Properties of ATP Tightly Bound to Catalytic Sites of Chloroplast ATP Synthase

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Under steady state photophosphorylating conditions, each ATP synthase complex from spinach thylakoids contains, at a catalytic site, about one tightly bound ATP molecule that is rapidly labeled from medium [32P]ATP. The level of this bound [32P]ATP is markedly reduced upon de-energization of the spinach thylakoids. The reduction is biphasic, a rapid phase in which the [32P]ATP/synthase complex drops about 2-fold within 10 s, followed by a slow phase, kobs = 0.01/min. A decrease in the concentration of medium [32P]P, to well below its apparent Kₐₜₚ for photophosphorylation is required to decrease the amount of tightly bound ATP/synthase found just after de-energization and before the rapid phase of bound ATP disappearance. The [32P]ATP that remains bound after the rapid phase appears to be mostly at a catalytic site as demonstrated by a continued exchange of the oxygens of the bound ATP with water oxygens. This bound [32P]ATP does not exchange with medium P, and is not removed by the presence of unlabeled ATP.

The levels of tightly bound ADP and ATP arising from medium ADP were measured by a novel method based on use of [β-32P]ADP. After photophosphorylation and within minutes after the rapid phase of bound ATP loss, the measured ratio of bound ADP to ATP was about 1.4 and the sum of bound ADP plus ATP was about 1/synthase. This ratio is smaller than that found about 1 h after de-energization. Hence, while ATP bound at catalytic sites disappears, bound ADP appears. The results suggest that during and after de-energization the bound ATP disappears from the catalytic site by hydrolysis to bound ADP and P, with subsequent preferential release of P.

These and related observations can be accommodated by the binding change mechanism for ATP synthase with participation of alternating catalytic sites and are consistent with a deactivated state arising from occupancy of one catalytic site on the synthase complex by an inhibitory ADP without presence of P.

Tightly bound nucleotides associated with ATP synthases have been studied with chloroplast, mitochondrial, and bacterial membranes in various laboratories (see reviews, Refs. 1-6). Present information is regarded by us as demonstrating presence of tightly bound ATP and ADP on both noncatalytic and catalytic sites of de-energized membranes and isolated ATPases. Such unusual retention of substrates at catalytic sites may be explained by alternating site participation in the binding change mechanism (7, 8). In this mechanism, the ADP and P, that are loosely bound at a catalytic site are converted, in a step requiring an energy-driven conformational change, to tightly bound ADP and P, capable of rapidly and reversibly forming bound ATP. In the same conformational transition, a transiently tightly bound ATP at an alternate catalytic site is converted to loosely bound ATP. The reverse of these binding changes occurs during ATP hydrolysis. When de-energization terminates net synthesis, some nucleotides appear to be retained at catalytic sites in tightly bound form.

Considerable clarification is needed as to the relationships between bound nucleotides found on isolated membranes and ATPases and the ATP and ADP transiently tightly bound to catalytic sites on ATP synthase on energized membranes. In the studies reported here, we investigated the fate of the ATP, ADP, and P, bound at catalytic sites when the protonotive force is collapsed by de-energization. The experiments were designed to address some important questions about the nature of nucleotides bound at catalytic sites of the chloroplast ATP synthase. Does the ATP that remains on the catalytic site continue to undergo rapid, reversible hydrolysis? Could some ATP migrate from catalytic to noncatalytic sites without mixing with medium ATP, as suggested by Aflalo and Shavit (9) and Kozlov and Skulachev (10)? During net photophosphorylation, do catalytic sites have a tightly bound ADP as well as ATP rapidly labeled from medium ADP present at catalytic sites, and, if so, how much tightly bound ADP is present? How much does the [32P]P, concentration need to be lowered before the amount of bound ATP rapidly labeled from medium P, is appreciably reduced?

Closely related to the above questions is the possibility of control of ATPase and ATP synthase activities by bound nucleotides. Several laboratories have presented evidence that ADP binding can cause loss of ATPase activity of isolated ATPases from mitochondria (11-13), chloroplasts (14-17), and bacteria (18). The ATPase synthase of de-energized membranes has been regarded as in a deactivated state that may correlate with a lack of ATPase activity (19). Our results also shed light on whether ADP binding without P, at catalytic sites may control ATPase activity (16, 20, 21) and on the nature of catalytic site deactivation that accompanies de-energization.

EXPERIMENTAL PROCEDURES

Materials—Hexokinase, adenylate kinase, glycerokinase, Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine, CF,, chloroplast coupling factor 1 or chloroplast coupling factor 1 ATPase.
Centrifuged and the supernatant neutralized with 0.6 M KOH. ATP was eluted from the Dowex 1 column with 2 M triethylamine carbonic acid and the supernatant neutralized with solid Tris base. The H2O was recovered by lyophilization. The thylakoid pellet was suspended and incubated in solution A for 10 to 15 min with H2O in solution A. Then, 0.35 ml of nearly 100% "0. The mixture was illuminated for 20 s using a 300-watt slide projector; the duration of the illumination was controlled by an electronic shutter. When the illumination was terminated, the following were added in the order indicated: 1.5 ml of the 2 M NH4Cl/Tris solution, 0.01 ml of a solution containing 50 pmol each of ADP and ATP, and 100 units of adenylate kinase. The mixture was incubated for 1 min to allow the enzyme-coupled reactions to remove nearly all medium ADP and ATP. The thylakoid membranes were filtered as in Ref. 7 except that they were washed with the NH4Cl/Tris solution in place of the EDTA wash, and both unlabeled ADP and ATP were present in the perchloric acid solution. [32P]ATP and [38P]ATP were separated by anion exchange chromatography as described above and counted.

