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 $[^32]P$. The level of this bound $[^32]P$ATP is markedly reduced upon de-energization of the spinach thylakoids. The reduction is biphasic, a rapid phase in which the $[^32]P$ATP/synthase complex drops by 2-fold within 10 s, followed by a slow phase, $k_{obs} = 0.01/min$. A decrease in the concentration of medium $[^32]P$ to well below its apparent $K_m$ 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 $[^32]P$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 $[^32]P$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 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 $[^32]P$ 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 ATP 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 (18, 20, 21) and on the nature of catalytic site deactivation that accompanies de-energization.

EXPERIMENTAL PROCEDURES

Materials—Hexokinase, adenylate kinase, glycero kinase, Tricine.$^1$

$^1$The abbreviations used are: Tricine, $N$-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; $CP$, chloroplast coupling factor 1 or chloroplast coupling factor 1 ATPase.
ADP, and ATP were purchased from Sigma. Chloroplast thylakoids were prepared from market spinach as described by Vinkler et al. (22) with the modification of Rosen et al. (7). Chlorophyll concentration was measured by the method of Arnon (23), and ATPase concentration (1.3 nmol of Mg$_2^+$/mg of chlorophyll) was estimated as previously described (2). [γ-32P]ADP was prepared from 0.1 M of γ-32P-ATP, incubated with unlabeled ATP, neutralized with 0.6 M KOH, and 10 μmol of Na/Tri, 25 mM MgCl$_2$, and 10 μmol of Na$_2$CO$_3$ at pH 8, with 100 units of adenylyl kinase for 1 min (final volume, 1 ml). [γ-32P]ADP was separated from ATP and AMP on a column (22).

**Time Course of [32P]ATP Disappearance**—The standard assay mixture at pH 8 contained total volume 10 mM MgCl$_2$, 25 mM NaCl, 2 mM Pi, 5 μM [γ-32P]ADP (10'000 cpnmol), 50 mM Na$_2$CO$_3$, 50 mM Na$_2$HPO$_4$, 200-500 units of hexokinase, and chloroplast thylakoids membranes equivalent to about 50 μg of chlorophyll. These conditions gave typical steady-state ATP synthesis rates of about 1200 μmol/h/mg chlorophyll. The mixture was illuminated for 20 s using a 300-watt slide projector; the duration of the illumination was controlled by an electronic shutter (22). When the illumination was terminated, 1 ml of 150 mM NaH$_2$PO$_4$ in 50 mM Tris-Cl, pH 8, (NaH$_2$PO$_4$/Tris) was added. At various times during the course of the reaction, bound nucleotides were extracted from the thylakoid membranes by the addition of 2 ml of 1 M perchloric acid containing 1-2 μmol of unlabeled ATP. [γ-32P]ATP was isolated by passage through a charcoal column followed by a Dowex 1 column as in Ref. 22 except in experiments where more than 10$^6$ cpnmol of ATP, were used per time point. In those cases, the perchloric acid solution contained 5 μmol 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 Pi, 0.1 M H$_2$PO$_4$, and 0.3 M 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, [γ-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 at increasing concentrations of hexokinase, with perchloric acid quench after a few seconds in the light (25). Extrapolation to infinite hexokinase, which gives a measure of 32P-labeled, bound ATP, showed that 95-90% of the [32P]ATP present was bound. Bound ATP levels reported are corrected for this portion of free ATP.

**ATP = H$_2$O Exchange Experiments**—In the oxygen exchange experiments, assay conditions similar to those described above were used except that 2.2 mM P$_i$ or [18O]P$_i$ (including 32P, at 7500 cpnmol, 200 μM ATP, about 7000 units of hexokinase, and chloroplast thylakoids membranes equivalent to 50 μg of chlorophyll) were used in place of unlabeled ATP in the assay mixture. A 96-ml solution of the assay mixture, containing 10 mg of chlorophyll was placed in a wide beaker (21.5 cm) and was illuminated through a CuSO$_4$ filter for 2 s with two 650-watt Lentar photo lamps. This procedure was repeated 5 times. After illumination, 3.75 ml of 2 M NaH$_2$PO$_4$ in 50 mM Tris-Cl, pH 8, were added to each aliquot, the solution incubated for 1 min, and 50 μmol of ATP/alloquet were added (2 nmol of EDTA were also added to the sample with [18O]P$_i$). The mixture was incubated for 5 min more and then centrifuged in 250-ml bottles at 10,000 rpm for 15 min. The pellets were homogenized in 5 ml of solution 150 mM Na$_2$/Tricine, 10 mM MgCl$_2$, 25 mM glucose, 75 mM Na$_2$CO$_3$, and 10 units of hexokinase/ml at pH 8.

