Role of AMP in Photophosphorylation by Spinach Chloroplasts*

Marjorie A. Tiefert and Evangelos N. Moudrianakis

From the Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

ATP formation was studied under limited turnover conditions during a postillumination period to ascertain what role, if any, AMP plays in this process. Spinach chloroplast fragments devoid of the conventional adenylate kinase (EC 2.7.4.3) were illuminated in the presence of [32P]orthophosphate and AMP at pH 7.0, and then immediately after the light was turned off, ADP was added. The amount and distribution of 32p label in the soluble nucleotides was determined. Among the findings are: (a) AMP must be present during the illumination period for ATP to be synthesized after ADP is added in subsequent darkness; (b) AMP appears to interact with the known high affinity nucleotide binding site previously considered to have only regulatory functions; (c) ADP has the same apparent affinity under these conditions as it does under conventional steady state photophosphorylation conditions; and (d) an uncoupler and an electron transport inhibitor prevent the reactions that occur in the light phase, while the energy-transfer inhibitor phlorizin appears to affect mainly the events that occur in the dark period after ADP is added.

These results provide strong support for the role of coupling-factor-bound ADP as a phosphorylated intermediate in ATP synthesis, as proposed by Roy and Moudrianakis ([1971] Proc. Natl. Acad. Sci. U. S. A. 68, 2720-2724). This bound ADP, formed by the direct phosphorylation of AMP in the light, donates its y-phosphoryl group to a substrate (acceptor) ADP, where it becomes the y-phosphoryl group of ATP. Thus, during steady state photophosphorylation the ATP synthesized is derived only from medium phosphate and medium ADP. The AMP moiety of the bound ADP is not incorporated into the ATP product but serves as a cofactor in the reactions leading to ATP synthesis.

The ATP synthetase (coupling factor 1) isolated from chloroplast membranes has at least two different types of sites for binding adenine nucleotides (1, 2). The first extensive study of nucleotide binding to CF1 was published 8 years ago (3).

That study showed that CF1 binds up to 2 mol of ADP/mol of enzyme noncovalently but tightly enough to survive nonequilibrium methods of analysis, and also that the CF1-bound ADP is partly converted to bound ATP and AMP. These findings led directly to studies of metabolizing chloroplast membranes, which suggested that a nucleoside monophosphate acts as a cofactor in photophosphorylation (3, 4). It was proposed at that time that AMPPi is phosphorylated to form ADP tightly bound to CF1, on the chloroplast membrane and that the terminal phosphoryl group of this special, bound ADP then is transferred to a substrate ADP to form ATP (4).

In the years since then, the binding of nucleotides to purified coupling factors and the behavior of the tightly bound adenine nucleotides associated with chloroplasts and with other energy-transducing membranes have been extensively studied by many workers. However, the function in ATP synthesis of the "tight binding" sites of coupling factors remains controversial (see Refs. 1 and 2 for specific references not cited here). It seems to be generally agreed that chloroplasts contain bound adenine nucleotides (1 to 4 nmol/mg of chlorophyll) and that the bound nucleotides are noncovalently associated with CF1. In darkness the nucleotides remain tightly bound even when CF1 is isolated from the membranes and purified to homogeneity (3, 4) or partly digested with trypsin (5). Bound nucleotides are released from CF1, when chloroplasts are illuminated, especially when P, and ADP are added. Also under energized conditions, chloroplasts bind added adenine nucleotides and incorporate [32p]Pi into the bound nucleotides. These three processes are generally similar to steady state photophosphorylation in their responses to changes in reaction conditions, although they are significantly less sensitive to energy-transfer inhibitors than is steady state phosphorylation.

The results of several studies appear consistent with the idea that CF1-bound ADP (formed from P, plus AMP) functions as a phosphorylated intermediate in ATP synthesis. For example, the formation of CF1-bound ADP from P, and AMP in chloroplasts and its discharge by medium ADP, as reported earlier (3, 4), was independently verified (1). In other experiments, [32p]Pi, was incorporated into both bound ADP and ATP by chloroplasts, rather than only into bound ADP, but still, substantial labeling of the y-phosphoryl groups of these nucleotides occurred (2). The initial rate of formation of bound nucleotide from P, and [32p]Pi is faster than the conversion of AMP to soluble ADP and ATP by chloroplasts (6). AMP affects electron transport (7, 8) and promotes an ATP-Pi exchange reaction (8) under certain conditions. Chromatophores from Rhodopseudomonas species convert [beta-32p]ADP into [beta, gamma-32p]ATP under the same conditions that [32p]Pi is incorporated into 90% y-labeled ATP (9). These findings, while suggestive, are of course not proof that the formation of bound ADP from P, and AMP is a required intermediate step in ATP synthesis, because of the possible or actual presence of the soluble, non-energy-dependent adenylate kinase (EC 2.7.4.3).
A majority of workers in this field presently appears to favor the view that P, directly adds to ADP to form ATP without the catalytic mediation of the tightly bound nucleotides. Under these views, the bound nucleotides are considered to act only as regulators of coupling factor function (e.g. Ref. 10). These conclusions have been reached mainly because, in most studies of steady state phosphorylation, [γ-32P]ADP, was incorporated into the ATP pool faster than into the ADP pool (11-15), although there have been exceptions (16, 17). These results thus raise the question of the kinetic competence of the bound ADP to function as an intermediate in ATP synthesis. We should note that these previous studies were carried out under conditions where the ATP-synthesizing system was turning over rapidly.