To check the catalytic competence of the [32P]ATP remaining bound to the thylakoid membranes, the experiment was repeated except that 2.2 mM P04, 57500 cpm/mmol, 200 mM ADP, about 7000 units of hexokinase, and chloroplast thylakoid membranes equivalent to 50 mg of chlorophyll were used except that 2.2 mM P04, or [32P]Pi (including 32Pi at 7500 cpm/mmol of "Pi were used per time point. In those cases, the perchloric acid containing 5 mu of unlabeled Pi in addition to the carrier ATP. Also, the charcoal column was washed with 20 ml of a solution containing 0.025 M P04, 0.1 M H3PO4, and 0.3 mM perchloric acid (22) after adsorption of ATP, and the Dowex column was washed with 25 ml of 60 mM HCl after ATP adsorption. In all experiments, [32P]ATP was quantitated by liquid scintillation counting in water using Cerenkov radiation.

Under the assay conditions, the total [32P]ATP present will consist mostly of bound ATP, but a low steady state level of medium [32P]ATP would be expected. To assess the amount of medium [32P]ATP, repeated estimates were made of the amount of bound ATP rapidly formed during illumination in the absence of hexokinase, with perchloric acid quench after a few seconds in the light. Extrapolation to infinite hexokinase, which gives a measure of [32P]Pi, labeled, bound ATP (7), showed that 85-90% of the [32P]ATP present was bound. Bound ATP levels reported are corrected for this portion of free ATP.

\[\text{ATP} = H_2O \text{ Exchange Experiments} \]

In the oxygen exchange experiments, assay conditions similar to those described above were used except that 2.2 mM P04 or [32P]Pi, including 2.2 mM P04, at 7500 cpm/mmol, 200 mM ADP, about 7000 units of hexokinase, and chloroplast thylakoid membranes equivalent to 50 mg of chlorophyll were used in the assay mixture. The assay mixture containing 10 mg of chlorophyll was placed in a 2.2 ml reaction mixture (22) to about 0.5 s. They were then diluted 10-fold with a similar dose of "Pi and incubated with the NH4Cl wash. The thylakoids were centrifuged and washed at 4°C, refrigerated at 7000 rpm, and the pellet resuspended in fresh wash solution, which contained 0.4 M sucrose, 5 mM MgCl2, 10 mM NaCl/Tris, and 0.2 M choline, pH 8. Part of the suspension was reserved for the determination of the chlorophyll concentration and the remainder was illuminated at room temperature in an unlabeled assay mixture for various times and quenched in 1 ml of the NH4Cl/Tris solution. After 1 min, the bound nucleotides were extracted by adding 225 μl of concentrated perchloric acid. In one experiment, 50 min after the first NH4Cl quench, the thylakoids were centrifuged and washed at 4°C with 2 M NaCl/Tris solution and incubated in the dark for 10 s. They were then diluted 10-fold with a similar assay mixture except with 500 units of hexokinase and without the "0. The mixture was quenched with 20 μl of the 2 M NH4Cl/Tris solution and incubated in the dark for 10 s. When the light was extinguished, 100 μl of the 2 M NH4Cl/Tris solution were added and samples incubated for 1 min. The nucleotides were extracted by adding 225 μl of concentrated perchloric acid. In one experiment, 50 min after the first NH4Cl quench, the thylakoids were centrifuged and washed at 4°C, reilluminated. When the light was extinguished, 100 μl of the 2 M NH4Cl/Tris solution were added and samples incubated for 1 min. The nucleotides were extracted by adding 225 μl of concentrated perchloric acid. In one experiment, 50 min after the first NH4Cl quench, the thylakoids were centrifuged and washed at 4°C, reilluminated. The [32P]Pi remaining labeled by "0 and inaccessible to cleavage by hexokinase. They presented evidence that for short periods following "0, tightly bound ATP (solution 1) were mixed with a 0.75-mM aliquot of the assay mixture (solution 2) and illuminated for 23 s. In experiments 2 and 3, chloroplasts were illuminated in a 0.75-mM aliquot of solution 1 for 20 s, then combined with a 0.75-mM aliquot of solution 2 and illuminated for 3 s more. Experiment 3 was as experiment 2 but contained no P04. When the illumination was terminated, the following were added in the order indicated: 1.5 ml of the 2 M NH4Cl/Tris solution, 0.01 ml of a solution containing 50 pmol each of ADP and ATP, and 100 units of adenylate kinase. The mixture was incubated for 1 min to allow the enzyme-coupled reactions to remove nearly all medium ATP and ATP. The thylakoid membranes were filtered as in Ref. 7 except that they were washed with the NH4Cl/Tris solution in place of the EDTA wash, and both unlabeled ADP and ATP were present in the perchloric acid solution. [32P]ATP and [32P]ATP were separated by anion exchange chromatography as described above and counted.

