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# Control of ATP Hydrolysis by ADP Bound at the Catalytic Site of Chloroplast ATP Synthase As Related to Protonmotive Force and $Mg^{2+}$ †

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ABSTRACT: The activation of the ATP synthesis and hydrolysis capacity of isolated chloroplast membranes by protonmotive force is known to be associated with the release of tightly bound ADP from the ATP synthase. Our data support the view that the activation requires only those structural changes occurring in the steady-state reaction mechanism. The trapping of ADP released during light activation or the chelation of $Mg^{2+}$ with EDTA effectively reduces the rate of decay of the ATPase activity. When the release of tightly bound ADP and $Mg^{2+}$ is promoted by light activation, followed by immediate dilution and washing to retard the rebinding of the ADP and $Mg^{2+}$ released, the ATPase activity remains high in the dark long after the protonmotive force has disappeared. After the addition of ADP and $Mg^{2+}$ the decay of the ATPase activity has the same characteristics as those of the unwashed chloroplast membrane. The results are interpreted as indicating that both $Mg^{2+}$ and ADP must be present prior to exposure to MgATP for the ATPase to be inhibited. However, in contrast to the isolated chloroplast ATPase, the steady-state activity of the membrane-bound ATPase is not inhibited by excess $Mg^{2+}$. The replacement of [$^3$H]ADP from catalytic sites during hydrolysis of unlabeled ATP or during photophosphorylation with unlabeled ADP occurs as anticipated if $Mg^{2+}$ and ADP bound at one catalytic site without $P_i$ block catalysis by all three enzyme sites. The inhibited form induced by $Mg^{2+}$ and ADP may occur only under laboratory conditions and not have an in vivo role.

The activation of the ATPase of chloroplast membranes by exposure to light is a well-established and studied phenomenon (Junge et al., 1970; Schlodder & Witt, 1981; Junesch & Gräber, 1987; Biaudet & Haraux, 1987). This light activation and subsequent inhibition of the ATPase in the dark are known to be closely associated with binding of ADP. A prominent characteristic of the $Mg^{2+}$-activated ATPase of the chloroplast membranes and of the isolated $CF_1$[1] ATPase is the strong inhibition by micromolar concentrations of ADP. Such inhibition has been well documented in important studies from several laboratories (Carmeli & Lifshitz, 1972; Nelson et al., 1972; Strotmann & Bickel-Sandkötter, 1977; Shoshan &

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[1] Abbreviations: $CF_1$, ATPase portion of the ATP synthase from chloroplasts; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; $P_i$, inorganic phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol.

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Selman, 1979; Bar-Zvi & Shavit, 1980, 1982; Dunham & Selman, 1981; Malyan, 1981; Anthon & Jagendorf, 1984; Schumann, 1984; Chernyak & Kozlov, 1986; Strotmann et al., 1987; Huchzermeyer, 1988). As suggested by accumulating evidence (Smith & Boyer, 1976; Feldman & Boyer, 1985; Chernyak & Kozlov, 1986; Leckband & Hammes, 1987, 1988), and shown more definitively by the use of 2-azido-ADP (Zhou et al., 1988), the inhibition results from binding of ADP at catalytic sites, probably without concomitant binding of $P_i$.

The light activation of the ATPase has been explained by the membrane-bound synthase undergoing changes in structure and functional properties, induced by the protonmotive force, that activate the enzyme for both synthesis and hydrolysis. Whether such changes are in addition to or are the same as those necessary for the steady-state catalysis by the synthase has been uncertain. That only those conformational changes associated with the catalytic mechanism may be involved is indicated by properties of bound inhibitory ADP. A tightly bound [$^3$H]ADP present on the synthase after exposure to light and medium [$^3$H]ADP is released during the first turnover time when the thylakoids are subjected to protonmotive force from an acid–base transition, as if the ADP were at catalytic sites (Smith & Boyer, 1976). A catalytic site location for such ADP has been confirmed (Zhou et al., 1988). The experiments reported in this paper show that the loss of ATPase activity of the synthase on the chloroplast membranes correlates closely with the presence of the inhibitory ADP at catalytic sites, but not with the presence or absence of protonmotive force. It is not necessary to postulate any structural changes induced by protonmotive force other than those for release of tightly bound nucleotides from catalytic sites as in the binding change mechanism for ATP synthesis.

The ATPase activity of chloroplast membranes upon exposure to light is higher if suitable reducing agents are present to cleave a disulfide bond in the $\gamma$ subunit (McCarty & Racker, 1968; Nalin & McCarty, 1984). Essentially all the ATPase activity is readily lost in the dark, even with the continued presence of thiol compounds, such as DTT. The studies in this paper are directed toward to a better understanding of the factors determining the activation and inactivation of the ATPase when the critical disulfide remains reduced.

