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NUCLEOTIDE EXCHANGE AND CONTROL OF ATPase ACTIVITY IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

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(1) Light-activated 'dark' ATPase in *Rhodospirillum rubrum* chromatophores is inhibited by preincubation with ADP or ATP (in the absence of Mg^{2+}). I_{50} values were 0.5 and 6 μM, respectively, after 20 s of preincubation. (2) In the absence of MgATP, the rate constant for dissociation of ADP or ATP from the inhibitory site was less than 0.2 min^{-1} in deenergized membranes. Illumination in the absence of MgATP caused an increase of over 60-fold in both rate constants. (3) In some experiments hydrolysis was performed in the presence of 10 μM Mg^{2+} and 0.2 mM MgATP. Under these conditions, the ADP or ATP inhibition was reversed within about 20 or about 80 s, respectively, after the onset of hydrolysis. This suggests that recovery from ADP or ATP inhibition (i.e., release of tightly bound ADP or ATP) in the dark is induced by MgATP binding to a second nucleotide-binding site on the enzyme. (4) Results obtained with variable concentrations of uncoupler suggest that in the absence of bound Mg^{2+} (see below), MgATP-induced release of tightly bound ADP or ATP does not require a transmembrane $\Delta\tilde{\mu}_{H^+}$. This, together with the inhibitor/substrate ratios prevalent during hydrolysis, suggests that these reactivation reactions involve MgATP binding to a high-affinity binding site ($K_d < 2$ μM). (5) At high concentrations of uncoupler, a time-dependent inhibition of hydrolysis occurred in the control chromatophores as well as in the nucleotide-pretreated chromatophores. This deactivation was dependent on Mg^{2+}. In addition, MgATP-dependent reversal of ADP inhibition in the dark was inhibited by Mg^{2+} at concentrations above 20–30 μM. By contrast, MgATP-dependent reversal of ADP inhibition occurs within 3–4 s, despite the presence of high concentrations of Mg^{2+} if the chromatophores are illuminated during contact with the nucleotides. Uncoupler abolishes the effect of illumination. A reaction scheme incorporating these findings is proposed. (6) The implications of these findings for the mechanism of lightactivation of ATP hydrolysis (Slooten, L. and Nuyten, A., (1981) Biochim. Biophys. Acta 638, 305–312) are discussed.

Introduction

In the previous article [1] it was shown that the membrane-bound ATPase enzyme is in a low-activity state in untreated chromatophores from *Rhodospirillum rubrum*. The enzyme could be activated by application of an (e.g., light-induced) electrochemical proton gradient across the membrane. The results could be summarized in the following scheme:

$$E^o \rightleftharpoons E \leftrightharpoons E'$$

in which E is the active form of the enzyme, E^o and E' are low-activity forms, and open arrows indicate energy-dependent reactions.

In chloroplasts [2,3] and *Rhodopseudomonas capsulata* chromatophores [4], light activation of ATPase is reversed [2,3] or inhibited [2–4] by prein-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; BChl, bacteriochlorophyll.

cubation with low concentrations of ADP. The results indicated that membrane energization was required for dissociation of ADP from an inhibitory site [4,5], a conclusion which is in agreement with earlier work on energy-dependent exchange reactions of labelled nucleotides [6–11]. In chloroplasts, Mg^{2+} accelerated the dark decay of the light-induced active state of the ATPase enzyme in the absence of substrate [12]. Similar results were obtained with *R. rubrum* chromatophores in the presence of substrate [13]. It was proposed that Mg^{2+}, when bound to a regulatory site, inhibits release of ADP, produced in the course of hydrolysis, from the catalytic site if the membrane is deenergized [13]. We decided to check this by looking at the Mg^{2+} dependence of light-activated ATPase without or after ADP preincubation. As shown below, the results are consistent with this hypothesis. However, it was not clear whether, in deenergized membranes, ADP binding was reversible in the absence of Mg^{2+} when MgATP was absent. For this reason, we resorted to a competition method based on our finding (shown below) that preincubation with ATP (in the absence of Mg^{2+}) causes similar inhibition of light-activated ATPase. It turned out that ATP preincubation affected the kinetics of the reaction in a different manner than did ADP preincubation; furthermore, ATP exhibit much less synergism with Mg^{2+} than ADP in causing inhibition of ATPase. We then looked at the effect of successive addition of the two nucleotides on the kinetics, and the extent of inhibition, of ATPase. It appeared that the first nucleotide added (whether ADP or ATP) was tightly bound in the absence of Mg^{2+}, thus preventing inhibition by the second nucleotide. This indicates that MgATP binding after pretreatment with ADP or ATP involves a different nucleotide-binding site. The nature of the tight binding site and the implications of these findings for the mechanism of light activation of the ATPase enzyme [1] are discussed.

Methods

R. rubrum cells were grown and chromatophores were prepared as described previously [1]. ATPase was measured as described earlier [1]. The medium used in the pH measurements contained 0.2 M sucrose, 50 mM KCl, 20 mM NaCl, 0.1 mM EDTA, 0.2–0.6 mM glycylglycine depending on the sensitivity required, NaOH to pH 8.0 and other additions as indicated. In the P_i measurements, NaCl was omitted whereas the glycylglycine concentration was raised to 20 mM.

Bacteriochlorophyll was estimated using an in vivo extinction coefficient of 140 $mM^{-1} \cdot cm^{-1}$ [14]. Mg^{2+} concentrations were calculated using the pK_a values for ATP and the stability constants for the Mg^{2+} complexes of ATP as given by Phillips et al. [15], corrected for an ionic strength of 75 mM. The stability constants for the K^+ and Na^+ complexes of ATP were taken from Ref. 16. From these data an overall stability constant of $20.2 \cdot 10^3$ M^{-1} for Mg complexation with ATP was calculated. The conditional stability constant for the Mg-EDTA complex, $2.22 \cdot 10^6$ M^{-1}, was calculated from Ref. 17.

The source of chemicals was as described previously [1].

Results

(1) Effects of ADP

Fig. 1 shows experiments in which uncoupler-stimulated ATPase was measured in the light. In agreement with earlier results [1], the reaction proceeded at a high rate only after the chromatophores had been illuminated for a few seconds in the absence of uncoupler (open circles). The open and solid circles show experiments in which MgATP and uncoupler (CCCP) were added simultaneously. In this case, light-activated ATPase was inhibited by ADP added prior to these compounds (solid circles), instead of together with them (open circles). This inhibition was not reversed during uncoupled ATP hydrolysis (not shown). However, the ADP inhibition was reversed within 4 s by MgATP added in the light prior to uncoupler (solid vs. open triangles).