The experiments with [18O]P$_i$ were similar except the mixture analyzed was 18O-depleted ATP. The thylakoids were allowed to react 10 to 15 min with H$_2$O$_2$ in solution A. Then, 0.35 ml of nearly 100% trichloroacetic 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 H$_2$O$_2$ was recovered by biphosphorylation transfer and the residue brought to 200 ml, pH 8.5. The ATP was isolated with use of a 3-ml Dowex column as described above. The recovered ATP was neutralized with 50% NaOH, desalted over Sephadex G-10, and treated with glycerokinase to form P$_i$ from the γ-phosphoryl group of ATP (24, 25). The P$_i$ was derivatized to the chloroplast thylakoids and treated with glycerokinase to form P$_i$ from the ATP. ADP was eluted from the Dowex 1 column with 2 M triethylamine carbon.
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 P$_i$ 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 sub saturating levels of P$_i$, 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$_i$. To characterize this phenomenon more carefully, the amount of bound $[^{32}P]$ATP remaining after de-energization was determined at different levels of P$_i$ in the light and in the dark. With 50 $\mu$M or more P$_i$, about 1 ATP/synthase was rapidly labeled. A decrease of P$_i$, 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 the dark value 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$_i$, 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$_i$, 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$_i$ 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$_i$ 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$_i$, bound at an alternate catalytic site. The off constant for this tightly bound ATP, calculated from the results of Table I, 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 P$_i$, characteristic of ATP formation catalyzed by the light-activated synthase. Measurements were made to test this possibility. Table I 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
TABLE I

Exchange of oxygens of bound ATP on de-energized thylakoids with water oxygens

Conditions and procedures were as outlined under "Experimental Procedures."

| Experiment | Reactant labeled with "O | Reaction time | Initial "O % in labeled reactant | Final "O % in γ-phosphoryl of ATP | γ-phosphoryl of ATP | Fraction exchange of γ-phosphoryl of ATP |
|------------|------------------------|--------------|--------------------------------|---------------------------------|-------------------|---------------------------------------|
| 1          | P<sub>1</sub>          | 40 min       | 85%                            | 9.2                             | 0.9               |                                       |
| 2          | H<sub>2</sub>O         | ~15 min      | 49                             | 32                             | 0.7               |                                       |
| 3          | H<sub>2</sub>O         | ~10 min      | 45                             | 26                             | 0.6               |                                       |

*Total time after de-energization: exchange may occur in both rapid and slow phases.

1<sup>3</sup>O<sub>2</sub> content of the γ-phosphoryl group estimated from the 1<sup>3</sup>O content of the P<sub>1</sub> used (98%) and the known extent of water oxygen incorporation into ATP during steady state phosphorylation under conditions used.

1<sup>2</sup>H<sub>2</sub>O added several minutes after light extinguished and uncoiler added.

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1<sup>14</sup>O<sub>2</sub> produced during net photochemistry (27, 28). Thus, the 1<sup>3</sup>O enrichment of the bound ATP formed in the light is expected to be not much less than the enrichment of the P<sub>1</sub> used in the experiment, and the marked decrease in the enrichment of 1<sup>3</sup>O observed in the ATP could only occur in the dark.

Hackney et al. (28) showed that under low light intensity, intermediate ATP = H<sub>2</sub>O 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 decreasing and the NH<sub>4</sub>Cl was mixing with the thylakoid reaction mixture. Experiments 2 and 3 were conducted to test this possibility. In these experiments, H<sub>2</sub>O added 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 1<sup>3</sup>O enrichment of the bound ATP formed in the light is expected to be not much less than the enrichment of the P<sub>1</sub> used in the experiment, and the marked decrease in the enrichment of 1<sup>3</sup>O observed in the ATP could only occur in the dark.

An important point from the data of Table I is that extensive exchange was observed when 1<sup>3</sup>O-labeled ATP was present at the time of de-energization or water H<sub>2</sub>O 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 ["P]<sub>1</sub>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 P<sub>1</sub> and Bound ["P]<sub>1</sub>ATP on De-energized Thylakoids.—Because the ["P]<sub>1</sub>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 ["P]<sub>1</sub>P<sub>i</sub>, is exchangeable with medium P<sub>1</sub>. To test if such an exchange might occur, unlabeled P<sub>1</sub> in the millimolar range was added 1 min after illumination of thylakoids in 20 μM "P<sub>1</sub>. Even after 1 h, no loss of "P from the bound ["P]<sub>1</sub>ATP was detectable. The experiment was repeated under conditions giving a higher initial bound ["P]<sub>1</sub>ATP concentration. Thylakoids were illuminated at higher initial P<sub>1</sub> concentrations, either 100 or 500 μM "P<sub>1</sub>, and the concentration of unlabeled P<sub>1</sub> present after quenching was 500 μM or 3 mM, respectively. An apparent slow exchange reaction occurred at 100 μM P<sub>1</sub>, (k<sub>obs</sub> = 0.002/min), and at a faster rate at 500 μM P<sub>1</sub>, (k<sub>obs</sub> = 0.006/min). However, we found that, when "P<sub>1</sub> at 100 μM or higher concentration was used, some "P<sub>1</sub> 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 "P<sub>1</sub>, and apparent wash out of label is measured.