## Experimental Procedures

*Isolation of Chloroplast Membranes.* Chloroplast membranes were isolated from spinach essentially as described by Shavit and Strotmann (1980); after an initial grinding in an isolation buffer containing 0.3 M sucrose, 50 mM NaCl, 1 mM $MgCl_2$, and 10 mM Tricine–NaOH, pH 7.8, they were collected by centrifugation at 3000$g$ and washed once by resuspension in the same solution. The membranes were further washed three times (by centrifugation at 10000$g$) in a solution containing 2 mM Tricine–NaOH, pH 8.0, 50 mM NaCl, and 1 mM $MgCl_2$. Finally, the membranes were resuspended in the isolation buffer to a chlorophyll concentration of about 3 mg/mL and used immediately or rapidly frozen and stored in liquid nitrogen. Chlorophyll concentration was measured according to Arnon (1949).

*Activation by Light and DTT and Measurement of Chloroplast ATPase Activity.* The chloroplast membranes were illuminated for 5 min at room temperature in a reaction mixture containing 25 mM Hepes, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 50 $\mu$M phenazine methosulfate, 20 mM DTT, and 0.2 mg of chlorophyll/mL. Five seconds after the light was turned off aliquots were removed at the times indicated in the figures and added to a solution containing ATP, phosphoenolpyruvate, and pyruvate kinase. The final concentrations were 5 mM ATP, 10 mM phosphoenolpyruvate, and 0.1 mg/mL pyruvate kinase. ATP hydrolysis was allowed to occur for 5 min in the dark. The sample was then quenched by the addition of trichloroacetic acid to a final concentration of 3% (w/v). $P_i$ formed was measured by a conventional method.

*Depletion of Endogenous ADP and $Mg^{2+}$.* The chloroplast membranes were exposed to light in the presence of DTT as described above for 5 min. To retard the rebinding of ADP and possibly of $Mg^{2+}$, the sample then was diluted just before the light was turned off with 10 volumes of a solution containing 100 mM KCl, 0.5 mM EDTA, and 5 mM Tricine–NaOH at pH 8.0. The membranes were collected by centrifugation for 2 min at 10000$g$, washed once with the same medium, and resuspended in it.

*Binding of [$^3$H]ADP to Chloroplast Membranes.* The isolated chloroplast membranes were exposed to light for 2 min as described by Shavit and Strotmann (1980) in a medium containing 25 mM Hepes, 50 mM KCl, 5 mM $MgCl_2$, 50 $\mu$M phenazine methosulfate, 10 $\mu$M [$^3$H]ADP (200000 cpm/nmol of ADP), and 0.2 mg of chlorophyll/mL at pH 8.0. After three washes, the amount of tightly bound [$^3$H]ADP on the chloroplast membranes is about 0.8–1.0 nmol of ADP/mg of chlorophyll. These conditions have been shown to result in the [$^3$H]ADP binding nearly exclusively to the catalytic sites (Zhou et al., 1988).

*Photophosphorylation and Release of Tightly Bound ADP.* ATP synthesis and [$^3$H]ADP release were measured in a reaction mixture containing 25 mM Hepes, 50 mM KCl, 5 mM $MgCl_2$, 50 $\mu$M phenazine methosulfate, 1 mM ADP, 10 mM $P_i$ (for [$^3$H]ADP release) or 10 mM [$^{32}$P]$P_i$, 1500 cpm/nmol (for ATP synthesis), and 0.2 mg of chlorophyll/mL at pH 8.0. The illumination time was controlled by an electronic shutter. ATP synthesis and [$^3$H]ADP release were stopped by trichloroacetic acid and centrifugation, respectively. [$^{32}$P]ATP was isolated by water-saturated isobutyl alcohol/benzene as described by Avron (1960). [$^3$H]ADP released was measured by counting the supernatant after removal of membranes by centrifugation.

## Results

*Decay of Light- and DTT-Activated ATPase Activity of the Chloroplast Membranes in the Dark Is Promoted by ADP.* As noted in the introduction, it is well-known that the light- and DTT-activated ATPase activity of chloroplast membranes decays rapidly after the light is turned off without the addition of ATP and that ADP promotes the decay process. Figure 1 shows a typical time course of the decay of ATPase activity after the light is turned off in the presence or absence of an added low concentration of ADP. The addition of 20 $\mu$M ADP greatly promotes the decay of the ATPase activity. The protonmotive force is known to decay with a half-time of less than 1 min after the light is turned off (Hind & Jagendorf, 1963). In the presence of ADP, the ATPase activity after 5 s in the dark is less than 10% of that observed without ADP addition. Such results are consistent with the rapid decay of ATPase activity in the dark being controlled primarily by the tight binding of ADP and not by the drop of protonmotive force. As noted in the insert to Figure 1, the ADP concentration for half-maximal effect is about 1 $\mu$M, as reported earlier (Strotmann & Bickel-Sandkötter, 1977; Dunham & Selman, 1981).