In all remaining experiments, hydrolysis was measured in the dark. Fig. 2 shows experiments in which chromatophores were illuminated in the presence of valinomycin; CCCP was added just after the switching off of the light. This treatment leads to a rapid dissipation of the light-induced $\Delta\tilde{\mu}_{H^+}$ (complete within 20 s), but the light-induced active state of the ATPase enzyme has a considerably longer lifetime (cf. Ref. 1). Preincubation with ADP (added after dissipation of the light-induced $\Delta\tilde{\mu}_{H^+}$) caused a severe inhibition of light-activated ATPase (open circles; I_{50} 0.5 μM),

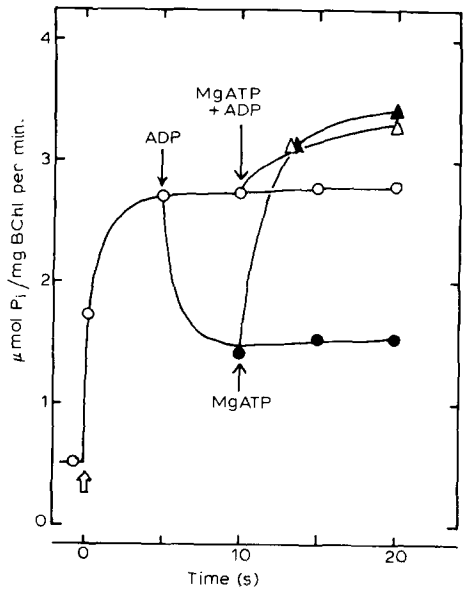

Fig. 1. MgATP-dependent, uncoupler-sensitive reversal of ADP inhibition of light-activated ATPase (pH method). The complete reaction mixture consisted of 5 ml medium supplemented with chromatophores, 0.1 μM nigericin, 20 μM ADP, 1 mM MgATP and 7.7 μM CCCP. Procedure: chromatophores and nigericin were added to 4.5 ml medium. The light was switched on at t = 0 (open arrow). The mixture was completed at the time indicated on the x-axis by addition of 0.5 ml medium containing CCCP and (if required) nucleotides. Rates were determined 4 s later. Each point represents one experiment. (○———○) MgATP and ADP were added along with CCCP. (●———●) ADP was added at t = 5 s; MgATP was added along with CCCP. (▲———▲) ADP and MgATP were added at t = 5 s and 10 s, respectively. (△———△) ADP and MgATP were both added at t = 10 s. The medium was as described in Methods but contained in addition 2 mM $MgCl_2$. MgATP was a mixture of equimolar amounts of $MgCl_2$ and ATP.

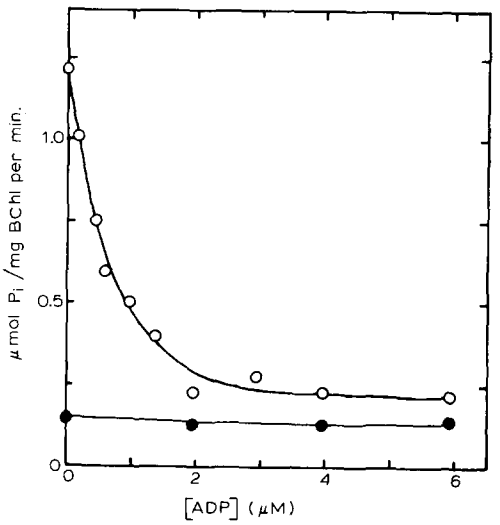

Fig. 2. ADP inhibition of light-activated ATPase (P_i method). Hydrolysis was carried out in 2 ml medium supplemented with 1.2 mM ATP, 3.2 μM ADP, 9.3 mM $MgCl_2$, 4.2 μM CCCP and 0.1 μM valinomycin. (○———○) Chromatophores were illuminated for 10 s in 1.3 medium in the presence of Valinomycin. CCCP was added just after the switching off of the light. The indicated concentrations of ADP were added 20 s after CCCP. The reaction mixture was completed 40 s after CCCP addition by addition of 0.7 ml medium containing $MgCl_2$, ATP and ADP such that in each experiment the ADP concentration at the onset of hydrolysis was 3.8 μM. (●———●) As ○———○ except that no light was given. Reaction time, 2 min.

but had no significant effect on the ATPase reaction catalyzed by the nonactivated enzyme (solid circles). Similar results were obtained in the absence of uncoupler (not shown).

In the above experiments ATPase was measured in the presence of high (millimolar) concentrations of Mg^{2+}. However, the effect of ADP preincubation was strongly dependent on the Mg^{2+} concentration during hydrolysis, as shown in Fig. 3. In these experiments $MgCl_2$ was added at the onset of hydrolysis. Preincubation with 5 μM ADP (10-times the I_{50} value as determined in Fig. 2) was inhibitory only if the ATPase reaction was performed at $MgCl_2$ concentrations close to, or above, the optimal concentration. Raising the ADP concentration above 5 μM had no further effect, apart from competitive inhibition (not shown). The concentration of free Mg^{2+} at the optimal $MgCl_2$ concentration was 20–30 μM, as calculated from the stability constants given in Methods.

The experiments in Fig. 3 were done with the colorimetric P_i technique. Experiments with the pH technique (Figs. 4 and 5) provided more insight into the role of Mg^{2+}. In Fig. 4, hydrolysis was performed in the presence of 10 μM ADP and 10 μM free Mg^{2+}. A transient inhibition of light-activated ATPase (in the presence of uncoupler) was observed when ADP was added before $MgCl_2$ and ATP. The inhibition was reversed within 13–20 s at CCCP concentrations ranging from 4.2 to 25 μM. Light-activated ATPase

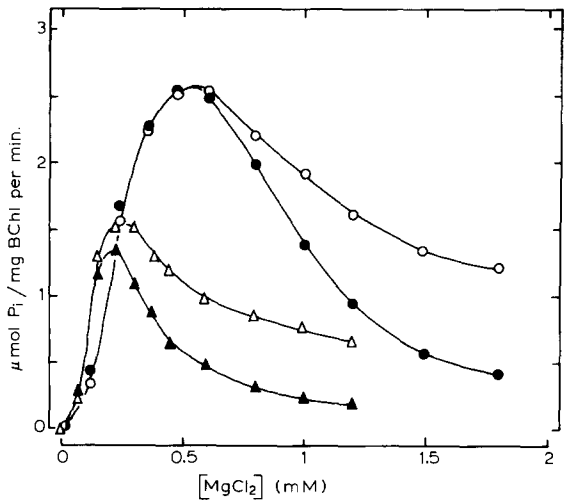

Fig. 3. Effect of ADP preincubation on light-activated ATPase at various $MgCl_2$ concentrations (P_i method). ATPase was measured in 2 ml medium supplemented with 0.3 mM ATP (△, ▲) or 1.2 mM ATP (○, ●), $MgCl_2$ as indicated, 3.2 µM ADP, 4.2 µM CCCP and 0.1 µM valinomycin. CCCP was added after a light trigger (see legend to Fig. 2). $MgCl_2$ and ATP were added, 30 s after CCCP, in 0.7 ml medium. ADP was added along with ATP (△, ○), or before light activation (▲, ●).

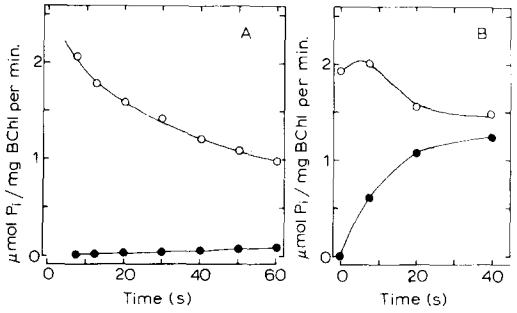

Fig. 5. Mg^{2+} inhibits MgATP-dependent recovery from ADP inhibition of light-activated ATPase (pH technique). Chromatophores were illuminated for 15 s in the presence of 0.1 µM nigericin. CCCP (6 µM) was added just after the switching off of the light. Hydrolysis was started 1 min later by addition of 1.2 mM ATP and 2.5 mM $MgCl_2$. ADP (10 µM) was added either at the onset of hydrolysis (○———○) or before light activation (●———●). Rates were determined from the slope of the traches at the indicated times after the onset of hydrolysis. (B) As A, except that 0.3 mM $MgCl_2$ was added along with ATP. The $MgCl_2$ concentration was raised to 2.5 mM at the indicated time after the onset of hydrolysis. The rates were determined 10 s later. Each point represents one experiment.