RESULTS

**Time Course of Disappearance of [32P]ATP Tightly Bound to the Chloroplast ATP Synthase Complex upon De-energization**

Rosen et al. (7) have shown that during net photosynthesis by substrates near saturating concentrations, the ATP synthase complex contains approximately one tightly bound ATP molecule/enzyme complex that is rapidly labeled by "0, and inaccessible to cleavage by hexokinase. They presented evidence that for short periods following "0, tightly bound ATP falls to about 0.27 within 1 min. They presented evidence that for short periods following "0, tightly bound ATP falls to about 0.27 within 1 min. The experiments with [32P]Pi were similar except the water of solution A contained 86% "0. The thylakoids were allowed to react 10 to 15 min with H2O in solution A. Then, 0.35 ml of nearly 100% trichloracetic acid was added and the mixture centrifuged at 10,000 rpm for 5 min. The supernatant (pale yellow) was collected and neutralized with solid Tris base. The H2O was recovered by bopyllization transfer and the residue brought to 200 ml, pH 8.5. The ATP was isolated with use of a 3-ml Dowex 1 column as described above. The recovered ATP was neutralized with 50% NaOH, desalted over Sephadex G-10, and dialyzed with glycerokinase to form ATP. As shown in Fig. 1, the decay of labeled ATP is biphasic. Under the experimental conditions used, the initial level of bound [32P]ATP during steady state photosynthesis was about 0.58 ATP synthase, and when the light was extinguished and NH4Cl added, the level fell to about 0.27 within 10 s. This rapid drop was followed by a much slower decrease of k2, 0 = 0.01/min. This biphasic
decay shows that two processes are occurring when the ATP synthase complex is de-energized. Aspects of both phases of bound $^{32}$P]ATP decay are given in this report.

Similar results were obtained in the absence of NH$_4$Cl, using only precautions to obtain essentially complete darkness for thylakoid de-energization. Thus, the behavior noted is not dependent upon presence of NH$_4$Cl.

**Effect of Pi on Levels of Rapidly Labeled $^{32}$P]ATP on Energized and Deenergized Thylakoids**—The results of Fig. 1 as compared to those of Rosen et al. (7) suggest that when thylakoids are illuminated at subsaturating levels of P$_n$, the amount of bound $^{32}$P]ATP remaining in the dark after an NH$_4$Cl quench is less than that remaining in the dark after an illumination at saturating levels of P$_n$. To characterize this phenomenon more carefully, the amount of bound $^{32}$P]ATP rapidly labeled from P$_i$/synthase complex was determined at different levels of P$_n$ in the light and in the dark. With 50 $\mu$M or more P$_n$ about 1 ATP/synthase was rapidly labeled. A decrease of P$_n$ to 2 $\mu$M, far below that required for half-maximal velocity of net ATP formation, was required to drop the bound $^{32}$P]ATP level during steady state phosphorylation to about 0.3/synthase. However, the amount of $^{32}$P]ATP remaining after de-energization decreased more than one-half by decreasing the medium P$_n$ from 2 mM to 50 $\mu$M. Thus, results show that the level of bound $^{32}$P]ATP remaining in the dark is more sensitive to the concentration of medium P$_n$ than is the steady state level of bound $^{32}$P]ATP rapidly labeled from $^{32}$P, during phosphorylation. Nonetheless, the biphasicity of the ATP decay occurs at all concentrations of P$_n$ tested.

To characterize better the effect of P$_n$ concentration on the level of hexokinase-inaccessible ATP during photophosphorylation, measurements were made while medium P$_n$ was consumed. Results are shown in Fig. 2. The reaction was carried out in the presence of glucose and hexokinase, hence, P$_n$ was ultimately converted to glucose 6-phosphate while the ADP concentration remained constant. At the hexokinase concentration used for this experiment and at the initial P$_n$ concentration of 50 $\mu$M, the total $^{32}$P]ATP found upon acid quench represents 2 species, free ATP plus enzyme-bound ATP. Both species are present while the P$_n$ concentration is above 20–30 $\mu$M because there is not enough hexokinase to remove nearly all of the $^{32}$P]ATP formed. This was shown in separate experiments with increasing hexokinase concentration at 20 $\mu$M P$_n$. As P$_n$ is consumed, ATP synthesis slows, and the level of free $^{32}$P]ATP declines. Below 15 $\mu$M P$_n$, nearly all of the $^{32}$P]ATP present is bound to the synthase, and the level of $^{32}$P]ATP present per synthase decreases at a faster rate than before until it reaches a lower level. This relatively rapid decrease is as would be expected if most of the bound $^{32}$P]ATP present earlier was at a catalytic site. The remaining low level of bound $^{32}$P]ATP is present mostly at noncatalytic sites on the synthase because, as shown in Fig. 2, most of it does not chase out. As shown later in this paper, at an earlier period with 100 $\mu$M P$_n$, nearly all $^{32}$P]ATP is, as expected, removed by a cold chase.