*Removal of Endogenous ADP and $Mg^{2+}$ Partly Prevents the Decay of ATPase Activity of the Chloroplast Membranes.* The isolated chloroplast membranes retain bound ADP even

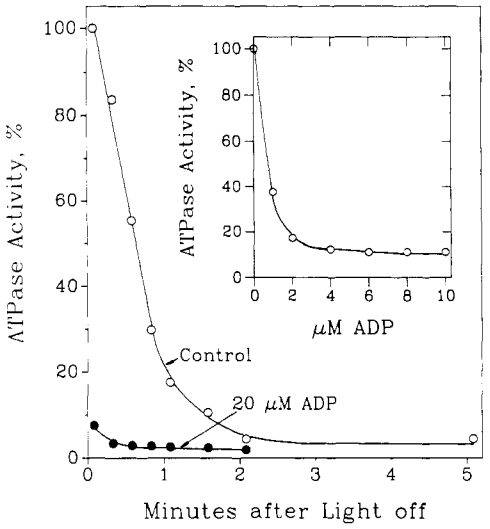


FIGURE 1: Decay of ATPase activity of chloroplast membranes after the light is turned off. Conditions were as described under Experimental Procedures. ADP was added during the light activation. After the light was turned off aliquots were removed at the times indicated. ATP, pyruvate kinase, and phosphoenolpyruvate were added to final concentrations of 5 mM, 0.1 mg/mL, and 10 mM, respectively. (Insert) Dependence of the decay of ATPase activity on ADP concentration. ATP was added 5 s after the light was turned off.

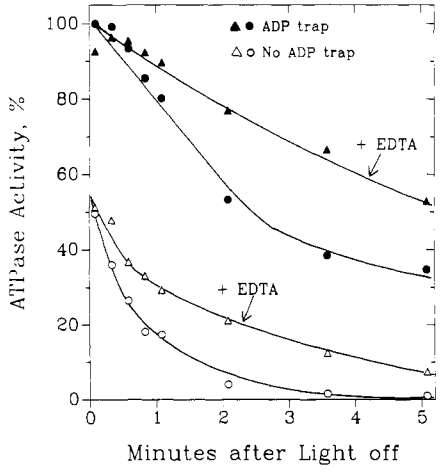


FIGURE 2: Trapping of endogenous ADP and $Mg^{2+}$ partly prevents the decay of ATPase activity. Pyruvate kinase and phosphoenolpyruvate were present during the light activation (ADP trap) or were added for the ATPase assay at the time of ATP addition (no ADP trap). Where indicated, 10 mM EDTA was added 10 s before the light was turned off. To make samples uniform during the ATPase assay, all samples were brought to 10 mM EDTA and a total of 15 mM $Mg^{2+}$ at the time that ATP was added for the ATPase assay.

after extensive washing. Such bound ADP may promote the activity loss noted in the Figure 1 curve without ADP addition. It seemed possible that in the light some of this ADP might be released and be available to pyruvate kinase and thus the subsequent inhibiting effect in the dark would be decreased. Also, particularly in view of the inhibition induced by $Mg^{2+}$ with $CF_1$ containing tightly bound ADP (Carmeli et al., 1981; Feldman & Boyer, 1985), tests were made on the effect of trapping of ADP by pyruvate kinase and chelating $Mg^{2+}$ with EDTA. Figure 2 shows that trapping of medium ADP or chelation of $Mg^{2+}$ considerably retards the decay of ATPase activity.

It should be noted that in the presence of phosphoenolpyruvate, pyruvate kinase, and EDTA there is still about 50% of the ATPase activity left even 5 min after the light is turned off. Most of the protonmotive force should have long disappeared, giving further evidence that the presence or absence of tightly bound ADP and $Mg^{2+}$, not protonmotive force, is determining whether the membrane-bound $CF_1$ is in an inactive form or active form for ATP hydrolysis.

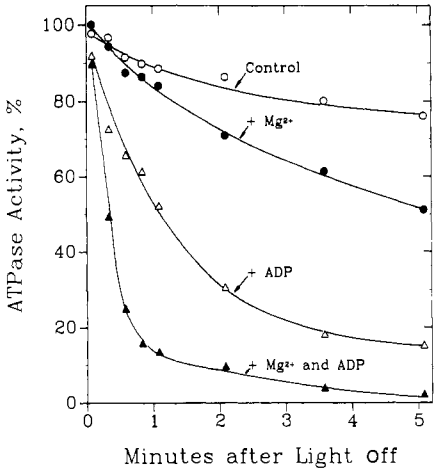


FIGURE 3: Dependence of the loss of the ATPase activity on $Mg^{2+}$ and ADP. The chloroplast membranes were depleted of endogenous ADP and $Mg^{2+}$ as described under Experimental Procedures. The washed chloroplast membranes were reexposed to light for 30 s with no additions (control) or in the presence of 5 mM $MgCl_2$, 1 μM ADP, or 5 mM $MgCl_2$ and 1 μM ADP, as indicated in the figure.