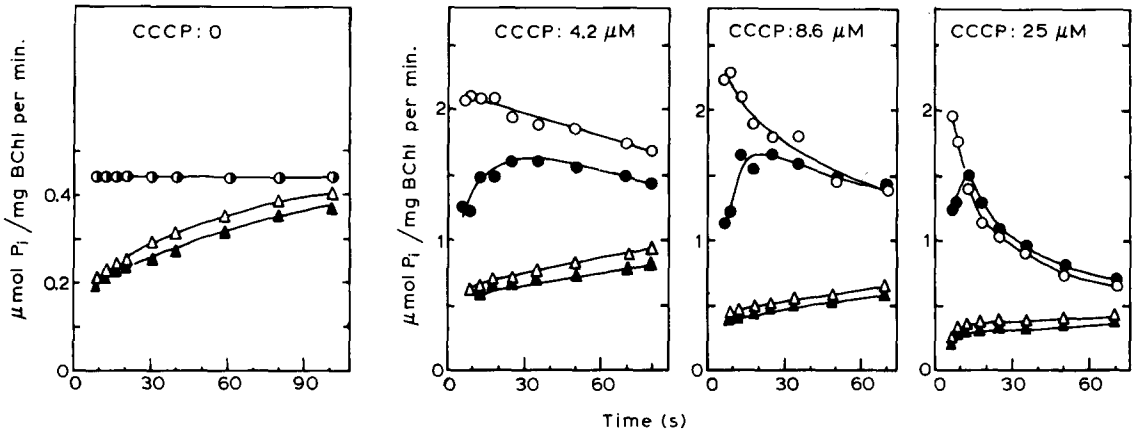

Fig. 4 Effect of ADP preincubation on the kinetics of ATPase with excess ATP (pH technique). ATPase was measured with 0.1 µM nigericin, CCCP as indicated, 10 µM ADP, 1.2 mM ATP and 0.3 mM $MgCl_2$. Rates were determined at the indicated times after the start of the reaction. (○———○) Chromatophores were illuminated for 15 s in the presence of gericin. CCCP was added just after the switching off of the light. Hydrolysis was started 1 min later, by addition of ADP, $MgCl_2$ and ATP. (●———●) As ○———○ except that ADP was added 30 s before the onset of hydrolysis. (△———△ and ▲———▲) As ○———○ and ●———●, respectively, except that no light was given.

measured without uncoupler was not affected by ADP preincubation. In this case, the inhibition was presumably reversed within about 5 s (the time required to obtain a stable baseline after addition of the reaction mixture). The ATPase reaction catalyzed by the nonactivated enzyme (triangles) was only slightly affected by ADP preincubation, whether or not uncoupler was present.

In Fig. 5A, light-activated ATPase was measured in the presence of 1.2 mM Mg^{2+} and 6 μM CCCP. Preincubation with 10 μM ADP elicited an almost complete inhibition, which was not appreciably reversed during the first minute of hydrolysis.

A preceding, short period of ATPase incubation with 10 μM Mg^{2+} restored the capacity for light-activated ATPase measured subsequently at 1.2 mM Mg^{2+} (Fig. 5B).

All this suggests that ADP added before MgATP is bound to an inhibitory site on the light-activated ATPase enzyme. At low Mg^{2+} concentrations, MgATP can rapidly displace ADP from the inhibitory site, even when added to deenergized membranes (Fig. 4). This leads to reactivation of ATPase measured subsequently at high concentrations of Mg^{2+} (Fig. 5). However, Mg^{2+} at concentrations above 20–30 μM inhibits the MgATP-dependent ADP release (Figs. 3 and 5). The Mg^{2+} inhibition in turn is abolished by energizing the membrane during contact with the nucleotides (Fig. 1).

The role of MgATP in these experiments was not quite clear. ADP binding might be reversible by itself (within about 20 s) in deenergized membranes at low Mg^{2+} concentrations. This would allow MgATP to 'pull' the equilibrium in the direction of MgATP binding and restoration of ATPase activity. Alternatively, binding or hydrolysis of MgATP at one site of the enzyme might lead to release of ADP from another site, and thus to restoration of ATPase activity in an energy-independent reaction (Fig. 4). Mg^{2+} would inhibit this reaction in deenergized membranes. Experiments aimed at discriminating between these alternatives will be described below.

(2) Effects of ATP

No hydrolysis was detected in the absence of Mg^{2+} (e.g. Fig. 3). However, addition of ATP prior to $MgCl_2$ led to inhibition of hydrolysis. An example is shown in the solid circles of Fig. 6. In these experiments, 2.1 mM ATP was added during light activation, in the absence of $MgCl_2$. The resulting inhibition of uncoupler-stimulated ATPase was saturated within 2 s (cf. open circles). The inhibition was reversed almost equally rapidly by subsequently adding $MgCl_2$ in the light (solid triangles). The control experiments (open triangles) show that $MgCl_2$ addition during illumination had, by itself, no effect. These experiments are analogues to those shown in Fig. 1 and suggest that MgATP (formed after addition of $MgCl_2$) can rapidly displace ATP, bound to the light-activated

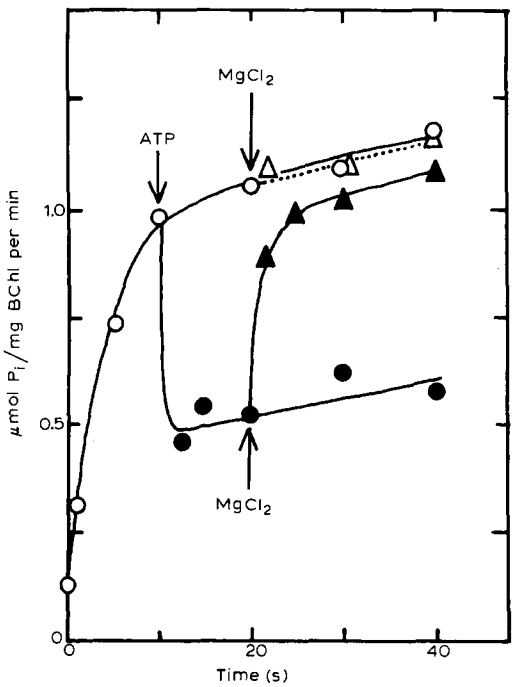

Fig. 6. Mg^{2+}-dependent, uncoupler-sensitive reversal of the ATP inhibition of light-activated ATPase (P_i method). Hydrolysis was carried out in 2 ml medium supplemented with 0.1 μM valinomycin, 4.2 μM CCCP, 1.2 mM ATP and 9.3 mM $MgCl_2$. Reaction time, 2 min. Each point represents one experiment. Procedure: chromatophores and valinomycin were added to 1.3 ml medium. The light was switched on at $t = 0$ and was switched off at the indicated time. The mixture was completed just before the switching off of the light, by addition of 0.7 ml medium containing CCCP, and other components as required. (o———o) ATP and $MgCl_2$ were added along with CCCP. (•———•) ATP was added at $t = 10$ s; $MgCl_2$ was added along with CCCP. (Δ———Δ) ATP and $MgCl_2$ were added at $t = 10$ and 20 s, respectively. (Δ- - - - -Δ) $MgCl_2$ was added at $t = 20$ s; ATP was added along with CCCP.