Strong new evidence, presented here, for the direct involvement of AMP in phosphorylation has been obtained from studies on limited-turnover postillumination ATP synthesis by chloroplast membranes depleted of adenylyl kinase. The work described here and in the following paper (18) allows further development of the reaction mechanism that was outlined in 1971 by this laboratory (4). Further, it provides explanations for some of the apparent conflicts between the behavior of bound nucleotides and their properties as anticipated by this mechanism. This paper is concerned with understanding the synthesis and discharge of the bound ADP intermediate, while the accompanying paper (18) deals with its stability.

EXPERIMENTAL PROCEDURES

Preparation of Fragmented, Washed Chloroplast Membranes—Chloroplasts were freshly prepared for each experiment from 0.56 kg (20 oz) of spinach leaves and were washed twice in sucrose/Tris/NaCl (pH 8.0) as described earlier (19). The membranes were suspended in 20 mM NaCl and were broken with a VirTis 23 homogenizer at top speed from 4.5 to 6 min or in a Lourdes homogenizer at half-speed for 40 to 60 s. (The longer shearing times were used for summer chloroplasts.) The suspension was centrifuged at 750 x g for 5 min and the firm portion of the pellet was discharged. The supernatant and the very soft portion of the pellet were combined and were centrifuged at 27,000 x g for 10 min. The pellet obtained in this step was washed twice by suspension in 20 mM NaCl and centrifugation. This protocol was sufficient to remove all detectable adenylyl kinase activity from the thylakoids (see "Results"). The final pellet was suspended in 50 mM Tricine/20 mM NaCl (pH 7.0) at a concentration equivalent to 2 mg of chlorophyll/ml (20) and was used in phosphorylation studies as soon as possible. All the preceding steps were performed at 0-4°C in darkness or under a dim green safelight.

Assay of Phosphorylation under Limited-turnover Conditions—The reaction components and their final concentrations were: chloroplasts equivalent to 0.4 mg of chlorophyll/ml, 50 mM Tricine (pH 7.0), 20 mM NaCl, 1 mM MgCl₂, 20 μM pyocyanine, 1 mM nucleoside monophosphate (pH 7.0), labeled with 2.4 μCi of [γ-32P]ADP, 0.5 mM AMP, and 1 mM ADP. The final volume was 5 ml, and the incubations were carried out at room temperature.

In general, 1.0 ml of the chloroplast suspension was removed from the stock (kept in darkness on ice) and was added to a 100- or 150-ml beaker containing all of the above components except ADP. The mixture was illuminated for 30 s with a Sylvania Sun Gun tungsten iodide photographic flood lamp (approximately 45 cm distant). A metal plate was used to cut off the after-glow when the lamp was switched off. ADP (in 50 μl) was added in darkness, usually immediately at the end of the illumination period, and the beaker was kept in darkness for 20 to 30 s. Then the reaction was ended by quickly adding 10 ml of a suspension of Celite filter-aid (Johns-Manville) in water (0.1 g/ml) and immediately vacuum-filtering the mixture through two Whatman GF/A or GF/C filters laid on top of one Whatman No. 1 filter in a Büchner funnel (5.5 cm diameter). The filtrate was collected into an ice-cold flask containing 1 ml of 70% perchloric acid, and then the mixture was added to 50 ml of Norit A charcoal in 2 ml of 6% perchloric acid/0.05 M NaH₂PO₄ and was kept at ice temperature for further processing. Perchloric acid was not added until after the chloroplasts were removed by filtration, because we wanted to study only the free nucleotides, and perchloric acid extracts bound nucleotides from CF₁ (3). Steady state phosphorylation was measured under the same conditions except that ADP was present during the illumination period. Labeled 32P was recovered by centrifuging the mixture and then transferring the charcoal to a small column plugged with glass wool. The column was washed with cold 5% perchloric acid/0.05 M NaH₂PO₄ and then with 0.01 M HCl to remove remaining [γ-32P]ADP. Nucleotides were eluted with an ammonia/ethanol solution and then were separated from one another by a Dowex 1-Cl column (21). About 5% of the [γ-32P]ADP trailed into the [γ-32P]ATP fractions and was mathematically corrected for. The amount of 32P in the γ position of ATP was determined by reaction with hexokinase and glucose-6-phosphate type P 200 was from Sigma Chemical Co.; Dowex AG 1-X4 was from Bio-Rad Laboratories; DCMU (Dianuron) was from DuPont; CCCP was from Calbiochem; and chloroplasts were from K & K Laboratories.

RESULTS

Rationale Governing the Design of the Experiments—In the mechanism of ATP synthesis envisioned by Roy and Moudrianakis (4), AMP acts as a cofactor during steady state phosphorylation. That is, it is continually phosphorylated to form CF₁-bound ADP and dephosphorylated through donation of its β-phosphoryl group to substrate ADP to form ATP (see also Fig. 2, this paper). The steady state level of coupling-factor-bound donor ADP on the chloroplast membranes, therefore, would depend on the relative rates of the reactions involved in both its formation (Step 1, Fig. 2) and its utilization (discharge) (Steps 2 to 4, Fig. 2). Past studies on the initial net rate of formation of [β,γ-32P]ADP by chloroplasts have yielded contradictory results; in some cases [γ-32P]ADP was formed more quickly than [γ-32P]ATP and, in others, more slowly (11-17). Thus, it seems likely that the relative values of the rate constants for the formation and discharge reaction steps may vary. These variations might be brought about by differences in reaction conditions, in the methods used to prepare the chloroplasts, or even in the way the plant material is grown or stored.