The rapid decrease in bound $^{32}$P]ATP after about 30 s, shown in Fig. 2, may allow an approximation of the rate of release of bound ATP from the catalytic site in the light under these conditions. In the binding change mechanism (7, 8), catalysis is regarded as involving participation of alternating sites and catalytic cooperativity of product release. Normally, ATP at one catalytic site is released only after substrates bind to an alternate catalytic site. At concentrations of P$_n$ far below that required for half-maximal velocity, the turnover rate decreases to the point that ATP is being released from the enzyme before P$_n$ binds to the alternate site. This results in a decrease in the concentration of bound $^{32}$P]ATP. The rate of $^{32}$P]ATP decrease calculated from the slope of Fig. 2 at 10 $\mu$M P$_n$ may be used to give a rough estimate of the off constant for the release of tightly bound ATP from one catalytic site with only ADP but not P$_n$ bound at an alternate catalytic site. The off constant for this tightly bound ATP, calculated from the rate at 10 $\mu$M P$_n$ is 6.8/s, about 40-fold lower than the potential maximum turnover rate (250/s) under the conditions used.

**ATP $\rightleftharpoons$ H$_2$O Exchange of the Bound $^{32}$P]ATP on Deenergized Thylakoids**—Fig. 1 shows that de-energized thylakoids retain a tightly bound ATP that was rapidly labeled with $^{32}$P, during brief illumination. Under the experimental conditions used, most of this bound ATP originated at the active site of the synthase and presumably remains at the catalytic site after de-energization. Therefore, the possibility existed that this ATP might be undergoing the rapid, reversible hydrolysis to ADP and Pi characteristic of ATP formation catalyzed by the light-activated synthase. Measurements were made to test this possibility. Table 1 shows 3 experiments that demonstrate that an ATP $\rightleftharpoons$ H$_2$O exchange does occur in the dark, and suggest that this may be at a much slower rate than that observed in the presence of light.
on De-energized Thylakoids—Because the $[^{32}P]$ATP tightly bound to the synthase on de-energized thylakoids under conditions of our experiments undergoes a reversible hydrolysis, as shown by the oxygen exchange, the question arises as to whether a required product of hydrolysis, bound $[^{32}P]$Pi, is exchangeable with medium Pi. To test if such an exchange might occur, unlabeled Pi in the millimolar range was added 1 min after illumination of thylakoids in 20 µM $[^{32}P]$Pi. Even after 1 h, no loss of $^{32}$P from the bound $[^{32}P]$ATP was detectable. The experiment was repeated under conditions giving a higher initial bound $[^{32}P]$ATP concentration. Thylakoids were illuminated at higher initial Pi concentrations, either 100 or 500 µM $^{32}$Pi, and the concentration of unlabeled Pi present after quenching was 500 µM or 5 mM, respectively. An apparent slow exchange reaction occurred at 100 µM Pi, ($k_{obs} = 0.002/min$), and at a faster rate at 500 µM Pi, ($k_{obs} = 0.006/min$). However, we found that, when $^{32}$Pi, at 100 µM or higher concentration was used, some $^{32}$P, is incorporated into a product that is not ATP but that coelutes with ATP during the usual isolation procedure. This side reaction complicates the interpretation of the experiments in which thylakoids are illuminated at relatively high concentrations of $^{32}$P, and apparent wash out of label is measured.

The exchange reaction did not greatly interfere with our measurements of the rate of labeled $[^{32}P]$ATP disappearance because the product isolated from ATP synthase at 2 mM Pi, only 1 min after the NH₄Cl quench was more than 85% ATP as tested by hexokinase cleavage. Therefore, any reaction to give the contaminating $^{32}$P species is too slow to affect appreciably the accuracy of the early time points of the ATP decay curve at high Pi concentrations. And at low Pi concentrations, this contaminating reaction does not interfere at any time points as indicated by a lack of effect of the addition of unlabeled Pi, to the medium containing 20 µM $^{32}$P.

To assess more readily if the tightly bound Pi, formed from hydrolysis of $[^{32}P]$ATP bound to thylakoids might be exchangeable with medium Pi, an inverse experiment was carried out i.e. illumination in 100 µM unlabeled Pi, followed by a quench and incubation in NH₄Cl and 500 µM $^{32}$Pi, for 1 h. Hexokinase was unable to cleave any of the radioactive compound isolated in the ATP fraction from this procedure (data not shown), demonstrating that radioactive ATP is not formed under these conditions. We thus conclude that the labeled ATP remaining bound does not undergo exchange with medium Pi, upon de-energization.