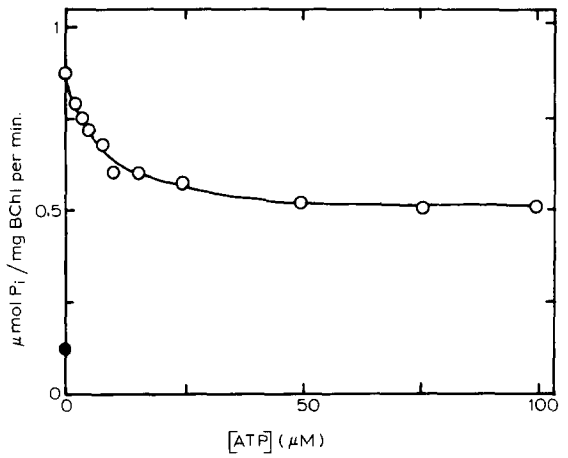

Fig. 7. ATP inhibition of light-activated ATPase (P_i method). The complete reaction mixture was as in Fig. 6. (o———o) Chromatophores were illuminated for 10 s in 1.3 medium in the presence of valinomycin. CCCP was added just after the light trigger. The indicated concentrations of ATP were added 20 s after CCCP. Hydrolysis was started 20 s after ATP addition by addition of 0.7 ml medium containing $MgCl_2$ and the amount of ATP required to complete the mixture. Reaction time, 2 min. (•) As o———o except that no light was given.

enzyme, if the membrane is sufficiently energized during contact with both compounds.

Fig. 7 shows experiments in which ATP was added after dissipation of the light-induced $\Delta\tilde{\mu}_{H^+}$.

After a preincubation time of 20 s, the inhibition caused by ATP was half-saturated at 6 µM. Thus, ATP binding does not require an energized membrane. Similar results were obtained when the Mg^{2+} concentration during hydrolysis was 10 µM (not shown), instead of 8 mM as in Figs. 6 and 7.

ATP hydrolysis catalyzed by the nonactivated enzyme was not strongly affected by ATP preincubation as shown, for example, in Fig. 8 (triangles).

In the course of the reaction, light-activated ATPase recovered partially or completely (depending on the conditions) from inhibition due to ATP preincubation. During assays at 10 µM Mg^{2+} (Fig. 8, circles) a slow but virtually complete recovery from ATP inhibition was observed when the CCCP concentration was less than about 6 µM. Up to this concentration, CCCP had no significant effect on the kinetics of the recovery from ATP inhibition (circles in Fig. 8, left and middle panels). Independently of the

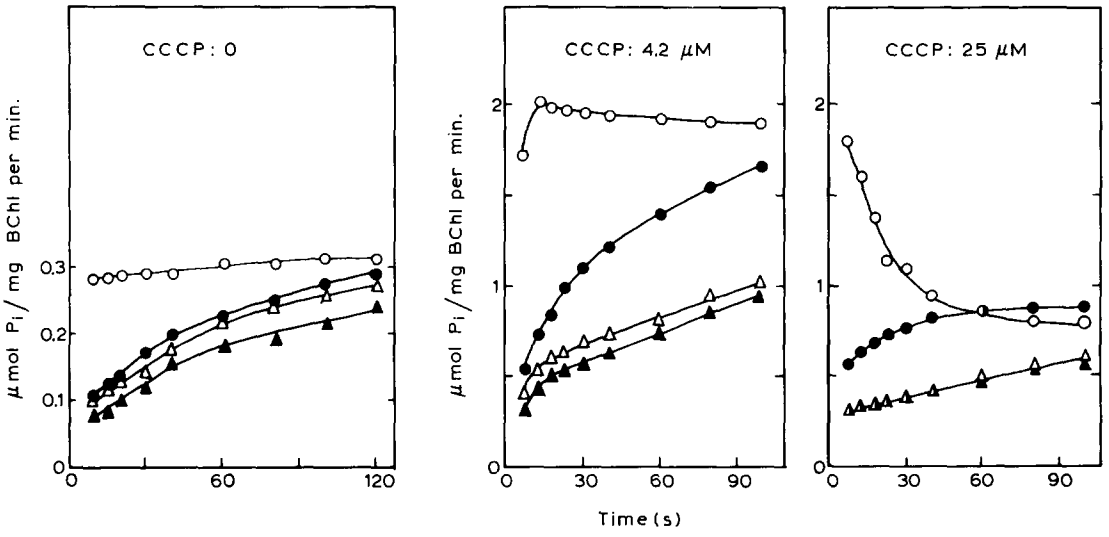

Fig. 8. Effect of ATP preincubation on the kinetics of ATPase with excess ATP (pH method). ATPase was measured with 0.1 µM nigericin, CCCP as indicated, 1.2 mM ATP and 0.3 mM $MgCl_2$. Rates were determined at the indicated times after completion of mixture. Each curve represents one experiment. Procedure: (o, •) chromatophores were illuminated 15 s in the presence of nigericin. CCCP was added just after the light trigger. $MgCl_2$ was added 1 min after CCCP. ATP was added along with $MgCl_2$ (o———o), or 30 s before $MgCl_2$ (•———•). (△———△ and ▲———▲) As o———o and •———•, respectively, except that no light was given.

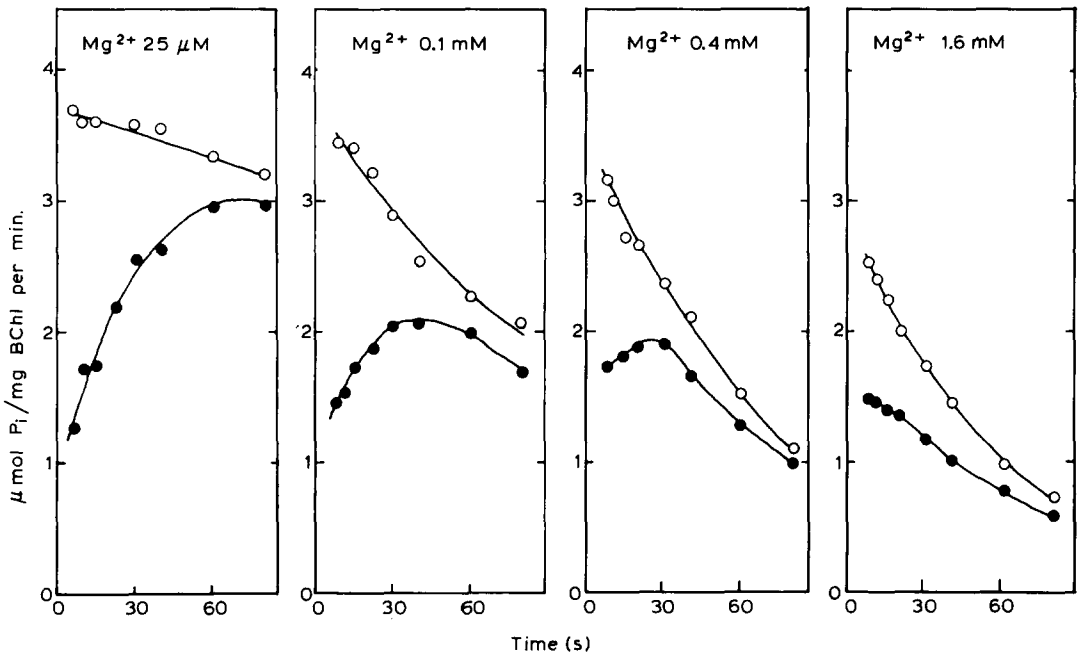

Fig. 9. Affect of ATP preincubation on the kinetics of light-activated ATPase (pH method). Hydrolysis was carried out with 0.1 μM nigericin, 6 μM CCCP, 0.3 mM MgATP and the indicated concentrations of Mg^{2+}. Amounts of $MgCl_2$ and ATP to be added were calculated using the stability constants given in Methods. Procedure: chromatophores were illuminated for 15 s in the presence of nigericin. CCCP was added just after the light trigger. $MgCl_2$ was added 40 s after CCCP. The rates shown were determined at the indicated times after $MgCl_2$ addition. (o———o) The full complement of ATP was added along with $MgCl_2$. (•———•) 0.3 mM ATP was added 20 s before $MgCl_2$; supplementary ATP was added along with $MgCl_2$.