Consequently, for the present study, phosphorylation was measured under non-steady-state conditions. The reactions were carried out in two phases. The first was designed to obtain maximal formation of CF₁-ADP, while preventing its utilization (4). Utilization of the CF₁-bound ATP was allowed to occur during a subsequent discrete step, in the absence of further CF₁-ADP formation. Thus, chloroplast membranes were illuminated in the presence of F, and AMP without any added ADP. CF₁-bound ADP is formed under these conditions (4, 18). If this bound ATP is the last phosphorylated intermediate, it then should be maximally available for donating its terminal phosphoryl group to acceptor ADP to form ATP when substrate (medium ATP) is added subsequently in the dark (second phase of the reaction). Even if the synthesis and discharge cycle were not completely halted at the point where medium ADP is required (Step 2, Fig. 2), general processes of endogenous acceptor ADP may be present, or the donor ADP may dissociate and rebind at an acceptor site), the discharge reaction should at least be rate-limiting under these conditions. Thus, under these limited-turnover conditions, added...
AMP would be expected to have a more significant effect on ATP synthesis than it does under conditions for steady state photophosphorylation, where its turnover is rapid.

To further ensure a limit to the number of catalytic turnovers, a pH shift was not imposed on the light/dark shift as is often done (23). The light and dark phases of the reaction were both carried out at pH 7.0 because this is the optimum pH for the net formation of CF1-bound ADP on chloroplasts (4) and because the magnitude of the transmembrane pH gradient that develops during illumination is relatively low and also is not affected by the addition of ADP when the pH of the medium is 7.0 (24). This experimental design does not allow the formation of an extensive pH gradient that would have promoted rapid and continued turnover after ADP was added in the postillumination phase.

The amount of $^{32}$P, converted into soluble adenine nucleotides in all the experiments on which these two publications are based (this paper and Ref. 18) ranged from 1.3 to 6.25 nmol/mg of chlorophyll when P, and AMP were in the light phase and ADP was added immediately in the dark phase (mean, 3.47 nmol/mg of chlorophyll; standard deviation, 1.36). For reference, the amounts of substrate P, AMP, and ADP usually added were 2500, 1250, and 2500 nmol/mg of chlorophyll, respectively. Independent experiments utilizing electron microscopy (25) and binding of the specific inhibitor tetroxine (26) indicate there is 1 mol of CF1 for every 500 mol of chlorophyll. Thus, 3.5 nmol of P$_i$ esterified/mg of chlorophyll represents 1.6 turnovers/adenylate molecule (not necessarily per active site). This is of course an average turnover number since it is possible that some coupling factor molecules may have turned over several times and others not at all (27).

A short-hand notation for the various reaction designs has been used to facilitate presentation. For example, when chloroplast membranes were illuminated with $[^{32}P]P_i$ and AMP, along with pyocyanine, Tricine, and NaCl, and then ADP was added immediately after the light was switched off (followed by filtration to recover the free nucleotides), this is indicated as "$[^{32}P]P_i$ + AMP → ADP". For some experiments the dark reaction phase was interrupted. For example, "$[^{32}P]P_i$ + AMP $\rightarrow$ CCP$P_i$ + ADP" indicates that CCP$P_i$ was added in the dark phase prior to ADP. A control for this experiment, where CCP$P_i$ was not added, would be written as "$[^{32}P]P_i$ + AMP $\rightarrow$ ADP". A single vertical line will be used to separate a second illumination stage from the preceding dark period. The duration of each reaction phase is noted in the figure and table legends if it differs from what is listed under "Experimental Procedures." An ellipsis (...) indicates a reaction phase whose duration was varied.

And a note on the terminology used here. "Donor" ADP is that formed from P, and AMP and bound in a special donor site on membrane-bound CF1, "Substrate" or "acceptor" ADP refers to ADP that is in ready equilibrium with the surrounding medium and binds in the acceptor site of CF1 (see Fig. 2). The ADP molecules bound in these two types of sites belong to two separate pools and do not ordinarily interexchange freely (4). "Discharge" will refer to the loss of bound donor ADP from chloroplasts via the transfer of its terminal phosphoryl group to added acceptor ADP, resulting in the formation and release of ATP (see Steps 2 to 4 of Fig. 2). Thus, discharge involves chemical change; "exchange," as used here, does not. Exchange (with reference to bound nucleotides) will mean simply the replacement of a bound nucleotide by a free one which becomes bound. Exchange may involve the same or different enzyme sites. "Exchange reactions" will have the usual meaning (16) in discussion of, for example, the ATP-P, or $[^{32}P]P_i$-$H_2O$ exchange reactions. "Binding" and "release" will be used without specifying whether the mechanism involves discharge, exchange, chase, or de novo binding.

Assay for Presence of Adenylate Kinase Activity—The isolated CF1 catalyzes an ADP:ADP transphosphorylation that cannot be attributed to contamination by the conventional adenylate kinase (EC 2.7.4.3) of chloroplasts (22). To decide whether this reaction is catalyzed also by the membrane-bound CF1, during ATP synthesis by chloroplasts, the membranes were prepared under conditions designed to remove adenylate kinase, while retaining CF1. Fragmented chloroplasts were washed several times with dilute NaCl since washes with hypotonic salt solutions remove adenylate kinase and other soluble enzymes from chloroplast membranes (28-30). The washing medium did not contain sucrose or mannitol, etc., because chloroplasts tend to retain their soluble enzymes when the osmotic strength is high (29).