*Exposure to Light Followed by Washing Increases the Dependence of ATPase Inactivation on Addition of ADP and $Mg^{2+}$.* The results given in the preceding section suggested that ATPase activity decay might be prevented by more effective removal of ADP and $Mg^{2+}$ from the chloroplast membranes. The tightly bound ADP on the chloroplast membranes is released to the medium when the membranes are exposed to the light (Strotmann & Bickel-Sandkötter, 1977). To find if this might provide a way to further reduce the endogenous ADP and $Mg^{2+}$ from the chloroplast membranes, the chloroplast membranes were exposed to the light in the presence of DTT, diluted 10-fold into an EDTA-containing solution just before the light was turned off, and then washed as outlined under Experimental Procedures. Figure 3 shows that after such treatment and brief reexposure of the chloroplast membranes to the light the decay of ATPase activity in the dark is almost completely prevented. Adding back $Mg^{2+}$ alone slightly accelerates the decay. The addition of ADP is somewhat more effective. Addition of both ADP and $Mg^{2+}$ restored an inhibitory behavior similar to that given in Figure 1. Exposure of the isolated $CF_1$ containing tightly bound ADP at catalytic sites to $Mg^{2+}$ is known to be required for an inhibitory effect to be observed (Carmeli & Lifshitz, 1981; Feldman & Boyer, 1985). Thus it seems likely that the inhibition observed with the membrane-bound enzyme without $Mg^{2+}$ addition probably results from the presence of quite low amounts of $Mg^{2+}$ from residual endogenous $Mg^{2+}$ of chloroplast membranes or from contamination of reagents. Similarly, the slight inhibition noted without ADP addition probably results from the presence of a small residual amount of ADP at catalytic sites. The data are thus consistent with the decay of ATPase activity of the chloroplast membranes in the dark being dependent upon binding of both ADP and $Mg^{2+}$.

It may be noted that the inhibition by addition of 1 μM ADP observed in Figure 3 is less than that reported in the inset of Figure 1. This is as expected due to the greater amount of endogenous ADP present under the conditions as used for Figure 1.

*Chloroplast Membranes Can Retain ATPase Activity in the Dark for Prolonged Periods after Removal of Residual*

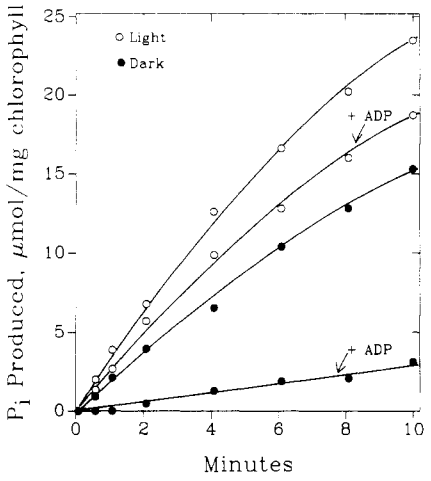


FIGURE 4: DTT-treated and washed chloroplast membranes show high ATPase activity without reactivation in the light. Chloroplast membranes depleted of endogenous ADP and $Mg^{2+}$ (see Experimental Procedures) were kept in the dark for at least 30 min before the experiments. The membranes were then incubated in the presence of 5 mM $Mg^{2+}$ with or without 1 μM ADP either for 1 min in the dark or for 30 s in the light. ATP was then added and the time course of $P_i$ formation measured.

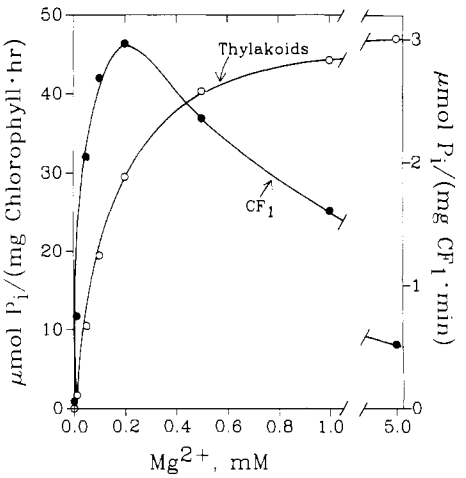


FIGURE 5: Effect of increasing $Mg^{2+}$ concentration on the ATPase activity of chloroplast membranes and of $CF_1$. The chloroplast membranes were prepared as indicated under Experimental Procedures for depletion of endogenous ADP and $Mg^{2+}$ and used without further light activation. The $CF_1$ was isolated and heat activated as reported previously (Zhou et al., 1988). $P_i$ formed was measured 5 min after the addition of the chloroplast membranes or $CF_1$ to the assay medium at pH 8.2 containing 50 mM Tricine, 50 mM KCl, 10 mM phosphoenolpyruvate, 0.1 mg/mL pyruvate kinase, 0.1 mM ATP, and $Mg^{2+}$ as indicated in the figure.

