposure to Mg^{2+}, azide does not affect the initial activity but strongly inhibitis the steady-state activity of CF_1. With the enzyme that has catalytic site ADP present and has been inhibited by prior exposure to Mg^{2+}, azide prevents the ATP-dependent reactivation of the inactive complex (Figure 1). The formation of the inactive complex results from a relatively slow binding of Mg^{2+} (Guerrero et al., 1990b). Additional insight into azide action is given by our demonstration that azide does not increase the rate of Mg^{2+} binding but decreases the rate of dissociation of Mg^{2+} from the inactive complex. Such behavior suggests that the binding site for azide does not exist until the inhibitory Mg^{2+} has bound.

The results given in Figure 7 show that CF_1 without ADP bound at the catalytic site does not form a detectable complex with Mg^{2+} that is rapidly inactivated by the binding of ADP. We conclude that the inhibitory effect of Mg^{2+} on CF_1 is shown only when such catalytic site ADP is present. This behavior is akin to that of nucleotide-depleted MF_1. Such an enzyme without ADP at a catalytic site is not inhibited by exposure to Mg^{2+} before being assayed (Minkov et al., 1979; Drobinskaya et al., 1985). These properties of MF_1 and CF_1 give evidence that the binding site for inhibitory Mg^{2+} does not exist unless the catalytic site ADP is present.

The results presented in Figures 3–6 revealed an unexpected characteristic of the Mg^{2+} inhibition. They show that inactivation of CF_1 by Mg^{2+} is a two-step process that can be described by the following minimal scheme, where *both* complexes A and B are inactive, and E·ADP is CF_1 with a catalytic site ADP present:

Scheme I

$$\text{E·ADP} + Mg^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{A}}{\overset{1}{\text{E·ADP·Mg}^{2+}}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{B}}{\overset{2}{*\text{E·ADP·Mg}^{2+}}}$$

Complex A is formed during the initial interaction of Mg^{2+} with E·ADP and has a dissociation constant of about 10–15 μM. This complex is relatively slowly converted to the complex B. The rate of this second step is independent of the medium Mg^{2+} concentration, but the complex shows an increased affinity for Mg^{2+}. The rate constants for forward reactions 1 and 2 are designated as k_1 and k_2 and for the reverse as k_{-1} and k_{-2}, respectively. The values of the rate constants were calculated from the results of the following figures: k_1, Figures 4 and 5, k_2, Figures 3 and 7, k_{-1} and k_{-2}, Figure 6. The estimated rate and equilibrium constants for steps 1 and 2 are given in Table I. The K_d for Mg^{2+} decreases to 2.6 μM in step 2 representing a 3–5-fold increase in affinity for Mg^{2+} (Figure 2). Carmeli and co-workers have reported previously that with increase in the time of incubation less Ca^{2+} is required for inhibition of CF_1 (Carmeli et al., 1979). They also mentioned, without giving data, that a similar behavior was shown by Mg^{2+} and Mn^{2+}. Our results thus confirm and extend their observations.

The results in Figures 2–4 show that azide affects neither the rate of formation of complex A nor the rate of its isomerization to complex B (see Scheme I). Importantly, azide decreases the rate of reactivation of both complexes (Figure 6). After Mg^{2+} has bound, a tight binding site for azide is present in both complexes.

Recently it was reported that the Ca-ATPase activity of activated CF_1 in the presence of 4 mM Ca^{2+} is inhibited very slowly by 2.5 mM azide (Andralojc & Harris, 1990). The authors concluded that azide binds to CF_1 slowly. It seems more likely to us that the slow inhibition of CF_1 by azide resulted from slow binding of some contaminating Mg^{2+} or similar cation present in the media used.

The two-step model, following Mg^{2+} addition to CF_1 with ADP at a catalytic site as presented here, appears to be analogous to the two-step reaction sequence found when ADP is added to nucleotide-depleted MF_1 in the presence of Mg^{2+} (Milgrom & Murataliev, 1989). In both instances, an inactive enzyme·ADP·Mg^{2+} complex is initially formed. Milgrom and Murataliev (1989) noted also that with continued incubation the affinity for ADP increased. In this paper, with CF_1 we noted an increase in affinity for Mg^{2+}. Likely, the affinities for both Mg^{2+} and ADP are increased with both CF_1 and MF_1 complexes.