Chloroplast membranes that were prepared in this manner (see "Experimental Procedures") were tested for the presence of adenylate kinase in two ways. First, they were incubated for 30 s in darkness with $[^{3}H]AMP$, $[^{3}H]ADP$, or $[^{3}H]ATP$. Except for darkness, the conditions were those routinely used in this study. No redistribution of label among the nucleotides was seen. Second and for a more rigorous test, chloroplast membranes were allowed to synthesize $[^{32}P]ATP$ in the light from $[^{32}P]P_i$ and ADP in the presence of AMP (reaction design: $[^{32}P]P_i$ + AMP + ADP ...). The $[^{32}P]ATP$ made was 97.5% γ-labeled when the reaction was stopped immediately (standard deviation for three experiments, 2.2) and remained completely γ-labeled when the chloroplasts were allowed up to 120 s of postillumination incubation in darkness (mean of six experimental points, 97.4% γ labeled; standard deviation, 2.8). The results were the same whether these experiments were done with (a) the usual concentrations of added adenine nucleotides (0.5 mM AMP, 1.0 mM ADP) so that unlabeled ADP would be in excess with respect to the $K_m$ (0.103 to 0.184) of chloroplast adenylate kinase (28), or (b) 1.0 mM AMP and 0.5 mM ADP to poise the adenylate concentrations near the $K_m$ of adenylate kinase (34 μM $[^{32}P]ATP$ was made in the light). In another experiment, chloroplasts were illuminated with $[^{32}P]P_i$ and AMP, then ADP was added immediately in the dark (i.e. $^{32}P_i$ + AMP $\rightarrow$ ADP ...). Again, the β/γ distribution of $^{32}P$ in ATP was no different whether the reactions were stopped after 30 or 120 s of darkness. The lower limit of detection in the most sensitive assay for adenylate kinase used here was approximately 0.4 nmol ATP/min/mg of chlorophyll, so it seems very unlikely that the classical, soluble adenylate kinase could be responsible for the results reported here. More importantly, the results themselves are simply not consistent with the characteristics of chloroplast adenylate kinase (see "Discussion") but do accord with the behavior of membrane-bound CF1.

Substrate Requirements for Postillumination ATP Synthesis under Limited-turnover Conditions—In previous experiments from this laboratory (3, 4), CF1-bound ADP was studied by direct analysis of the bound nucleotides after isolation and purification of CF1. In the present work, the free (soluble) nucleotides were analyzed instead because the reactions could be terminated more quickly and more variables could be tested in each experiment. The knowledge that CF1-bound ADP is formed from P, plus AMP and is discharged by added soluble ADP under these conditions (1, 3, 4, 18) aided in interpreting the results.

Table I shows the substrate requirements for postillumination ATP formation under the conditions used here (see "Experimental Procedures" and "Rationale Governing the Design of the Experiments" above). When chloroplasts are illuminated with $[^{32}P]P_i$, in the absence of AMP and then incubated in darkness with added ADP, only a small amount
Role of AMP in Photophosphorylation

Substrate requirements for phosphorylation under limited turnover postillumination conditions

Chloroplast membranes were incubated with MgCl₂, pyocyanine, Tricine, and NaCl and the substrates indicated below as described under "Experimental Procedures," except for Experiment 3, in which the chloroplasts were never illuminated. The components listed to the left of the double vertical line were present during the illumination phase, and those to the right were added immediately in the dark (see "Rationale Governing the Design of the Experiments" under "Rationale"). Experiments 1, 2, and 5 were performed on separate days. Each of the reaction designs listed below has been done two to three times with the same general results.

The time required to saturate membrane-bound CF₁ with ADP, under limited-turnover conditions, was shown earlier to be rather long (4), probably because it takes some time for the entire population of CF₁ molecules on the membranes to become activated during illumination (27). The rate of discharge was studied here by illuminating chloroplast membranes in the presence of [³²P]P₁ and AMP, then adding ADP immediately in the dark, and finally stopping the reaction at various times after the ADP was added. Formation of [³²P]ATP is fast enough that the maximum amount is formed within 2 s or less (Table II). The small decrease in the amount of [³²P]ATP that occurs by 20 s can be attributed to a slow enzymatic ATP hydrolysis occurring in the dark after illumination (13).

Enzyme Kinetics—In the experiment described in this section, the concentrations of added AMP and ADP were independently varied to determine their effects on the amounts of free [³²P]ADP and [³²P]ATP formed (Fig. 1). Chloroplasts were illuminated with [³²P]P₁ and AMP, and then ADP was added immediately during a subsequent dark period. Fig. 1A shows an experiment where the amount of added AMP was varied, and the ADP concentration was kept at saturation. Conversely for the experiment shown in Fig. 1B, a saturating concentration of AMP was used, and then various amounts of ADP were added. Two major trends can be recognized immediately: First, the total amount of [³²P]esterified unmistakably depends on the amount of AMP present during the light phase of the reaction. Second, under these conditions, the amount of unlabeled ADP added in the dark affects only the relative proportions of [³²P]ADP and [³²P]ATP and has no effect on the amount of [³²P]P₁, esterified. With increasing amounts of substrate ADP added in the dark, the yield of free [³²P]ADP is reduced, while there is a corresponding increase in the yield

Table I

<table>
<thead>
<tr>
<th>Reaction design</th>
<th>Free [³²P] labeled nucleotides</th>
<th>ATP</th>
<th>β Position</th>
<th>γ Position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADP</td>
<td>nmol/mg chlorophyll</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. [³²P]P₁ + ADP</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.40</td>
</tr>
<tr>
<td>2. [³²P]P₁ + AMP + ADP</td>
<td>0.00</td>
<td>0.01</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>3. [³²P]P₁ + AMP</td>
<td>2.34</td>
<td>0.04</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[³²P]P₁ + ADP</td>
<td>1.18</td>
<td>0.62</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>[³²P]P₁ + ADP + AMP</td>
<td>0.01</td>
<td>0.00</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Effect of length of dark incubation time on amounts of [³²P]ADP and [³²P]ATP formed

The reaction design was [³²P]P₁ + AMP + ADP. Chloroplast membranes were illuminated for 30 s with [³²P]P₁ and AMP, and then ADP was added immediately in the dark. Celite was added and the reaction mixtures were filtered after the indicated periods of time in darkness.