*ADP.* If the ATPase activity of the chloroplast membranes is controlled primarily by the bound $Mg^{2+}$ and ADP but not the protonmotive force, then after removal of endogenous ADP and $Mg^{2+}$ by light exposure and subsequent washing in the dark, the membranes might show high ATPase activity without repeated light exposure. That this is the case is shown in Figure 4. After removal of the endogenous ADP and $Mg^{2+}$ from the chloroplast membranes, high ATPase activity is found even without reexposure to the light. The data of Figure 4 were obtained at least 30 min after the light exposure to promote endogenous ADP release. Clearly any protonmotive force is long gone. The reexposure to the light does stimulate the ATPase activity of the membranes somewhat (Figure 4); this might be because some ADP remained tightly bound to catalytic sites of the ATP synthase after the first light exposure and washing.

The high ATPase activity remaining under the conditions of Figure 4 showed sensitivity to prior exposure to ADP and $Mg^{2+}$ quite similar to that for the light-activated ATPase reported in Figure 3; the half-time for the decay of ATPase activity with 1 μM added ADP and 5 mM $Mg^{2+}$ was about 15 s.

There is a slow decline (about 5% per minute) in ATPase activity during the time of assay in the dark, as seen in Figure 4. A similar time course is observed whether or not the ADP formed is removed by pyruvate kinase. This suggested that the steady-state rate of the chloroplast membrane ATPase might not be subject to $Mg^{2+}$ inhibition. That this is so is shown in the next section.

*Chloroplast Membrane ATPase Is Not Inhibited by Excess $Mg^{2+}$.* A well-known characteristic of the ATPase activity of $CF_1$ is the pronounced inhibition of the steady-state rate by excess $Mg^{2+}$. The chloroplast membranes show a striking difference in this regard. Figure 5 shows that dependency on $Mg^{2+}$ concentration of the ATPase activity of membrane and $CF_1$ preparations as measured under identical conditions. The membrane ATPase activity shows no inhibition even if up to 4–5 mM excess $Mg^{2+}$ over ATP is used. In contrast, the $CF_1$ is strongly inhibited at higher $Mg^{2+}$ concentrations.

In the experiment of Figure 5 a pyruvate kinase trapping system was present to remove ADP. In similar experiments without ADP removal, the velocity of the membrane-bound ATPase declined some as ADP was produced, but no inhibitory effect of excess $Mg^{2+}$ was noted. Also, the presence of 5 mM $NH_4Cl$ to reduce protonmotive force did not result in any $Mg^{2+}$ inhibition (data not shown). With $CF_1$, the inhibitory effect of excess $Mg^{2+}$ during ATP hydrolysis is observed even when medium ADP is removed by pyruvate kinase; bound ADP may be responsible (Zhou et al., 1988).

*Correlation of ATPase Activity with the Amount of Tightly Bound ADP at Catalytic Sites.* As reported in a recent paper from our laboratory (Zhou et al., 1988), the results from labeling experiments with 2-azido-ATP and 2-azido-ADP add convincing evidence to earlier suggestions that the ADP which controls the ATPase activity of the synthase on the chloroplast membranes and of the isolated $CF_1$ ATPase is bound at a catalytic site without $P_i$. If this is so, the loss of ATPase activity with $Mg^{2+}$ present after the light is turned off should correlate with the amount of catalytic site ADP that is not readily replaced during ATP hydrolysis. By addition of a trace amount of [$^3$H]ADP to the reaction mixture during light activation, [$^3$H]ADP interchanges with ADP at catalytic sites but not at noncatalytic sites. We tested the replacement of such bound [$^3$H]ADP when ATP was added at different times after the light was turned off. The $P_i$ formed by ATP hydrolysis and the [$^3$H]ADP remaining bound to the chloroplast membranes were measured after 5 min of ATP hydrolysis. The results are presented in Figure 6. They demonstrate that the loss of ATPase activity correlates quite well with the amount of tightly bound ADP that remains at catalytic sites on the chloroplast membranes.

After the light-activated ATPase activity of the chloroplast membranes decays, as in Figure 6 after 5 min in the dark, it is no longer activated by prolonged incubation with MgATP. This is unlike $CF_1$ which, when inhibited by catalytic site bound ADP and $Mg^{2+}$, will regain ATPase activity within a few minutes in the presence of MgATP (Feldman & Boyer, 1985).