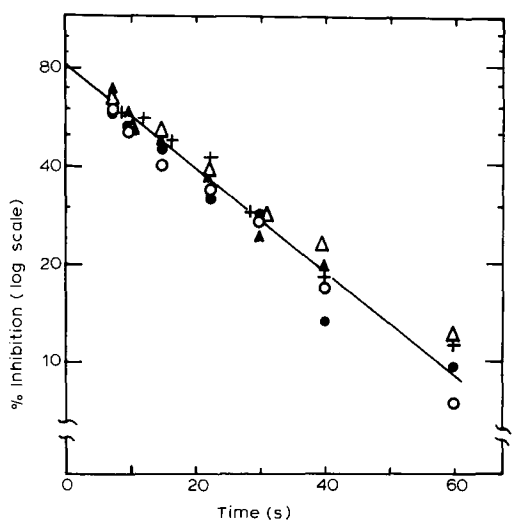

Fig. 10. Kinetics of the recovery from ATP inhibition during light activated ATP hydrolysis. Conditions were as in Fig. 9. The inhibition at time t was determined from the ratio of the rates at that time, in samples in which ATP preincubation was carried out or omitted, respectively. Hydrolysis starts at $t = 0$. Mg^{2+} concentrations were (in μM) 6 (o); 12 (•); 25 (△); 50 (▲) and 100 (+).

CCCP concentration (even up to 25 μM), the activity in the light-activated and ATP-pretreated chromatophores increased to the level observed in the control chromatophores within 60–80 s after the onset of hydrolysis (Fig. 8, circles). However, the activity in the control chromatophores declined in the course of hydrolysis at a rate which was determined by the uncoupler concentration (open circles; see also Fig. 4, open circles).

The experiments in Fig. 9 are similar to those shown in Fig. 8, but in Fig. 9 the CCCP concentration was kept fixed (6 μM) and instead the Mg^{2+} concentration was varied. The ATPase-induced deactivation of the enzyme was dependent on Mg^{2+} (it did not occur at 6 μM Mg^{2+}, not shown). This effect was most clear-cut in the control chromatophores (open cir-

cles). In the ATP-pretreated chromatophores (solid circles), the activity changed in a biphasic manner. In th first phase, recovery from ATP inhibition was relatively rapid, so that the activity increased with time. But after some time, recovery from ATP inhibition slowed down, and the time-dependent Mg^{2+} inhibition of ATPase became manifest. Fig. 9 shows that the duration of the first phase was progressively shortened by raising the Mg^{2+} concentration. We will return to this in the Discussion.

From experiments such as those shown in Fig. 9, we calculated the inhibition due to ATP preincubation as a function of the reaction time. Some results are shown in Fig. 10. Substrate-induced recovery from ATP inhibition behaved (at 0.3 mM MgATP) approximately as a first-order reaction ($t_{1/2} \simeq 19$ s), which was not significantly influenced by the Mg^{2+} concentration, provided this was kept below 0.2 mM. The inhibition extrapolated back to about 80% at zero time.

(3) Effect of combinations of ADP and ATP

The experiments described above can be explained most easily with the assumption that inhibition of ATPase by preincubation with ADP or ATP involves binding of these nucleotides to an inhibitory site (or sites) on the ATPase enzyme. The experiments shown below were based on (and strongly support) this hypothesis. The rationale of these experiments was as follows. The data shown in sections 1 and 2 provide the means to distinguish between ATP and ADP inhibition of light-activated ATPase. Now, if for instance, ADP binding is reversible (within 15–20 s) in deenergized membranes in the absence of Mg^{2+} (cf. Figs. 4 and 5), then we should be able to induce ATP inhibition by adding an excess of ATP after ADP in deenergized membranes. Whether or not ATP is added in excess can be determined from competition experiments, or predicted from a comparison of the I_{50} values for ADP (Fig. 2) and ATP (Fig. 7); the values shown in these figures were determined under identical conditions. By the same reasoning we can investigate the reversibility of ATP binding.

In the experiments shown in Figs. 11 and 12, Mg^{2+} was present at 10 μM during hydrolysis. Uncoupler was added just after a light trigger given in the presence of nigericin. The time interval between dissipation of the light-induced $\Delta\tilde{\mu}_{H^+}$ and the onset of

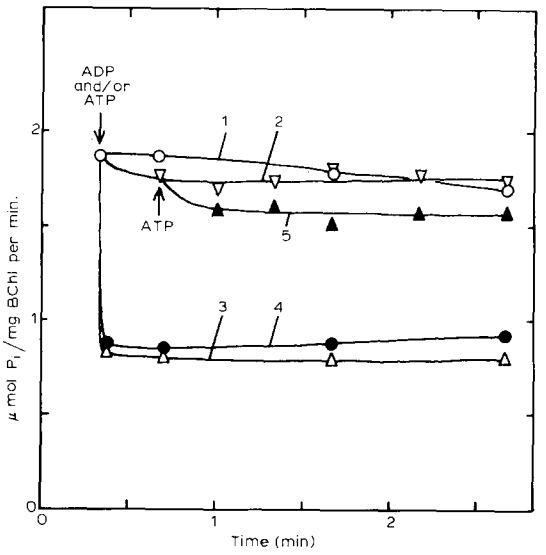

Fig. 11. Tight binding of ADP in the absence of Mg^{2+} (pH method). ATPase was measured with 0.1 μM nigericin, 8.7 μM CCCP, 0.3 mM $MgCl_2$, 1.2 mM ATP and 10 μM ADP. Procedure: chromatophores were illuminated for 15 s in the presence of nigericin. CCCP was added just after the switching off of the light (at $t = 0$). ADP (curve 2), ATP (curve 3), ADP + ATP (curve 4) or ADP followed by ATP (curve 5) were added at the times indicated by the arrows. The reaction mixture was completed at the time indicated on the x-axis and the rates were determined 15 s later. Each point represents one experiment.