Free [³²P] labeled nucleotides

<table>
<thead>
<tr>
<th>Duration of dark period</th>
<th>ADP</th>
<th>[³²P]ATP</th>
<th>β Position</th>
<th>γ Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>s</td>
<td>nmol/mg chlorophyll</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>~2</td>
<td>1.03</td>
<td>0.63</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.18</td>
<td>0.62</td>
<td>1.22</td>
<td></td>
</tr>
</tbody>
</table>
of free [32P]ATP. This is the expected behavior of the ATP-synthesizing apparatus operating via the transphosphorylation mode (4) but not via the direct phosphorylation of substrate ADP by P_i.

The kinetic parameters derived from linear replots of the data shown in Fig. 1 are contained in Table III (see legends of Fig. 1 and Table III for methods of calculation). The substrate concentrations (C_i/2) yielding half the maximum amounts of [32P]ATP product are approximately 10 μM for AMP and 70 μM for ADP. This suggests that in these experiments AMP is interacting with the high affinity, high specificity site known to exist on membrane-bound CF_1,12 while ADP is combining with the lower specificity, low affinity site that also functions in ATP synthesis; and one with lower affinity for AMP (K_m, approximately 10 μM), where the bound [32P]ADP made from AMP is preferentially used as a phosphoryl donor in ATP synthesis; and one with lower affinity for AMP (K_m, approximately 30 μM), where the [32P]ADP is more likely to be released unaltered under these conditions. (An explanation is offered under “Discussion” in Ref. 18.)

In Fig. 1B the free [32P]ADP has an apparent precursor-product relationship with the [32P]ATP. That is, the amount of [32P]ATP formed as a function of the ADP concentration (saturating AMP concentration) is equal to the amount of [32P]ADP depleted, and the C_i/2 values are the same for both processes. However, the free [32P]ADP is not itself the precursor of [32P]ATP, since the β/γ labeling pattern in [32P]ATP implies that the [32P]ADP (formed from [32P]P_i and AMP) does not equilibrate with the added unlabeled medium ADP (Table I). Evidently, if a given molecule of bound [32P]ADP is not used as a phosphoryl donor, then it can subsequently dissociate, unaltered, from CF_1. Even at saturating levels of unlabeled substrate ADP, a significant amount of free [32P]ADP remains as such. This remaining [32P]ADP may represent donor ADP that completely dissociates from the membranes during the light phase and becomes lost to the surrounding bulk medium. No cooperativity is seen in the effects of either AMP or ADP. Lineweaver-Burke and Hill plots of the results shown in Fig. 1 are all linear, and the slopes of the Hill plots are near 1.0 (Table III).

**Effects of Inhibitors and Uncouplers** The formation of

![FIG. 1](image-url) Effect of varied AMP and ADP concentrations on limited turnover postillumination phosphorylation. Reaction design: [32P]P_i + AMP → ADP. In A, AMP was varied from 0 to 100 μM, while ADP was 1 mM. In B, AMP was 0.5 mM, and various concentrations (0 to 300 μM) of ADP were present. The points represent the actual experimental values for the amounts of free [32P]ADP (△) and free [32P]ATP (●) formed and for their sum (■). In A the lines were calculated from the values for C_i/2 and for maximum amounts of products formed (listed in Table III), which were computed on the assumption that endogenous nucleotide equivalent to 1.5 μM AMP was present. This value (to the nearest 0.5 μM) gave the best fit of lines to points and also the best agreement between the kinetic parameters derived by least squares analysis from two types of replots of the data (i.e. Lineweaver-Burke and Eadie-Scatchard plots). In B, the lines were calculated from the kinetic parameters shown in Table III. The best fit to the points above 15 μM added ADP was obtained by assuming that there was no interfering endogenous ADP present in the dark stage and that the [32P]ADP present in the absence of added ADP amounted to 3.15 nmol/mg of chlorophyll (to within 0.05 nmol). The curve for [32P]ADP extrapolates to a minimum of 0.98 nmol/mg of chlorophyll.

The total amount of [32P] in free ATP.

A. With AMP as varied substrate and ADP saturating [32P]ATP

<table>
<thead>
<tr>
<th>Product</th>
<th>C_i/2 for varied substrate (μM)</th>
<th>Maximum product (nmol/mg chlorophyll)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. With AMP as varied substrate and ADP saturating</td>
<td>[32P]ATP</td>
<td>9.18</td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td>[32P]ADP</td>
<td>28.7</td>
<td>1.64</td>
</tr>
<tr>
<td>B. With AMP saturating and ADP as varied substrate</td>
<td>[32P]ATP</td>
<td>67.6</td>
<td>2.11</td>
</tr>
<tr>
<td></td>
<td>[32P]ADP</td>
<td>69.8</td>
<td>2.17</td>
</tr>
</tbody>
</table>

The total amount of [32P] in free ATP.