The comparison of the rate constants found with CF_1 with those for a two-step process for MF_1 (Milgrom & Murataliev, 1989) shows (Table I) close coincidence of the values k_{-1}, k_2, and k_{-2} (the only constants that are intrinsic feature of the enzyme·ADP·Mg^{2+} complex) for the enzymes from plant and animal sources. To us this favors the view that in both cases the same process is involved. It seems likely that F_1 ATPase from mitochondria or chloroplasts, and with ADP at a catalytic site, becomes inactive during the initial combination with inhibitory Mg^{2+}. The initial inactive complex is converted to a more stable and still inactive complex.

Added in Proof

It has recently been reported (Bulygin & Vinogradov, 1991) that there is a behavior of MF_1 ATPase similar to that recorded here for CF_1 ATPase.

References

Andralojc, P. J., & Harris, D. A. (1990) *Biochim. Biophys. Acta 1016*, 55–62.

Binder, A., Jagendorf, A., & Ngo, E. (1978) *J. Biol. Chem. 253*, 3094–3100.

Bruist, M. F., & Hammes, G. G. (1981) *Biochemistry 20*, 6298–6305.

Bulygin, V. V., & Vinogradov, A. D. (1991) *Biochem. J. 276*, 149–156.

Carmeli, C., Lifshitz, Y., & Gutman, M. (1979) in *Cation Flux across Biomembranes*, pp 249–259, AP, Inc.

Carmeli, C., Lifshitz, Y., & Gutman, M. (1981) *Biochemistry 20*, 3940–3944.

Chernyak, B. V., Dukhovich, V. F., & Khodjaev, E. Y. (1988) *FEBS Lett. 230*, 159–162.

Drobinskaya, I. E., Kozlov, I. A., Murataliev, M. B., & Vulfson, E. N. (1985) *FEBS Lett. 182*, 419–423.

Duggett, S. D., Tomaszek, T. A., & Schuster, S. M. (1985) *Arch. Biochem. Biophys. 236*, 815–824.

Ebel, R. E., & Lardy, H. A. (1975) *J. Biol. Chem. 250*, 190–196.

Feldman, R. I., & Boyer, P. D. (1985) *J. Biol. Chem. 260*, 13088-13094.
Futai, M., Noumi, T., & Maeda, M. (1989) *Annu. Rev. Biochem. 58*, 111-136.
Guerrero, K. J., Ehler, L. L., & Boyer, P. D. (1990a) *FEBS Lett. 270*, 187-190.
Guerrero, K. J., Xue, Z., & Boyer, P. D. (1990b) *J. Biol. Chem. 265*, 16280-16287.
Hackney, D. D. (1979) *Biochem. Biophys. Res. Commun. 91*, 233-238.
Harris, D. A. (1978) *Biochem. Biophys. Acta 463*, 245-273.
Harris, D. A. (1989) *Biochem. Biophys. Acta 974*, 156-162.
Hiller, R., & Carmeli, C. (1985) *J. Biol. Chem. 260*, 1614-1617.
Hiller, R., & Carmeli, C. (1990) *Biochemistry 29*, 6186-6192.
Lien, S., & Racker, E. (1971) *Methods Enzymol. 23*, 547-555.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Milgrom, Y. M., & Murataliev, M. B. (1989) *Biochim. Biophys. Acta 975*, 50-58.
Milgrom, Y. M., & Boyer, P. D. (1990) *Biochim. Biophys. Acta 1020*, 43-48.
Milgrom, Y. M., Muntjan, M. S., & Skulachev, V. P. (1988) *Biol. Membr. 5*, 565-572.
Milgrom, Y. M., Ehler, L. L., & Boyer, P. D. (1990) *J. Biol. Chem. 265*, 18725-18728.
Milgrom, Y. M., Ehler, L. L., & Boyer, P. D. (1991) *J. Biol. Chem. 266*, 11511-11558.
Minkov, I. B., & Strotmann, H. (1989) *Biochim. Biophys. Acta 973*, 7-12.
Minkov, I. B., Fitin, A. F., Vasilyeva, E. A., & Vinogradov, A. D. (1979) *Biochem. Biophys. Res. Commun. 89*, 1300-1306.
Minkov, I. B., Vasilyeva, E. A., Fitin, A. F., & Vinogradov, A. D. (1980) *Biochem. Int. 1*, 478-485.
Moroney, J., Lopresti, L., McEwen, B. F., McCarty, R. E., & Hammes, G. G. (1983) *FEBS Lett. 158*, 58-62.
Moyle, J., & Mitchell, P. (1975) *FEBS Lett. 56*, 55-61.
Ohnishi, S. T., & Gall, R. S. (1978) *Anal. Biochem. 88*, 347-356.
Penefsky, H. (1977) *J. Biol. Chem. 252*, 2891-2899.
Shapiro, A. B., & McCarty, R. E. (1990) *J. Biol. Chem. 265*, 4340-4347.
Strotmann, H. (1986) in *Photosynthesis III: Photosynthetic Membranes and Light Harvesting Systems* (Staehelin, L. A., & Arntzen, C. J. eds.) pp 584-594, Springer-Verlag, Heidelberg.
Vasilyeva, E. A., Fitin, A. F., Minkov, I. B., & Vinogradov, A. D. (1980) *Biochem. J. 188*, 807-815.
Vasilyeva, E. A., Minkov, I. B., Fitin, A. F., & Vinogradov, A. D. (1982) *Biochem. J. 202*, 15-23.
Wei, J., Howlett, B., & Jagendorf, A. T. (1988) *Biochim. Biophys. Acta 934*, 72-79.
Xue, Z., & Boyer, P. D. (1989) *Eur. J. Biochem. 179*, 677-681.
Xue, Z., Melese, T., Stempel, K. E., Reedy, T. J., & Boyer, P. D. (1988) *J. Biol. Chem. 263*, 16880-16885.
Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1977) *J. Biol. Chem. 252*, 3480-3485.
Zhou, J.-M., Xue, Z., Du, Z., Melese, T., & Boyer, P. D. (1988) *Biochemistry 27*, 5129-5135.