*Removal of Inhibitory ADP upon Initiation of Photophosphorylation.* When the chloroplast membranes are exposed to the light under conditions for photophosphorylation, a constant rate of ATP synthesis is attained after a short lag

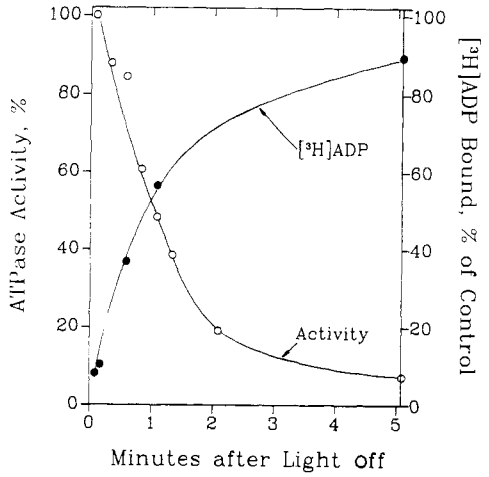


FIGURE 6: Loss of ATPase activity correlates with the amount of tightly bound [$^3$H]ADP. The chloroplast membranes were activated by light and DTT in the presence of a trace amount of [$^3$H]ADP, and ATP hydrolysis was followed as outlined in the text and under Experimental Procedures. 5 mM ATP was added at the times indicated after the light was turned off. After 5 min in the dark, the reaction was stopped either by addition of trichloroacetic acid for the measurement of $P_i$ formed or by centrifugation at 10000$g$ followed by extensive washing for the measurement of bound [$^3$H]ADP.

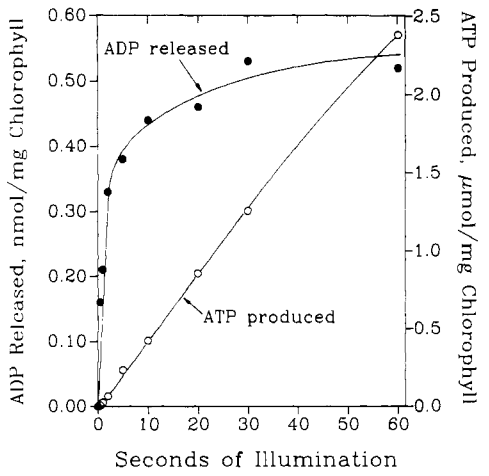


FIGURE 7: ATP synthesis and the release of tightly bound [$^3$H]ADP during photophosphorylation. Conditions for loading [$^3$H]ADP to the chloroplast membranes and for the measurement of ATP synthesis and [$^3$H]ADP release were as given under Experimental Procedures.

time. To test the release of tightly bound [$^3$H]ADP from catalytic sites under these conditions, chloroplast membranes were labeled by a short exposure to medium [$^3$H]ADP in the light, followed by washing. Photophosphorylation is initiated with an automatic shutter apparatus for short illumination times. Figure 7 shows the time course of ATP formation and the release of tightly bound [$^3$H]ADP in the light. The first three points of the plot in Figure 7 represent illumination times of 0.5, 1, and 2 s. Photophosphorylation appears to reach a near maximal rate within 2 s, at which time most but not all of the bound [$^3$H]ADP has been replaced. These results are similar to those noted earlier when an acid–base transition was used to activate photophosphorylation and the release of most of the [$^3$H]ADP was attained in a few milliseconds (Smith & Boyer, 1976). With acid–base transition no time lag is necessary to create the protonmotive force.

## Discussion

Our results give strong evidence that the changes associated with the tight binding of both ADP and $Mg^{2+}$ are responsible for the inhibition of ATPase when illumination of chloroplast membranes ceases. They also show that the ATPase can be active in the presence or absence of protonmotive force, although the initial conversion from the inhibited state to an active state depends on the protonmotive force. These characteristics are shown by the delay in onset of inhibition unless ADP is added (Figure 1), the retardation of the onset of inhibition when ADP is removed by pyruvate kinase or $Mg^{2+}$ is chelated by EDTA (Figure 2), the marked delay in onset of the inhibition when endogenous ADP and $Mg^{2+}$ are reduced (Figure 3), the ability to retain activity for a prolonged period in the dark after the chloroplast membranes are once light activated and washed (Figure 4), the demonstration that loss of ATPase activity is paralleled by an increase in the fraction of catalytic site ADP that becomes irreplaceable by MgATP (Figure 6), and the release of most of the tightly bound catalytic site ADP with the onset of photophosphorylation (Figure 7).

The requirement of both $Mg^{2+}$ and ADP for the inhibition of the ATPase activity of chloroplast membranes is in harmony with their need for an inhibition of ATPase activity observed with heart (Vasilyeva et al., 1982) and liver mitochondria (Chernyak et al., 1988). Such requirement is also shown by the isolated $CF_1$ (Carmeli & Lifshitz, 1981; Feldman & Boyer, 1985).

Bakker-Grunwald and Van Dam (1974), who some time ago noted that $Mg^{2+}$ promoted the onset of inhibition of membrane-bound ATPase, suggested that $Mg^{2+}$ might catalyze conversion to a more stable inhibited form. A requirement for binding of $Mg^{2+}$ seems probable, however. As shown by Hochman and Carmeli (1981), $Mn^{2+}$ has effects like $Mg^{2+}$ and binds tightly to $CF_1$. Also, the inhibition of $CF_1$ by $Mg^{2+}$ is reversed by exposure to EDTA (Feldman & Boyer, 1985), a result not expected if the $Mg^{2+}$ had a catalytic role.