Free [32P]ADP present at zero added ADP minus the free [32P]ADP present at each other concentration of unlabeled ADP.
Affect the discharge of bound donor ['2P]ADP by added uncouplers, and energy-transfer inhibitors of the type that acceptor ADP (4). In general, electron transport inhibitors, interact with the membrane sector of the ATP synthetase nucleotides by chloroplasts and the incorporation of [9'P]Pi into bound nucleotides (2, 32). However, energy-transfer inhibitors that interact with CF1 (e.g. phlorizin, Dio-9) have little or no effect on these processes (2).

Tables IVA and VA show the effects of DCMU, the uncoupler CCCP, and phlorizin when added in the light phase of postillumination phosphorylation assays. Their effects on steady state esterification of Pi, under these conditions are included for comparison (Table IB). All three compounds inhibit the appearance of free [32P]-labeled nucleotides under limited-turnover and steady state phosphorylation conditions about equally well.

When DCMU, CCCP, and phlorizin are added in darkness immediately after the light phase but before unlabeled ADP is added, they can no longer inhibit the formation of bound donor ADP but can interfere with its discharge. CCCP and DCMU have no effect on the amount of ['32P]ADP released under these conditions (Table VB). The amount of ['32P]ATP is decreased, but, significantly, this inhibition is not complete (compare with Table VA). Even after correction for the low amount of ['32P]ATP that seems to be synthesized in the light (see Table I), the inhibition by CCCP and DCMU is only approximately 50%. Two explanations are possible. A proton gradient may stimulate but not be absolutely required for the discharge stage of ATP synthesis. Or, the uncoupler may stimulate or induce a low level of ATPase activity (1, 14, 33), thereby decreasing the amount of ['32P]ATP after it has been formed. FCCP and other uncouplers added in darkness also do not affect postillumination binding of ADP to chloroplast membranes (34, 35).

Phlorizin inhibits to nearly the same extent whether it is added in the light or dark phase of the reaction (Table V). The decrease in free ['32P]ATP caused by phlorizin cannot be attributed to a stimulation of ATP hydrolysis, because phlorizin inhibits the ATPase activity of chloroplasts (33).

Thus, it seems that energy input derived from proton translocation is needed for phosphorylation of AMP to take place at the donor site of membrane-bound CF1. However, it is not directly required for discharge, i.e. for ATP synthesis per se. In contrast, phlorizin apparently interferes with some step in the discharge reaction involving the acceptor site.

### DISCUSSION

Fig. 2 shows a model for the mechanism of steady state ATP synthesis that is supported by the results presented here. This mechanism was proposed by this laboratory in 1971 (4), but the diagram has been redrawn here to emphasize the cyclic nature of the role of AMP during steady state phosphorylation and to show the points where various inhibitors seem to act. The nucleotide-binding sites on CF1 have specialized functions; that is, ADP bound in one type of site serves as a phosphoryl donor, whereas ADP bound in the other site is expected, the adenine moiety of tightly bound (i.e. donor) AMP is converted to ATP. Thus, the ATP product should be, and is (36), derived from medium Pi and medium ADP. Also as expected, the adenine moiety of tightly bound (i.e. donor) ADP is not incorporated into ATP in the presence of added acceptor (substrate) ADP (36). Enzyme kinetics studies with submitochondrial particles support the idea that the reaction is ordered, with Pi adding to the enzyme complex before ADP (37).

The decrease in free ['32P]ATP caused by phlorizin cannot be attributed to a stimulation of ATP hydrolysis, because phlorizin inhibits the ATPase activity of chloroplasts (33).

Since AMP would be continually regenerated during ongoing phosphorylation by this mechanism (Fig. 2), added AMP is not necessarily expected to affect ATP synthesis under steady state conditions. However, added AMP ought to stimulate ATP formation by well-washed chloroplast membranes under limited-turnover postillumination conditions. Such an AMP effect cannot be accommodated by models for ATP synthesis that entail direct combination of Pi with ADP except by being attributed to contamination by the classical soluble adenylate kinase.

Table V shows the labeling patterns expected under postillumination conditions under two possible mechanisms. In mechanism A (detailed in Fig. 2) AMP has a direct role in phosphorylation. In an energy-dependent event, AMP is converted to bound ADP, which then serves as a phosphorylated inter-
mediate\(^2\) and the source of the $\gamma$-phosphoryl group of ATP. In mechanism \(B\), where ATP synthesis involves direct phosphoryl transfer of ADP, any effects of AMP are indirect, i.e. due to its conversion to ADP via the conventional adenylate kinase. A number of findings are consistent with mechanism \(A\) but serve to disprove mechanism \(B\), as follows.

(a) For mechanism \(A\), the $[^{32}\text{P}]\text{ATP}$ synthesized should ideally be 100% $\gamma$-labeled. However, if reaction series 1 and 2 each occurs once (Fig. 3A) under these conditions (18), then the $[^{32}\text{P}]\text{ATP}$ synthesized would have 67% of its label in the $\gamma$ position. For mechanism \(B\), the pool of $[^{32}\text{P}]\text{ATP}$ made under these conditions would be equally labeled in the $\beta$ and $\gamma$ positions. The mean value actually obtained in these experiments for the $\gamma$ labeling of the $[^{32}\text{P}]\text{ATP}$ made in the dark was 64.6%, a value within 2 standard error units of 67% and (more important) substantially greater than 50%.