**Tests of Catalytic Competence of Tightly Bound ATP Remaining on Deenergized Thylakoids**—The results of Afalo and Shavit (9) suggest that some tightly bound ATP can shift from a catalytic to a noncatalytic site without equilibrating with medium ATP during net photophosphorylation or during or shortly after de-energization of thylakoids. Most of the bound $[^{32}P]$ATP remaining shortly after deenergization is at a catalytic site in view of the exchange of the γ-phosphoryl oxygens with water. As another means of testing for binding to a catalytic site and for the change in binding with time, the following experiments were conducted. Thylakoids were illuminated in an assay mixture containing 100 µM $^{32}$Pi, and subsequently incubated in NH₄Cl in the dark for 10 s. Then the thylakoids were diluted in fresh assay mixture without the radiolabel and reilluminated. Table II shows that time course of the disappearance of $[^{32}P]$ATP that was bound to de-energized thylakoids in the dark. After 2 s, about 80% of the bound ATP disappeared, and after 10 s only about 3% of the counts were left. The disappearance of $[^{32}P]$ATP did not

$^{3}$ This product is not a nucleotide triphosphate (R. I. Feldman, unpublished results).

**TABLE I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Reaction labeled with $^{18}$O</th>
<th>Initial $^{18}$O in labeled reactant</th>
<th>Final $^{18}$O in $\gamma$-phosphoryl of ATP</th>
<th>Fraction exchange of $\gamma$-phosphoryl</th>
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<td>1</td>
<td>P₄</td>
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<td>85°</td>
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</tr>
<tr>
<td>2</td>
<td>H₂O</td>
<td>~15°</td>
<td>49</td>
<td>32</td>
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<tr>
<td>3</td>
<td>H₂O</td>
<td>~10°</td>
<td>45</td>
<td>26</td>
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</tbody>
</table>

*Totai time after de-energization; exchange may occur in both rapid and slow phases. $^{18}$O content of the γ-phosphoryl group estimated from the $^{18}$O content of the P₄ used (98%) and the known extent of water oxygen incorporation into ATP during steady state phosphorylation under conditions used.

$^*$H₂O added several minutes after light extinguished and uncoupler added.

**Rate** than during ATP synthesis in the light. These experiments were carried out in the presence of hexokinase to trap medium ATP, allowing us to follow only the bound ATP $=$ H₂O exchange. In the first experiment, $[^{18}$O]P₄ was used as substrate and $[\gamma-^{18}$O]ATP was produced in the light. The light was extinguished and the $[^{18}$O]ATP $=$ H₂O exchange reaction was allowed to proceed for about 40 min in the dark. At high substrate concentrations, considerably less than 1 water oxygen appears in each ATP molecule made during rapid net photophosphorylation (27, 28). Thus, the $^{18}$O enrichment of the bound ATP formed in the light is expected to be not much less than the enrichment of the P₄ used in the experiment, and the marked decrease in the enrichment of $^{18}$O we observed in the ATP could only occur in the dark.

Hackney et al. (28) showed that under low light intensity, intermediate ATP $=$ H₂O exchange still occurs during ATP synthesis. Hence, the possibility existed that the exchange observed in experiment 1, Table I, had occurred only while the light intensity was deceasing and the NH₄Cl was mixing with the thylakoid reaction mixture. Experiments 2 and 3 were conducted to test this possibility. In these experiments, H₂O was added to the reaction mixture several min after the light was extinguished. Again, a slow exchange rate was observed at a rate approximately equal to that observed in which P₄ was labeled in experiment 1.

An important point from the data of Table I is that extensive exchange was observed when $^{18}$O-labeled ATP was present at the time of de-energization or water H₂O added several minutes after de-energization. This gives convincing evidence that most of the ATP, at the time of and for a number of minutes after de-energization, is bound at catalytic sites.

The exchange of water oxygens with oxygens of bound ATP is not as complete as would be expected if the exchange were as rapid as during net photophosphorylation and if all the bound ATP were at catalytic sites. From this and other considerations discussed later in this paper, it appears clear that part of the bound $[^{32}P]$ATP remaining after de-energization is not at catalytic sites. Further, if the exchange was continuing at a uniform rate up until the time of assay, then the actual exchange rate is much slower in the de-energized state. Each of the single time experiments reported in Table I required considerable experimental investment, and at this stage it did not appear worthwhile to attempt to firmly establish the exchange rate.

**Lack of Exchange between Medium P₄ and Bound $[^{32}P]$ATP**
follow first order kinetics, as if a slow and fast component were present. In addition, all of the product isolated was authenticated as ATP by hexokinase cleavage (data not shown). In separate experiments, under similar conditions, i.e. with 100 μM P₄, the photophosphorylation rate after the NH₄Cl addition and dilution is about 200 μmol/h/mg of chlorophyll. The phosphorylation rate is reduced from our usual value of about 1000–1200 μmol/h/mg because of the slow rate of ATP release. Presumably, this is due to disappearance of ATP for several minutes in the dark with NH₄Cl before the second illumination. Additional details are given under “Experimental Procedures.”