As indicated under Results, the small amount of $Mg^{2+}$ present in the experiments reported in Figure 3 suffices for the occurrence of greater than 80% inhibition in 5 min of incubation. This shows that the inhibited form has a high affinity for $Mg^{2+}$. The ability of higher $Mg^{2+}$ concentrations to speed the onset of the inhibition with $CF_1$ (Feldman & Boyer, 1985) suggests that the delay in the onset of inhibition as noted in the present experiments results from a slow $Mg^{2+}$ binding, a possibility that merits additional investigation.

In an experiment similar to that reported in Figure 3, Dunham and Selman (1981) noted that washing of chloroplast membranes after light exposure, as done by Bakker-Grunwald and Van Dam (1974), increased the dependency of the inhibition of ADP. However, Dunham and Selman (1981) did not note the requirement for the incubation with $Mg^{2+}$ prior to the MgATP addition for the inhibition to be observed. They suggest that only the ADP binding is required for the inhibition. Also, the membranes treated by their procedures required reexposure to light to acquire ATPase activity. The conditions we used for preparation of the membranes for the experiments of Figure 3 gave membranes that retained ATPase activity in the dark.

An important characteristic of the $Mg^{2+}$- and ADP-induced inhibition of chloroplast membrane-bound ATPase is that, once it has become established, the inhibition is not reversed by MgATP even in high concentrations. But if ATP is added before the MgADP-induced inactivation reaches completion, the residual ATPase activity only slowly declines with time (e.g., Figure 4) even though the rate of hydrolysis may be well below that necessary to sustain sufficient protonmotive force for photophosphorylation. Such behavior is clearly not as anticipated for a typical competitive inhibition by product. The

binding change mechanism provides a plausible explanation in that the ADP and $P_i$ tightly bound at the catalytic site just after ATP hydrolysis become loosely bound by the cooperative interactions resulting from ATP binding at another catalytic site. As long as the ATP concentration is sufficient, the active enzyme may seldom have tightly bound ADP at a catalytic site without $P_i$, a necessary condition for $Mg^{2+}$ to induce inhibition. If medium ATP is removed and illumination ceases, any tightly bound ATP remaining at a catalytic site can still form tightly bound ADP and $P_i$. This $P_i$ can slowly dissociate leaving catalytic site ADP. Then in the presence of $Mg^{2+}$, the enzyme can form the inhibited state which is not a normal catalytic intermediate. Once this has happened, MgATP can no longer reactivate the membrane-bound enzyme. The intact ATP synthase differs in this respect from the isolated $CF_1$ (Feldman & Boyer, 1985).

How exposure to light can remove the inhibitory MgADP can be explained by the binding change mechanism, without invoking other effects of protonmotive force. When chloroplast membranes with ADP tightly bound at the catalytic site without $P_i$ are exposed to the protonmotive force created by an acid–base transition, the ADP is released to the medium as expected if the site had contained ATP (Smith & Boyer). Even at very low ADP concentrations, very slow turnover of the catalytic sites continues in the light (Stroop & Boyer, 1987), which would thus be expected to cause ADP release as observed in the present experiments. When tightly bound ADP is discharged to the medium, the synthase becomes activated for hydrolysis or for formation of ATP. Such behavior underlies the well-documented light activation of ATPase and photophosphorylation (Junge, 1970; Gräber et al., 1977; Schlodder & Witt, 1981; Junesch & Gräber, 1987). No additional conformational changes other than those the synthase undergoes in the sequence of catalytic steps are necessary for the synthase activation. It also seems probable that the structural changes causing release of the ADP also suffice to facilitate reduction of disulfide bonds in $\gamma$ subunits.

The lack of complete replacement of the bound [$^3$H]ADP in the exposure time necessary for a near maximal rate of photophosphorylation can be attributed to one or more factors. These are the probability that the synthase is not rate limiting for photophosphorylation and thus that maximum synthesis rates can be attained while some synthases are still inoperative, the probable presence of some poorly coupled vesicles so that those with weak synthesizing capacity are delayed in activation and ADP release and do not contribute appreciably to the total rate of photophosphorylation, and the possible presence of slight amounts of the [$^3$H]ADP at noncatalytic sites.

The lack of inhibition of the thylakoid membrane ATPase by excess $Mg^{2+}$ is in contrast to the behavior of $CF_1$. Two explanations for this difference warrant consideration. One possibility, as mentioned above, is that during turnover the membrane-bound enzyme does not lose $P_i$ from the catalytic site as readily as $CF_1$. Another is that ATP hydrolysis may induce a localized energization of the synthase or of the membrane that can reverse or prevent the $Mg^{2+}$-induced inhibition. This would allow activation analogous to the light activation of the ATPase but not necessarily requiring delocalized protonmotive force.