(b) $[^{32}\text{P}]\text{ATP}$ is not converted to $[\alpha,\gamma-^{32}\text{P}]\text{ATP}$ by washed chloroplast membranes in the presence of AMP and ADP, even with dark-incubation times much longer than those routinely used here (see "Results").

(c) In the experiment presented in Table Ii (reaction design: $[^{32}\text{P}]\text{Pi} + \text{AMP} \rightarrow \text{ADP}$), the amount and distribution of esterified $^{32}\text{P}$ are about the same whether the reaction is ended at 2 or 20 s after the addition of ADP. If the results are due to adenylate kinase, then the reaction has reached equilibrium within 2 s. At this point, $[\text{AMP}] / [\text{ATP}] / [\text{ADP}] = 1$ is approximately 0.000025. This value is 3 orders of magnitude below the $K_m$ for chloroplast adenylate kinase (28) and, thus, the reaction cannot be attributed to this enzyme.

(d) Postponing the time at which ADP is added in the dark phase (i.e. $[^{32}\text{P}]\text{Pi} + \text{AMP} \rightarrow \text{ADP}$; ADP) should not affect the activity of adenylate kinase, yet it does decrease the ratio of $[^{32}\text{P}]\text{ATP}$ to $[^{32}\text{P}]\text{ADP}$ (18).

(e) For either mechanism \(A\) or \(B\), increasing the amount of unlabeled substrate ADP added in the dark should decrease the amount of free $[^{32}\text{P}]\text{ADP}$ and increase that of $[^{32}\text{P}]\text{ATP}$. The apparent $K_m$ for substrate ADP in this process is approximately 70 $\mu$M (Table III). In contrast, the apparent $K_m$ for ADP of isolated chloroplast adenylate kinase is in the millimolar range. The kinetics of the adenylate kinase reaction show apparent positive cooperativity, unlike the results shown in Table III. In addition, the amount of free $[^{32}\text{P}]\text{ADP}$ would extrapolate to zero at infinite concentrations of unlabeled ADP if mechanism \(B\) were operating, but it does not (Fig. 1).

(f) Phlorizin should not affect the dark stages of mechanism \(B\), yet it does inhibit $[^{32}\text{P}]\text{ATP}$ formation when added in the dark phase before unlabeled ADP (i.e. $[^{32}\text{P}]\text{Pi} + \text{AMP} \rightarrow \text{ADP}$) (Table VB).

(g) In mechanism \(B\), the initial rate of formation of $[^{32}\text{P}]\text{ADP}$ should never be faster than that of $[^{32}\text{P}]\text{ATP}$, yet it is under some conditions (16, 17) (see "Rationale Governing the Design of the Experiments" under "Results" for suggestions why ADP is not always formed sooner than ATP). In addition, when chloroplasts do form $[^{32}\text{P}]\text{ADP}$ rapidly (i.e. in <5 ms (17)) in the presence of added unlabeled ADP, the labeled ADP consistently levels off at an amount approximately stoichiometric to that of membrane-bound CF\(_1\) (16, 17), as would be expected for mechanism \(A\).

\(^2\) A mechanism involving AMP was proposed earlier by Ozawa (38), based on studies with submitochondrial particles. We were unaware of this work at the time our paper was published (4), but nonetheless regret the failure to cite this work previously. However, it should be emphasized that the earlier work was done with submitochondrial particles containing high levels of adenylate kinase (38) and can be questioned on this basis. The chloroplast work was done in the absence of interfering adenylate kinase (3; i.e. "unpublished paper).
Donor ADP as an Intermediate in ATP Synthesis—Because of the above findings, it is reasonable and likely that P, esterification during ATP synthesis by chloroplasts occurs via combination of P, with AMP to form ADP bound in a donor site on CF, (Fig. 2). The formation of donor ADP requires energy input since it is inhibited by electron transport inhibitors and uncouplers (Refs. 1 and 4; and Tables IV and V, this paper). Whether the need for energy input should be assigned primarily to the formation per se of donor ADP, the induction of an “energized” state in CF, or some other step in the probably complicated process summarized by Step I of Fig. 2, has not yet been determined.

It is important to note that the donor nucleotide probably exists only very transiently as AMP. Consistent with this idea, washed chloroplast membranes contain little or no endogenous AMP (1, 2). Added AMP does not bind significantly to membrane-free CF, and, when bound AMP is formed by purified CF, through ADP:ADP transphosphorylation, it is readily released from the enzyme (3, 39). The adenosine moiety of AMP seems to bind to membrane-bound CF, only when it is converted to ADP (3, 4). The bound donor ADP is then stable, in that it remains associated with CF, in the dark. Thus, membrane-bound CF, can decay to its “dark” state from the state containing bound donor ADP (A in Fig. 2). Under some conditions, a population of purified CF, contains both endogenous ADP and ATP (2), so some CF, molecules also may decay to a “dark” state from the state containing AMP and ATP (C, Fig. 2), with loss of the bound AMP.

During continuous illumination in the presence of acceptor ADP, the donor ADP is continually used as a phosphoryl donor and then immediately regenerated by re-phosphorylation of the AMP thus formed, perhaps without its ever leaving the donor site of CF,. However, when chloroplasts are illuminated in the absence of substrate ADP, some of the donor ["^32P"]ADP apparently finds its way to acceptor sites on CF,. This donor-to-acceptor migration does not involve equilibration of the released donor with the bulk phase of the solution because (a) during the light period this ["^32P"]ADP would be far below the Ks of the acceptor site (Table III) and (b) during the dark period any free ["^32P"]ADP would be diluted by the approximately 1000-fold excess of added unlabeled ADP. The amount of ["^32P"] in the β position of ATP is expected to be a function of [ADP] donor/[ADP] acceptor and, thus, is relatively high when acceptor (substrate) ADP is not added during the light phase.