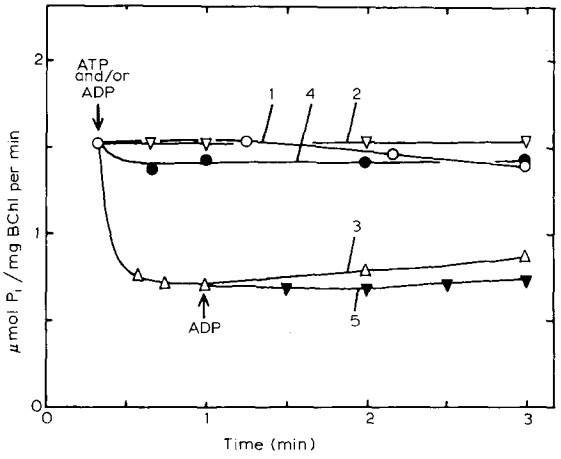

Fig. 12. Tight binding of ATP in the absence of Mg^{2+} (pH method). The reaction mixture was as in Fig. 11 but with 50 μM ADP. The procedure was as in Fig. 11, except that ADP (curve 2), or 50 μM ATP (curve 3), or ADP + 50 μM ATP (curve 4), or 50 μM ATP followed by ADP (curve 5) were added at the times indicated by the arrows. Each point represents one experiment.

hydrolysis was varied. This had no appreciable effect on light-activated ATPase initiated by simultaneous addition of $MgCl_2$ and nucleotides (curve No. 1 in Figs. 11 and 12). The rates of hydrolysis were determined at 15 s after the start of the reaction. At that time the inhibition due to ADP preincubation was largely reversed (curve No. 2; cf. Fig. 4), but the inhibition due to ATP preincubation was still extensive (curve No. 3; cf. Fig. 8). In Fig. 11, ATP and ADP were added at 1.2 mM and 10 μM, respectively, during preincubation. Simultaneous addition of ATP and ADP (curve 4) had nearly the same effect as addition of ATP alone (curve 3), indicating that competition was in favor of ATP binding. (This is in agreement with results shown in Figs. 2 and 7.) But when ADP was added before ATP, the latter compound caused only a slight further inhibition (curve 5 vs. curve 2). This inhibition did not increase when the contact time with ATP was prolonged from 20 s to 2 min. The ATP inhibition in the absence of ADP developed within 2 s (curve 3). This indicates that in curve 5, the rate-limiting step in the development of ATP inhibition during preincubation was ADP release.

In Fig. 12, ADP and ATP were both added at 50 μM during preincubation. In this case, simultaneous addition of ADP and ATP (curve 4) had nearly the same effect as addition of ADP alone (curve 2), indicating that competition was in favor of ADP binding. (This is in agreement with results shown in Figs. 2 and 7.)

However, when ATP was added before ADP, the latter compound did not reverse the inhibition caused by ATP preincubation (curve 5), even during a contact time of 2 min. ADP binding in the absence of ATP is quite rapid (Fig. 13 below). This indicates that ATP (like ADP, Fig. 11) is tightly bound to the light-activated enzyme when the membrane is deenergized in the absence of Mg^{2+}.

The last question we addressed was whether tightly bound ADP would be exchanged more rapidly with ATP (and vice versa) when the membrane was energized.

The experiments shown in Table I are similar to those shown in Fig. 11. Again, when after light activation ADP and ATP were added simultaneously (row 2), ATP was bound in preference to ADP; the resulting inhibition was not readily reversed (cf. row 1).

TABLE I

ENERGY-DEPENDENT EXCHANGE OF BOUND ADP WITH ADDED ATP

The reaction mixture was as in Fig. 11. Expt. 1. Chromatophores were illuminated for 15 s in the presence of nigericin. 0.1 μM CCCP was added just after the switching off of the light (at $t = 0$). ADP and ATP were added at the indicated time. At $t = 110$ s, 8.6 μM CCCP was added. The reaction mixture was completed at $t = 120$ s. Rates were determined 13.5 s later. Expt. 2. As Expt. 1 except that an additional 10 s light trigger was given just before addition of 8.6 μM CCCP.

Additions		ATPase (μmol P_i/mg BChl per min)	
$t = 60$ s	$t = 90$ s	Expt. 1	Expt. 2
ADP	–	1.86	2.05
–	ADP, ATP	0.96	1.11
ADP	ATP	1.73	1.14

But when ATP was added after ADP, the latter protected the enzyme against inhibition by the former (row 3). These control experiments ensured that ADP was already tightly bound by the time that ATP was added. Column 2 shows experiments in which the chromatophores were illuminated once more after addition of the two nucleotides. In this case it made no difference whether ADP was added along with

TABLE II

ENERGY-DEPENDENT EXCHANGE OF BOUND ATP WITH ADDED ADP

The complete reaction mixture was as in Fig. 12, except that $MgCl_2$ was present at 3.3 mM. Expts. 1 and 2 were carried out as were the corresponding experiments in Table I, except that at the indicated times, 50 μM ATP and 50 μM ADP were added.

Additions		ATPase (μmol P_i/mg BChl per min)	
$t = 60$ s	$t = 90$ s	Expt. 1	Expt. 2
–	–	2.17	2.58
–	ADP	0.11	0.06
ATP	–	0.81	1.37
ADP + ATP	–	0.17	0.17
ATP	ADP	0.86	0.13

(row 2) or before ATP (row 3): the ATP inhibition was observed anyway. This indicates that displacement of tightly bound ADP by ATP is energy dependent (In these experiments, CCCP was present at 0.1 μM during the second illumination. In combination with 0.1 μM nigericin, 0.1 μM CCCP causes a rapid dark decay of the light-induced $\Delta\tilde{\mu}_{H^+}$, but is unable to prevent light activation of the ATPase enzyme, not shown.)

The experiments shown in Table II (column 1) are similar to those shown in Fig. 12, except that hydrolysis was measured with 2 mM, instead of 10 μM, Mg^{2+}. Under these conditions, preincubation of deenergized membranes with 50 μM ADP elicited a stronger inhibition of light-activated ATPase (rows 1 and 2) than preincubation with 50 μM ATP (row 3). When ADP and ATP were added simultaneously, ADP was bound in preference to ATP (row 4). The inhibition caused by ADP alone was largely saturated within 1 s after its addition to deenergized membranes (Fig. 13). But when ATP was added before ADP, the latter compound caused no further inhibition (Expt. 1, rows 5 and 3), unless the chromatophores were illuminated once more during contact with both nucleotides (Expt. 2, rows 3 and 5). This indicates that displacement of tightly bound ATP by ADP was energy dependent. The same conclusion was reached when hydrolysis was performed with 10 μM Mg^{2+} (not shown).

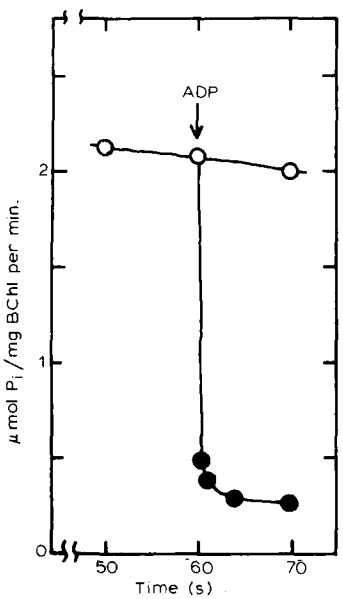

Fig. 13. Kinetics of the ADP-induced deactivation of the ATPase enzyme (pH method). The reaction mixture was as in Table II. Chromatophores were illuminated for 15 s in the presence of nigericin. CCCP was added just after the switching off of the light (at $t = 0$). $MgCl_2$ and ATP were added at the indicated time. ADP was added along with $MgCl_2$ and ATP (o———o), or at the time indicated by the arrow (•———•). Rates were measured 7 s after the onset of hydrolysis.