The inability of excess $Mg^{2+}$ to induce inhibition in the membrane-bound ATPase even in the presence of medium ADP raises some question about the possible physiological significance of the ADP- and $Mg^{2+}$-induced inhibited state. It has been suggested that the inhibition may function to preserve ATP in the intact plant in the dark. But our data indicate that when illumination ceases, and even though ADP and excess $Mg^{2+}$ may be present, ATP hydrolysis will continue. It may be that the pronounced inhibition that can be attained from presence of ADP and $Mg^{2+}$ only occurs under special laboratory conditions. It is also tempting to consider that the combination with $Mg^{2+}$ when both ADP and $P_i$ are bound to a catalytic site is an essential step of the catalytic mechanism. In the absence of bound $P_i$, the aberrant inhibitory complex could arise.

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**Registry No.** ATPase, 9000-83-3; ADP, 58-64-0; Mg, 7439-95-4; ATP synthase, 37205-63-3.

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# Characterization of Cytochrome $b_5$ Reconstituted with a Ferric Chlorin and a Ferric Oxochlorin†

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ABSTRACT: The role of the electronic properties of the heme group of rat cytochrome $b_5$ in biological electron transfer was investigated by substituting chlorin analogues for the native protoporphyrin IX prosthetic group. The resultant purified proteins displayed physical and chemical properties distinct from those of the native enzyme. Optical spectroscopy of the ferric chlorin substituted cytochrome $b_5$ revealed a blue-shifted Soret at 404 nm and a band at 586 nm characteristically red-shifted from the protohemin absorption band. The reduced, reconstituted protein displayed maxima at 406, 418, 455, 563, and 600 nm. The oxidized cytochrome $b_5$ containing the oxochlorin analogue produced a red-shifted Soret with maxima at 338, 416, and 602 nm. The reduced species differed only in the visible region with absorption maxima at 508, 554, and 600 nm. Characterization by EPR spectroscopy of the oxochlorin-substituted cytochrome $b_5$ yielded $g$ values of 2.566, 2.375, and 1.756 and respective axial $\Delta/\lambda$ and rhombic $V/\lambda$ components of 2.857 and 3.287, indicating significant electronic distortion in the chlorin ring and an increase in electron donation from the axial histidine ligands. A decrease in the reduction potential of 52 ± 5 mV (50 mM $KP_i$, pH 7.0, 25 °C) for the chlorin-reconstituted cytochrome $b_5$ was determined with respect to that of native cytochrome $b_5$. The reduction potential for the oxochlorin-containing cytochrome $b_5$ was unchanged from that of the native system. Both of the reconstituted proteins were found to be capable of transferring electrons to cytochrome $c$ in a reconstituted system dependent on NADH and cytochrome $b_5$ reductase, thus simulating the activity of native cytochrome $b_5$.

Cytochrome $b_5$, a low molecular weight heme protein, is a native component of liver microsomes (Ito & Sato, 1968) and functions in hepatic electron transfer systems. Specifically, cytochrome $b_5$ has been found to be active in fatty acid hydroxylation in association with cytochrome P-450 and NADPH-cytochrome P-450 reductase (Morgan & Coon, 1984; Chiang, 1981; Hildebrandt & Estabrook, 1971) and in stearoyl-CoA[1] desaturation involving cytochrome $b_5$ reductase and desaturase (Strittmatter et al., 1974; Shimakata et al., 1972; Oshino & Sato, 1971). The amphipathic protein is composed of two distinct regions, a hydrophobic tail which is imbedded in the endoplasmic reticulum (Spatz & Strittmatter, 1971; Rogers & Strittmatter, 1974) and a soluble catalytic core. The hydrophobic tail may be proteolytically clipped with trypsin yielding the soluble unit which retains fully activity (Ito & Sato, 1968). A soluble form of cytochrome $b_5$ lacks the COOH-terminus membrane anchor domain and is active as a methemoglobin reductase in erythrocytes (Hultquist & Passon, 1971).

The prosthetic group, ferric protoporphyrin IX (Figure 1), is liganded by bis(imidazole) coordination to axial histidines-39 and -63. The $b$-type heme is held rigidly in a hydrophobic crevice in an orientation such that the peripheral vinyl substituents are buried and the propionic acid groups are exposed to the aqueous environment (Mathews et al., 1972). The X-ray crystal structure of ferric cytochrome $b_5$ has revealed that one propionic acid interacts freely with solution, while the other is partially buried and forms hydrogen bonds with the hydroxyl group of serine-64 and a peptide amide (Mathews & Argos, 1975). It has been proposed that this acidic carboxyl group may form a salt bridge with the iron center of the heme,

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[1] Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide phosphate; CoA, coenzyme A; cytochrome $b_5$/chl, cytochrome $b_5$ reconstituted with iron(III) chlorin; cytochrome $b_5$/ochl, cytochrome $b_5$ reconstituted with iron(III) oxocholorin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $KP_i$, potassium phosphate; NADH, reduced nicotinamide adenine dinucleotide.

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