Under steady state phosphorylation conditions in the presence of P, and ADP, added AMP has no detectable effect on ATP synthesis⁴ (2, 12). Since only a small, catalytic amount of AMP is needed as a cofactor, it could be supplied in several ways, e.g. from free AMP or bound donor ADP endogenous to the chloroplast membranes or from a low amount of AMP contaminating the added ADP (AMP equal to 1 to 2% of the input ADP would be sufficient). Additionally, added ADP may bind in both the acceptor and any vacant donor sites on CF, (as it does with the isolated enzyme (3, 39)), so that the first turnover of ATP synthesis could begin with transphosphorylation (Step 3, Fig. 2) rather than with P, esterification (Step 1, Fig. 2).

In the earlier experiments from this laboratory, it was shown that it takes 20 to 30 s to obtain the maximum amount of CF,-bound ADP formed from P, and AMP (4). This has been taken by others as an indication that the rate of this reaction is slow (e.g. Refs. 6 and 12). However, those experiments (4) were designed not to obtain maximum rates of synthesis of CF,-bound ["^32P"]ADP but instead to trap the maximum possible amount of this intermediate by not allowing the reactions to turn over. In addition, 20 to 30 s is not the intrinsic time it takes to form bound ADP even with limited turnover, but instead represents the time needed to load the entire population of membrane-bound CF, molecules with ADP. Work from many laboratories suggests that, upon illumination of chloroplasts, CF, undergoes a conformational change that similarly takes approximately 30 s to reach its maximum extent in the population (1, 2). This conformational change seems involved in the conversion of CF, from a “dark” state to a form capable of catalyzing phosphorylation. Apparently each CF, molecule can convert to the phosphorylating state within milliseconds, but it takes much longer for the entire CF, population to become activated (27).

ATP Synthesis per se—The discharge of bound donor ADP resulting in the formation of free ATP seems to require little continuing net energy input since it will occur in postillumination darkness (4) even in the presence of CCCP (Table V). However, the ability of membrane-bound CF, to form ATP decays rather quickly in the dark (18). Thus, this reaction evidently requires CF, to be in a conformation that is induced by illumination of chloroplast membranes and remains stable for a short time when energy input ceases. The isolated, purified CF, also catalyzes (very slowly) an ADP:ADP transphosphorylation (3, 22, 39). This implies that either (a) this same or a very similar CF, conformation occurs spontaneously at a low frequency in the absence of coupled energy input, or (b) CF, in a “non-energized” conformational state also catalyzes the terminal step of ATP synthesis, but with very low efficiency. Of probable relevance to point (a) are findings that isolated coupling factors exhibit interconvertible conformational polymorphism (40) and that soluble CF, is apparently structurally dynamic but stabilized by the binding of adenine nucleotides (41).

Phlorizin inhibits a dark process in postillumination ATP synthesis (Table V). This compound also inhibits ATP hydrolysis by chloroplasts and by isolated CF, (28, 33). However, it does not inhibit transphosphorylation by isolated CF, (39) or incorporation of ["^32P"]P, into bound nucleotides by chloroplasts (2). It also does not affect binding or release of nucleotides by the high affinity site on chloroplast membranes (2). Consequently, the major effect of phlorizin appears to be on the release of synthesized ATP from CF, (i.e. Step 4, Fig. 2).

Others have suggested that the combination of P, with ATP during ATP synthesis does not require energy input, while binding of the released of the ligands do (2). Boyer's group, the major proponents of this view, reached these conclusions from studies on the uncoupler sensitivity of various exchange reactions in submitochondrial particles (16, 42). If the esterification of acceptor ADP is indirect as shown in Fig. 2 rather than direct as assumed by Boyer's group, then the esterification of P, and the formation of the β-γ-phosphoryl bond in ATP occur in two separate chemical steps, and the results of the studies on exchange reactions would need reinterpretation. For example, the available "O-exchange results do not differentiate whether "the" major energy-requiring step is P, binding or its esterification to form donor ADP. The finding that the intermediate P,-H₂O exchange reaction that occurs during ATP hydrolysis is independent of energy input from the mitochondrial membranes (42) is actually rather inconclusive, because ATP also will maintain membrane-bound coupling factors in an “energized” conformation (1, 2). ATP alters coupling factor conformation even with the soluble enzymes isolated from bacteria, chloroplasts, and mitochondria⁴ (1, 2, 40).

Conclusion—It seems clear from the results presented here

⁴ E. N. Moudrianakis, J. E. Barnes, and M. A. Tiefert, unpublished experiments.
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that CF₁-bound ADP can function as a phosphorylated intermediate during ATP synthesis under limited-turnover photolllumination conditions. The following paper presents results that are consistent with the functioning of bound ADP as a phosphorylated intermediate also under more usual conditions for studying ATP synthesis (18). In addition, the model presented in Fig. 2 does not contradict what is known so far about steady state photophosphorylation. Thus, this mechanism probably presents a relatively accurate picture of steady state ATP synthesis. This mechanism has some advantages. For example, because the formation of ATP is broken into steps, more opportunities exist for fine control of ATP synthesis by the chloroplast. Of perhaps more importance to bioenergetists, a catalytic function for the previously mysterious "regulatory" sites with high affinity and high specificity for nucleotides has been found. In addition, sites with dual types of functions (catalysis as well as regulation) would make more economical use of the enzyme structure.

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