Results obtained are given in Table III. In experiment 1, thylakoid ATP synthase was incubated with only 5 μM [β-³²P]ADP and P₄ in the light for 23 s and then quenched. Under these conditions, the ratio of bound ADP to bound ATP is about 1.2 and the total amount of nucleotide bound per synthase molecule is about 1.1. In experiment 2, thylakoids were illuminated with unlabeled substrates for 20 s and then pulsed with [β-³²P]ADP for 3 s before the NH₄Cl quench. Under the assay conditions used, the ADP concentration is only 2- or 3-fold below its Kₘ so the photophosphorylation rate is fairly rapid. The short labeling time insures preferable labeling of nucleotides at the catalytic site during steady state photophosphorylation, while minimizing labeling of non-catalytic sites (7). The decrease in levels of ATP and ADP/ synthase molecule in experiment 2 when compared to experiment 1 is as expected for some noncatalytic site labeling in experiment 1. Also, the ratio of ADP to ATP is about 1.4, slightly increased, compared to that in experiment 1, indicative that noncatalytic sites become labeled more rapidly with ATP than ADP. In similar experiments, with 100 μM P₄, the assay mixture, Vinkler et al. (22) showed that after extensive washing of thylakoids the ATP to ADP ratio was about 3, although, as in the present experiments, they showed that the amount of ADP plus ATP was about 1/synthase. These results indicate that the decrease in ATP correlates with an increase in ADP. Therefore, hydrolysis of bound ATP to bound ADP and P₄, and loss of that P₄ probably occurs.

Reproducibility of the values for bound ADP present was only fair in repeated experiments; results varied by as much as ±20%. The data do suffice to show the important point that both tightly bound ADP and ATP are formed from medium ADP with only a few turnovers of the synthase, and to give a fair estimate of the amounts of those transitorily tightly bound nucleotides at the catalytic site. It is of interest that a considerable amount of bound ADP and even some bound ATP were in the absence of added P₄ (experiment 3, Table III). The tight binding of ADP may reflect a quite rapid energy-dependent exchange (see Refs. 1 and 30) of ADP at a catalytic site with medium ADP. Whether or not this is accompanied by an equally fast or faster exchange of bound P₄ with a very low level medium P₄, present is uncertain from present data. A possible explanation for the presence of [³²P]ATP is that it resulted from the synthase reaction due to endogenous P₄, or from a reaction

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Before</th>
<th>After</th>
<th>P₄ concentration</th>
<th>Illumination time</th>
<th>Ratio</th>
</tr>
</thead>
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<tr>
<td></td>
<td>µM</td>
<td>µM</td>
<td>[³²P]ATP/synthase</td>
<td>[³²P]ADP/synthase</td>
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<tr>
<td>1</td>
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<td>3</td>
<td>0.22</td>
<td>0.35</td>
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</tr>
</tbody>
</table>

Table II
Release of bound [³²P]ATP from de-energized thylakoids when photophosphorylation is reinitiated

Table III
Levels of ADP and ATP rapidly labeled from [β-³²P]ADP during photophosphorylation and remaining on de-energized thylakoids

Samples were incubated in the standard assay mixture except 5 µM [β-³²P]ADP was used. Illumination was stopped when NH₄Cl was added for de-energization. Additional details are given under “Experimental Procedures.”
with a contaminating adenylate kinase that would catalyze the conversion of \([\beta,\gamma-^{32}P]\)ADP to \([\beta,\gamma-^{32}P]\)ATP and AMP. The doubly labeled ATP would then be transferred to a site on the synthase. The first possibility is more likely since no labeled ATP was formed in the absence of light.

**Inability of Medium ATP to Remove the Bound \([^{32}P]ATP—** Chloroplast thylakoids have been shown to possess the light-induced capacity for ATPase in the dark when they do not have ATP available to maintain a proton motive force (see Refs. 14, 30, and 31). Experiments were conducted to determine whether the site containing the bound \([^{32}P]ATP rapidly labeled from \(^{32}P\), during photophosphorylation may participate in a slow ATPase after de-energization. Less than 20% of the \([^{32}P]ATP disappeared when unlabeled ATP (final concentration 100 \(\mu M\)) was added with an NH\(4\)Cl quench. Also, when thylakoids were washed with 75 mM NH\(4\)Cl, then mixed with 4 mM unlabeled ATP and 10 mM Mg\(^{2+}\), no disappearance of \([^{32}P]ATP was observed. The catalytic site with bound \([^{32}P]ATP in the de-energized thylakoids appears to be indeed tightly closed to medium ATP.

**DISCUSSION**

Our results show that de-energization of chloroplast thylakoid membranes causes a biphasic decrease in amount of a tightly bound ATP that was at the active site of the ATP synthase during net ATP formation. The data in this report together with those of Vinkler et al. (22) give evidence that this decrease in bound ATP is accompanied by a corresponding increase in bound ADP. These findings and other data to be discussed below are consistent with a rapid hydrolysis of part of the bound, catalytic ATP upon de-energization with release to the medium of the Pi, and probably some of the ADP formed. The ATP remaining on the enzyme after the rapid decay phase appears to be largely at catalytic sites shown by the continued exchange of the \(\gamma\)-phosphoryl oxygens of most of the ATP with water oxygens. But the catalytic site is in a deactivated state as shown by lack of exchange with medium Pi, or ATP.