Discussion

(1) The mechanism of nucleotide exchange

The data of Figs. 11 and 12 suggest strongly that in deenergized membranes, the nucleotide added secondly is (in the absence of Mg^{2+}) unable to displace the nucleotide added first from an inhibitory site on the light-activated ATPase enzyme. Apparently, the rate constants for release of ADP or ATP from this site are very low (less than 0.2 min^{-1}). Yet, the enzyme recovers from ADP inhibition within 15–20 s after MgATP addition (Figs. 4 and 5), and it recovers from ATP inhibition within about 1 min after MgATP addition (Figs. 8 and 9). This suggests that at least two types of nucleotide-binding sites are involved in (regulation of) ATPase. The simplest explanation is that binding of ADP or ATP to one of these sites (site 1) results in inhibition of light-activated ATPase. In deenergized membranes the binding is tight, so that competition for binding occurs only when the nucleotides are added simultaneously, but (on a time scale of several minutes) not when they are added successively. The tight binding does not require the presence of Mg^{2+}. The second site (site 2) can bind MgATP; this results in release of ADP or ATP from site 1, and hence in reactivation of hydrolysis. In terms of this hypothesis, the data in Tables I and II indicate that in illuminated chromatophores, release of ADP or ATP from site 1 is not dependent on the presence of MgATP. In these experiments, the exchange reactions were virtually complete within 10 s of illumination. A comparison with Figs. 11 and 12 shows that illumination causes an increase of over 60-fold in the rate constants for dissociation of ADP or ATP from site 1 (from less than 0.2 min^{-1} to over 0.2 s^{-1}). This is in agreement with the energy dependence of nucleotide exchange in chloroplasts [6–10,

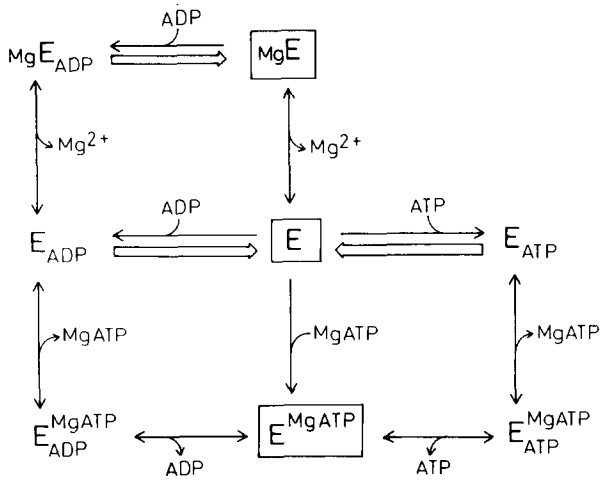

Fig. 14. Reaction scheme for the deactivation/reactivation reactions taking place with the light-activated ATPase enzyme (E) in *R. rubrum* chromatophores. Open arrows indicate energy-dependent reactions. Conformations within boxes are high-activity states; the remaining states are inactive or exhibit a low activity. Fog further details, see text.

18] and chromatophores [11]. Similar conclusions can be drawn from Figs. 1 and 6; in these experiments, membrane energization facilitated the exchange of bound ADP (Fig. 1) or ATP (Fig. 6) with added MgATP.

This interpretation is summarized in the scheme shown in Fig. 14 (Reactions leading to and from the conformations MgE and MgE_{ADP} will be discussed in section 2).

Under the conditions used here, the enzyme is in the state E after light activation. Subscripts and superscripts refer to binding reactions taking place at sites 1 and 2, respectively. Binding of either ADP or ATP at site 1 of the 'empty' enzyme is essentially irreversible in deenergized membranes and leads to deactivation. Binding of MgATP at site 2 of the empty enzyme may also be essentially irreversible (see below). The scheme also shows pathways for MgATP-dependent reversal of ADP inhibition (Fig. 4) or ATP inhibition (Fig. 8), via intermediate stages in which both sites 1 and 2 are occupied.

Admittedly, we have no direct evidence for tight binding or release of nucleotides at an inhibitory site (site 1). However, results obtained recently with chloroplasts support the above explanation. It was found that ADP binding to the light-activated enzyme follows the same kinetics as ADP-induced deactivation of the enzyme [19]. In addition, activation of ATPase [5] or phosphorylation [20–22] was correlated with release of tightly bound nucleotides. However, the scheme of Fig. 14 is likely to be an oversimplification, since we measured the effect of tight binding of ADP or ATP, instead of binding itself. For example, the formation of tight enzyme-nucleotide complexes presumably proceeds via intermediate stages in which the nucleotide is reversibly bound; this is also the case with ADP binding in chloroplasts [18,19].

The uncoupler insensitivity of MgATP-dependent recovery from ADP inhibition (Fig. 4) indicates that (at least in the absence of bound Mg^{2+}, see section 2 below) this reactivation reaction does not require a transmembrane $\Delta\tilde{\mu}_{H^+}$. This is supported by the fact that the ADP-induced inhibition of ATPase catalyzed by the isolated, mitochondrial F_1 was similarly reversed in the course of hydrolysis [23]. The dissociation constant (K_d) for ADP was less than 0.5 μM in the absence of Mg^{2+} (Fig. 2). But during hydrolysis catalyzed by uncoupled chromatophores, recovery from ADP inhibition occurred even in the presence of 10–50 μM ADP with only 200 μM MgATP present, provided the Mg^{2+} concentration was low (e.g., Fig. 12). If we assume that release of ADP from site 1 is, somehow, reversibly coupled to MgATP binding, then it follows that recovery from ADP inhibition involves MgATP binding to a high-affinity site ($K_d < 2$ μM).

The K_m (MgATP) value during the first few seconds of light-activated ATP hydrolysis was 30–40 μM, depending on the Mg^{2+} concentration (Slooten, L., unpublished data). However, Baltscheffski and Lundin [24] reported a very low K_m (1.3 μM) for MgATP during flash-activated ATP hydrolysis in *R. rubrum* chromatophores in the presence of phosphate. In addition, it was found recently that the catalytic site of the isolated, mitochondrial F_1-ATPase has an extremely high affinity ($K_d \simeq 10^{-10}$ M) for MgATP [25, 26]. These data allow us to propose a relatively simple mechanism in which MgATP-dependent recovery from ADP inhibition is due to tight binding of MgATP at the catalytic site (site 2). This would lead to release of ADP from site 1.

The same mechanism is thought to be responsible for MgATP-dependent recovery from ATP inhibition

in deenergized membranes (Figs. 8–10). The results of Fig. 8 suggest that this too is an energy-independent process. (At very high uncoupler concentrations recovery from ATP inhibition is to some extent masked by an ATPase-dependent deactivation reaction. This will be discussed in section 2.) An alternative explanation for this type of reactivation would be that the enzyme-ATP complex chelates an Mg^{2+} after addition of $MgCl_2$: this would result in the formation of an enzyme-MgATP complex with unimpaired activity. However, this is unlikely, since at concentrations below 0.2 mM, Mg^{2+} had no significant effect on the rate of recovery from ATP inhibition (Fig. 10): in fact, Mg^{2+} concentrations above 0.4 mM inhibited reactivation (Fig. 9).