Kinetic Behavior of the Monodehydroascorbate Radical Studied by Pulse Radiolysis

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Received March 4, 1991; Revised Manuscript Received May 24, 1991

ABSTRACT: The reactions of the monodehydroascorbate radical ($As^{\bullet -}$) with various biological molecules were investigated by pulse radiolysis. $As^{\bullet -}$ reacted with both fully reduced and semiquinone forms of hepatic NADH-cytochrome b_5 reductase with second-order rate constants of 4.3×10^6 and 3.7×10^5 M^{-1} s^{-1}, respectively, at pH 7.0. In contrast, no reaction of $As^{\bullet -}$ with ferrous cytochrome b_5 could be detected by pulse radiolysis, whereas the oxidation of cytochrome b_5 by $As^{\bullet -}$ was observed by ascorbate–ascorbate oxidase method. This suggests that the rate constant of $As^{\bullet -}$ with the ferrous cytochrome b_5 must be several orders in magnitude smaller than that of the disproportionation of $As^{\bullet -}$. On the other hand, $As^{\bullet -}$ reduced Fe^{3+}EDTA with a second-order rate constant of 4.0×10^6 M^{-1} s^{-1} but did not reduce ferric hemoproteins such as metmyoglobin, methemoglobin, and cytochrome b_5 by either the pulse radiolysis or the ascorbate–ascorbate oxidase method.

Ascorbate (AsH^-)[1] plays physiologically important roles in various metabolic reactions involving the biosyntheses of collagen (Prockop et al., 1976; Kivirikko & Myllyla, 1980; Myllyla et al., 1984) and norepinephrine (Rosenberg & Lovenberg, 1980). In addition, AsH^- is critically involved in cellular defense against oxidative injury, serving as a reductant in scavenging reactive oxygen and radical species (Packer et al., 1979; Galaris et al., 1989; Rose et al., 1990; Pietri et al., 1990). In these processes monodehydroascorbate radical ($As^{\bullet -}$) is produced by univalent oxidation of AsH^-. Its production has been shown in enzymatic and nonenzymatic reactions (Yamazaki & Piette, 1961; Skotland & Ljones, 1980;

[1] Abbreviations: AsH^-, ascorbate; $As^{\bullet -}$, monodehydroascorbate; As, dehydroascorbate; OM-cytochrome *b*, cytochrome *b* of the outer mitochondrial membrane; e_{aq}^-, hydrated electron; E-FAD, oxidized NADH-cytochrome b_5 reductase; E-$FAD^{\bullet -}$, semiquinone enzyme; E-$FADH^-$, fully reduced enzyme; EDTA, ethylenediaminetetraacetic acid.

0006-2960/91/0430-8310$02.50/0