These and other data pertinent to how the biphasic disappearance of bound, catalytic ATP may occur can be usefully discussed as related to the binding change mechanism for ATP synthesis (7, 8). Using rapid mixing-chase experiments, Smith and Boyer (32) showed that during steady state photophosphorylation there is about 1 ADP/synthase at catalytic sites. In accord with the binding change mechanism of ATP synthesis, the total ADP may represent the sum of two species of ADP, one of which is tightly bound at the site where reversible ATP formation occurs and the other loosely bound at another catalytic site of the same synthase complex. Similarly, for bound ATP, Rosen et al. (7) demonstrated the presence of about 1 bound ATP/synthase at catalytic sites during photophosphorylation, and the total bound ATP is the sum of that at the site where reversible hydrolysis is occurring and that at sites where interconversion no longer occurs. The presence of bound reactants at more than one catalytic site/synthase molecule during net ATP formation seems reasonable since there are 2 or 3 catalytic subunits/synthase complex (see Refs. 6, 30, and 33). An explanation for events occurring during and after de-energization needs to consider different species of bound ADP and bound ATP that may be present at the catalytic sites. Also, the relatively facile medium ATP \(\rightleftharpoons\) P, exchange that occurs in the light (see Ref. 30) and the transient ATPase activity observed when illumination ceases need to be considered.

A sequence of events consistent with the above observations and with results reported here is as follows. When illumination is terminated and NH\(4\)Cl is added, a transient ATPase activity occurs; net catalysis is now in the reverse direction. The sites containing loosely bound ATP that was just about to be released are changed to tight binding sites where a reversible hydrolysis of the bound ATP occurs. And the tight sites where ATP \(\rightleftharpoons\) ADP + P, interconversion was occurring in the light are converted, in the dark, to sites where only loosely bound ADP and P can be present. The ADP and P, would then dissociate to the medium. At most, only a few such turnovers would be expected to occur because of the removal of the substrate ATP from the medium by hexokinase. The net result would be that some of the catalytic sites that contained bound ADP and ATP during synthesis would now be empty. The sites retaining bound ATP and ADP on the de-energized enzyme complexes would be the tight binding sites where reversible hydrolysis occurs. This expected continued turnover of the enzyme upon de-energization could account for why Afalo and Shavit (9) observed decreases in bound \([^{32}P]ATP from \(^{32}P\), when unlabeled ATP is added with uncoupler.

The initial release of bound ATP and bound ADP and P, upon de-energization would be expected to be rapid because of the relatively high potential ATPase activity. Thus, the rapid phase of disappearance of bound catalytic ATP when illumination ceases and NH\(4\)Cl is added can be accounted for. The slow phase would reflect the properties of the remaining tightly bound ATP, ADP, and P. From the earlier results of Harris and Slater (29) showing that membrane bound CF, labeled with ATP in the light retains bound ADP and not Pi, from the ATP when isolated, and the data of Smith and Boyer (32) and Vinkler et al. (22), it is apparent that the de-energized enzyme preferentially loses Pi, and retains bound ATP. This release of Pi is not rapid because a continued oxygen exchange is observed. The slow phase of bound ATP disappearance we observe can thus be accounted for by the continued reversible hydrolysis of some of the bound ATP, and a slow preferential loss of the P, formed to the medium.

Our observation that a higher level of medium Pi, can increase the amount of \([^{32}P]ATP bound to the catalytic site found shortly after de-energization may reflect reversal of the P, release step that participated in the ATP \(\rightleftharpoons\) P, exchange while some promotive force still remains.

The above model invokes only two catalytic sites/synthase participating in the binding change mechanism. Whether two or three sites are present on each synthase complex is at present uncertain, but the relations to data reported here would be essentially the same even if three sites were operational. The stable end product when de-energization causes net ATP synthesis to cease is the synthase complex containing a tightly bound ADP, but not P, at a catalytic site that had been in the conformation which catalyzed rapid reversal hydrolysis during net photophosphorylation.

It needs emphasis that in this model the principal change from a quiescent state to the state active for net phosphorylation is one where the \(E\text{-ATP} \rightleftharpoons E\text{-ADP}\) equilibrium is not shifted more than a factor of 10 and is still close to unit; rates of both hydrolysis and synthesis are markedly increased. Two possible reasons for this change in properties appear to us to warrant consideration. One is that the decreased catalytic rates observed reflect changes in properties of the site due to the lack of occupancy of another catalytic site by medium ATP. Another is that there is a change in the properties of the synthase complex associated with the drop in promotive force. In either case, the quiescent state is not representative of a conformation that is present as part of the usual catalytic cycle.