This side reaction did not greatly interfere with our measurements of the rate of bound ["P]<sub>1</sub>ATP disappearance because the product isolated from ATP synthase at 2 mM P<sub>1</sub>, only 1 min after the NH<sub>4</sub>Cl quench was more than 85% ATP as tested by hexokinase cleavage. Therefore, any reaction to give the contaminating "P<sub>1</sub> species is too slow to affect appreciably the accuracy of the early time points of the ATP decay curve at high P<sub>1</sub> concentrations. And at low P<sub>1</sub> concentrations, this contaminating reaction does not interfere at any time points as indicated by a lack of effect of the addition of unlabeled P<sub>1</sub> to the medium containing 20 μM "P<sub>1</sub>.

As a test of more readily if the tightly bound P<sub>1</sub>, formed from hydrolysis of ["P]<sub>1</sub>ATP bound to thylakoids might be exchangeable with medium P<sub>1</sub>, an inverse experiment was carried out i.e. illumination in 100 μM unlabeled P<sub>1</sub>, followed by a quench and incubation in NH<sub>4</sub>Cl and 500 μM "P<sub>1</sub>, 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 P<sub>1</sub>, 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 ["P]<sub>1</sub>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 "P<sub>1</sub>, and subsequently incubated in NH<sub>4</sub>Cl 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 ["P]<sub>1</sub>ATP that was bound to de-energized thylakoids in the dark. After 2 s, about 90% of the bound ATP disappeared, and after 10 s only about 3% of the counts were left. The disappearance of ["P]<sub>1</sub>ATP did not
follow first order kinetics, as if a slow and fast component were present. In addition, all of the product isolated was chlorophyll. The phosphorylation rate is reduced from our

**Table II**

| Illumination time | [³²P]ATP/synthase remaining |
|-------------------|---------------------------|
| s                 | mol/mol CF₁ | % |
| 0                 | 0.30         | 100 |
| 2                 | 0.086        | 22 |
| 5                 | 0.078        | 18 |
| 10                | 0.065        | 12 |
| 20                | 0.037        | 2.7 |

bound [³²P]ATP was formed by a 15-s incubation in the light, under similar conditions but with only 100 μM ATP. Thylakoids were then incubated in the dark for 10 s in 5 mM NH₄Cl, followed by 10-fold dilution to 5 ml in assay mix with 106 μM unlabeled P, and reilluminated for the times indicated.

Results obtained are given in Table III. In experiment 1, thylakoid ATP synthase was incubated with only 5 μM [³²P]ADP and P, 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 preferential 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 P, in the assay mixture, Vinkler et al. (22) showed that after extensive washing of thylakoids the ADP to ATP 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 P, and loss of that P, 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 formed in the absence of added P, (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 P, with a very low level medium P, 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 P, or from a reaction

**Table III**

| Experiment | Illumination time | Before | After | P, concentration | [³²P]ATP | [³²P]ADP | ATP/
|------------|-------------------|--------|--------|------------------|---------|----------|---|
| s          | mM                | added  | added  | [³²P]ATP/synthase | [³²P]ADP/synthase | Ratio | ATP |
| 1          | 0                  | 23     | 2      | 0.51             | 0.62    | 1.2      | --- |
| 2          | 20                 | 3      | 0      | 0.39             | 0.54    | 1.4      | --- |
| 3          | 20                 | 3      | 0      | 0.22             | 0.35    | 4        | --- |
with a contaminating adenylate kinase that would catalyze the conversion of $[\beta,\gamma-^3P]ADP$ to $[\beta,\gamma-^3P]ATP$ 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 $[^3P]ATP$—** Chloroplast thylakoid membranes have been shown to lose readily the light-induced capacity for ATPase in the dark when they do not have ATP available to maintain a protonotive force (see Refs. 14, 30, and 31). Experiments were conducted to determine whether the site containing the bound $[^3P]ATP$ rapidly labeled from $^{32}P$, during photophosphorylation may participate in a slow ATPase after de-energization. Less than 20% of the $[^3P]ATP$ 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 $[^3P]ATP$ was observed. The catalytic site with bound $[^3P]ATP$ in the de-energized thylakoid 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 P$_i$ 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 P$_i$ 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$_i$ can be present. The ADP and P$_i$ 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 Afaflo and Shavit (9) observed decreases in bound $[^3P]ATP$ formed from $^{32}P$; when unlabeled ATP is added with uncoupler.

The initial release of bound ATP and bound ADP and P$_i$ 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$_i$. 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 P$_i$ 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 P$_i$ and retains bound ATP. This release of P$_i$ 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$_i$, formed to the medium.

Our observation that a higher level of medium P$_i$, can increase the amount of $[^3P]ATP$ bound to the catalytic site found shortly after de-energization may reflect reversal of the P$_i$ release step that participated in the ATP $\rightleftharpoons$ P, exchange while some protonotive 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 operative. 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$_i$, 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 $E_{-ATP} \rightleftharpoons E_{ADP}$ equilibrium is not shifted more than a factor of 10 and is still close to unity; 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 protonotive 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 Afaflo and Shavit (9) suggesting that $[^3P]ATP$ 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 on 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, non-catalytic 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 relatively rapid disappearance 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 conformation 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 10⁻⁶, 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 Scholz und 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 likely is 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 (39), submitochondrial particles (40), and heart F1 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 photo-phosphorylation assays.

Our results are pertinent to control of ATPase activity. Light activation of ATPase 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.

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