The evidence that ATP is tightly bound in deenergized membranes in the absence of Mg^{2+} (Fig. 12; Table II, Expt. 1) was unexpected in view of the rather high I_{50} value (6 μM) for ATP inhibition as shown in Fig. 7. This suggests a slow step in the formation of an inactive enzyme-ATP complex. A rate constant of about $4 \cdot 10^3\ M^{-1} \cdot s^{-1}$ would explain the data of Fig. 7 if ATP binding is the rate-limiting step. However, this is about 10^4 times slower than would be expected for diffusion-controlled binding. This could mean that the rate-limiting step is the formation of an inactive state after ATP binding. One might argue that this inactive state is induced by ADP, produced from ATP by hydrolysis. But this seems unlikely because preincubation with saturating amounts of ADP does not have the same effect as preincubation with saturating amounts of ATP (e.g., cf. Figs. 8 and 4; Figs. 11 and 12, curves 2 and 3; Figs. 7 and 2; Table II, Expt. 1).

We have no data concerning the nature of site 1. However, data on the nucleotide specificity of a tight binding site on the chloroplast ATPase enzyme [27, 28] suggest the presence of a noncatalytic site which is capable of ADP binding as well as ATP binding [27]. This may correspond with site 1 in our hypothesis.

(2) The effects of Mg^{2+}

In chloroplasts [12] and in *R. rubrum* chromatophores [13] Mg^{2+} was found to inhibit ATP hydrolysis only when the ATPase-induced $\Delta\tilde{\mu}_{H^+}$ was rapidly dissopated by uncouplers. The inhibition was noncompetitive. It was proposed that under these conditions, Mg^{2+} inhibits release of ADP produced in the course of hydrolysis [29]. A synergism between Mg^{2+} and ADP was indeed observed in the isolated, mitochondrial F_1, where preincubation with Mg^{2+} caused inhibition of ATPase only when the membrane contained tightly bound ADP [30]. In *R. rubrum* chromatophores at low uncoupler concentrations, Mg^{2+} at concentrations which apparently did not inhibit ATPase still protected the enzyme against Ca^{2+} inhibition [13]: this suggests that when the membrane is energized, Mg^{2+} binding still occurs but does not cause inhibition of ATPase.

These findings, and the data presented here, have been summarized in Fig. 14. According to this scheme, at least two forms of the light-activated enzyme, viz., E and E_{ADP}, are capable of Mg^{2+} binding to a regulatory site. We suggest that Mg^{2+} binding does not lead to a significant change in activity, i.e., MgE is active (cf. Ref. 13) and MgE_{ADP} is virtually inactive. However, unlike E_{ADP}, the form MgE_{ADP} cannot be reactivated by addition of MgATP in the dark; instead hydrolysis is blocked at, or after, the level of MgATP binding. The form MgE_{ADP} can, however, be reactivated by membrane energization. This leads to release of ADP. This would explain why Mg^{2+} increases the dependence of ATP hydrolysis on the $\Delta\tilde{\mu}_{H^+}$ which is generated by the reaction itself [13]. According to Fig. 14, two competing reactions will occur when a mixture of $MgCl_2$ and ATP is added in the dark to the ADP-pretreated enzyme: MgATP binding, leading to reactivation of the enzyme; and Mg^{2+} binding which inhibits reactivation by MgATP. The results of Fig. 3 suggest that the latter reaction begins to predominate over the former at Mg^{2+} concentrations above 20–30 μM. This explains why Mg^{2+} was found to inhibit MgATP-dependent recovery from ADP inhibition of light-activated ATPase (Fig. 5). This Mg^{2+} inhibition was observed during ATP hydrolysis in the presence of uncoupler (Fig. 5) as well as in its absence (not shown). A period of illumination in the absence of uncoupler was required to reverse the ADP inhibition of light-activated ATPase at high concentrations of Mg^{2+} (Fig. 1). By contrast, under the conditions of Fig. 4, with only 10 μM Mg^{2+} present, MgATP-induced reactivation of E_{ADP} in the dark was apparently more rapid than Mg^{2+} binding. Finally, the time dependence of the Mg^{2+} inhibition of light-activated ATPase (Fig. 9) can

be attributed to the ADP requirement of this inhibition.

Our data do not allow to decide whether the ATP-pretreated enzyme (E_{ATP}) reacts with Mg^{2+}; if it does, this apparently does not strongly interfere with MgATP-dependent reactivation of the enzyme during the first few seconds of hydrolysis. Thus, the 'initial' rate of light-activated ATPase after ATP preincubation was virtually independent of the Mg^{2+} concentration (above 25 μM) (Fig. 9). This is in marked contrast with results obtained after ADP preincubation (Figs. 4 and 5). This would explain why, when hydrolysis was measured at high Mg^{2+} concentrations, ATP preincubation was less inhibitory than ADP preincubation (cf. Figs. 2 and 7; Table II). At Mg^{2+} concentrations above 0.4 mM, MgATP dependent recovery from ATP inhibition was arrested within a few seconds the onset of hydrolysis (Fig. 9). This may be attributed to the influence of the feedback system. During hydrolysis at high Mg^{2+} concentrations, recovery from ATP inhibition will be followed by Mg^{2+}- and ADP-dependent deactivation. In the control chromatophores, only the latter reaction will occur. This deactivation reaction is retarded by the ATPase-induced $\Delta\tilde{\mu}_{H^+}$. However, this $\Delta\tilde{\mu}_{H^+}$ will be lower in the ATP-pretreated chromatophores than in the control chromatophores. Therefore, the Mg^{2+}- and ADP-dependent deactivation will occur more rapidly in the reactivated enzyme molecules of the ATP-pretreated chromatophores, than in the initially active enzyme molecules of the control chromatophores.

(3) The mechanism of light activation

In the first article [1], it was shown that the reactions associated with light activation, and subsequent deactivation, of the ATPase enzyme in the absence of substrates, could be represented as

$$E^o \Longrightarrow E \rightleftharpoons E'$$

in which E^o and E represent the nonactivated and the activated ATPase enzyme, respectively. In the absence of substrates, E is, in the dark, slowly transformed into the state E′. Like E^o, the state E′ has a low activity, but unlike E^o, the form E′ was rapidly reactivated by addition of MgATP (within about 20 s). Mg^{2+} at above 20–30 μM inhibited this substrate-dependent reactivation. E′ could also be transformed back to E by illuminating the membrane once more. In all these respects, E′ resembles the light-activated enzyme after ADP preincubation (Results, section 1; Table I). This suggests that release of endogenous ADP from a tight binding site is one of the factors involved in light activation. Rebinding of this ADP would correspond with the transition $E \rightarrow E'$, so that the activation/deactivation reactions may be written as:

$$E^o_{ADP} \Longrightarrow E + ADP \rightleftharpoons E_{ADP}$$

The half-time for the transition $E \rightarrow E'$ was of the order of 1.5–4 min in deenergized membranes [1], i.e., roughly 10-times longer than the ADP preincubation time used in Fig. 2 above. If we assume that ADP binding is essentially irreversible in deenergized membranes, then under the conditions used in Fig. 2 above and in Fig. 7 of Ref. 1, rebinding of about 0.1 μM ADP (i.e., about 1 mol ADP/200 mol BChl) would be sufficient to account for the observed transition $E \rightarrow E'$. This is in good agreement with the amount of tightly bound ADP estimated to be present in *R. rubrum* chromatophores [31]. It has been shown that among the tightly bound nucleotides present in *R. rubrum* chromatophores, especially ADP was susceptible to displacement by added nucleotides during illumination [31]. ADP release has also been implicated in the activation of the ATPase enzyme in chloroplasts [5,20,21].

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