Our data are in accord with those of Afalo and Shavit (9) suggesting that \([^{32}P]ATP formed from \(^{32}P\), can shift rapidly...
or preferentially to noncatalytic sites, either in the light or during de-energization. This shift could occur through a space not accessible to hexokinase as suggested by Aflalo and Shavit. Such behavior would also harmonize with evidence from Kozlov's laboratory (10) for some preferential access of catalytic ATP to noncatalytic sites. Evidence suggests that noncatalytic sites on α-subunits are close to catalytic sites on β-subunits (34), and such proximity or even surface migration could favor noncatalytic site capture of ATP once released from a catalytic site. Noncatalytic sites may become emptied during continued incubation with hexokinase and glucose present. The con constant for the tight binding at noncatalytic sites may be several orders of magnitude greater than that for hexokinase binding of ATP, and, in addition, some binding by hexokinase may be reversible before formation and release of ADP. Thus, even though present in relatively large amounts, hexokinase might compete poorly with avid, noncatalytic sites for ATP binding on the synthase complex.

Our demonstration that most [32P]ATP remaining tightly bound to the synthase after de-energization can undergo oxygen exchange gives strong evidence that, at least initially, such an ATP is bound to catalytic sites. Further evidence for this comes from the rapidly disappearing character of the bound [32P]ATP present in the light as medium 32P is depleted (Fig. 2). The presence of tightly bound ATP at catalytic sites was not supported in a recent report of Aflalo and Shavit (9). Their experiments and earlier results (35, 36) on characteristics of tightly bound nucleotide exchanges upon energization did not provide a critical assessment of participation of a transitorily tightly bound ATP as a catalytic intermediate. The considerable evidence for such ATP as a catalytic intermediate is summarized elsewhere (7). The view of Shoshan and Selman (35) from their studies that, "...energy requiring conformational changes might be involved in the release of tightly bound nucleotide as a step in the catalytic sequence," is quite in accord with the earlier and present data supporting the binding change mechanism. An appropriate interpretation is that on the ATP synthase two types of tightly bound ATP may be present at relatively high levels, that at the catalytic and that at the noncatalytic sites. Under some conditions, labeling of noncatalytic sites can be quite rapid (this paper and Refs. 1, 7, 9, 15, and 35–37). Further, rapid exchange of noncatalytic tight nucleotides with medium nucleotides is feasible. Even if dissociation constants were of the order of 10^{-9}, rebinding by a diffusion controlled process could result in exchange within a second or less. Experimental distinction of whether a label is at a catalytic or noncatalytic site can be difficult.

The tightly bound ADP that remains bound to the de-energized synthase likely represents that observed by Schlodder and Witt (37) which is released when the synthase is activated by electric pulses and the ADP that Smith and Boyer (32) observed to be rapidly released upon acid-base transition of thylakoid membranes. Also, it is likely that same type of ADP that accounts for the formation of bound ATP from medium P, and isolated CF1 as observed by Feldman and Sigman (38). Several laboratories have demonstrated that bound ADP inhibits the ATPase activity of both isolated and membrane bound CF1 (14–17, 34–36, 39) as well as F1 ATPases (31–13, 18). This inhibition may result from a tightly bound ADP present at a catalytic site without accompanying P (21). That the tightly bound ADP is at catalytic sites has been suggested by results with thylakoid membranes (32, 35) CF1 ATPase (39), submitochondrial particles (40), and heart F, ATPase (41–43).

Brief comment may be helpful on interactions between ADP and P binding. Dunham and Selman (44) have observed that P, can inhibit tight binding of ADP by light-activated spinach thylakoids. Binding of P, at a catalytic site may decrease ability of ADP to bind at the same or another catalytic site; their binding is anticooperative. The binding of P, and formation of bound ATP from bound ADP obtained by Feldman and Sigman (38) requires much higher P, concentrations and lower pH than that normally used for phosphorolytic assays.

Our results are pertinent to control of ATPase activity. Light activation of ATPase and of P, = ATP and P, = HOH exchange activities has sometimes been considered to result from a special conformational change induced by protonotive force. This remains feasible, but present data favor an alternate possibility, namely that inhibition of ATPase and other activities is an expression of occupancy of one catalytic site by ADP alone. The decay of ATPase activity after light is extinguished would result from formation of tightly bound ADP and P, at a catalytic site followed by preferential loss of P, Similarly, the well documented inhibition of ATPase by ADP binding mentioned earlier may result from ADP binding at an unoccupied site. The retention of ATPase activity after illumination ceases and even in presence of NH4Cl, could reflect coordinated departure of both ADP and P, by alternating site participation. A lone, tight ADP on isolated CF1, not activated by heat or other treatment, or on membrane bound CF1, may be only slowly displaced following ATP binding at an alternate site. Weak ATPase activity by turnover of single, nonalternating sites is possible, but when one catalytic site retains both P, and ADP and the other only ADP turnover of the former needs to be extremely slow to account for data presented here.

In conclusion, in our model when the light is extinguished, chloroplast thylakoids are transformed into a deactivated state where reversible ATP hydrolysis still occurs but net ATP hydrolysis and interchange of bound ATP with medium P, or ATP is nearly completely blocked. Eventually, all of the ATP is hydrolyzed to enzyme bound ADP and P, when all of the P, is released to the medium, the enzyme is in an inactive state with ADP but no P, at tight binding catalytic sites.

REFERENCES
Chloroplast ATP Synthase


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