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Interactions between ADP and the Coupling Factor of Photophosphorylation

HARRY ROY AND EVANGELOS N. MOUDRIANAKIS

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

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ABSTRACT **The coupling factor of photophosphorylation, which carries out the terminal steps in the light-dependent synthesis of ATP in spinach chloroplasts, forms tight complexes with [^{14}C]ADP *in vitro*. The bound [^{14}C]ADP undergoes a transphosphorylation reaction to give [^{14}C]AMP and [^{14}C]ATP. The [^{14}C]ATP remains tightly bound, and can be recovered conveniently only by denaturation of the enzyme·nucleotide complex. If spinach membranes are illuminated in the presence of pyocyanine and [^{3}H]AMP or [^{32}P]P_i, the enzyme can be recovered as a tight complex with [^{3}H]ADP or [^{32}P]ADP. The evidence indicates that AMP is an earlier acceptor of phosphate than is ADP, in a light-driven phosphorylation reaction. It also suggests that AMP serves as a cofactor in photophosphorylation.**

The coupling factor of photophosphorylation in spinach chloroplasts is a 13S protein bound to photosynthetic lamellae and required for the light-dependent synthesis of ATP. It has been shown (2) in this laboratory to reconstitute the phosphorylation of ADP in pre-illuminated, previously uncoupled chloroplast membranes when added in the dark, terminal step of a two-stage (light-dark, X_E-type) reaction. When activated by the presence of dithiothreitol (2, 3, 5) or by the action of trypsin (6), the enzyme exhibits a strong, Ca^{++}-dependent ATPase activity. These facts strongly suggest that the coupling factor ("13S enzyme") is the terminal enzyme in the pathway leading to photosynthetic ATP formation (2, 6). As such, it represents the same functional entity as the chloroplast coupling factor 1 (CF1) first described by Vambutas and Racker as the product of a different isolation method (6).

These considerations suggested that a study of the binding of adenine nucleotides to the coupling factor might contribute information relevant to the mechanism of photophosphorylation. With the results presented here it will be demonstrated that:

(*a*) ADP binds very strongly to the 13S enzyme *in vitro*, at two sites.

(*b*) Once the ADP molecules are bound, the 13S enzyme molecule carries out *in vitro* a stoichiometric transphosphorylation, linking the β-phosphate of one bound ADP molecule to the β-phosphate of the other bound ADP molecule. The products, AMP and ATP, are not readily released in solution.

(*c*) In chloroplast membranes supplied with the electron-transport cofactor pyocyanine, low levels of AMP and [^{32}P]-phosphate, and light, the AMP is rapidly phosphorylated at a site contiguous with, or identical to, the coupling factor. The product, [^{32}P]ADP, remains tightly bound to the 13S enzyme throughout the normal isolation procedure, and can be quantitatively recovered from that protein.

These results may be interpreted to suggest that ADP, bound to the coupling factor, is derived through a light-dependent phosphorylation of AMP, and may serve as the last stable "high-energy" intermediate in photophosphorylation.

MATERIALS AND METHODS

Enzyme isolation

Pure preparations of the coupling factor were obtained from EDTA extracts of previously salt-washed spinach chloroplast membranes by ammonium sulfate precipitation and sucrose gradient centrifugation, as described earlier (1, 2). Strict adherence to that protocol, especially in regard to pH and ionic strength, is essential for high yields of pure enzyme. In control experiments, CF1 was prepared by the methods of Vambutas and Racker (6) and Bennun and Racker (7), and similar results were obtained. In experiments with Swiss chard, chloroplasts were isolated as for spinach, but 0.02 M ascorbate was added to the buffered sucrose isolation medium.

Radionucleotides

[8-^{3}H]AMP and [8-^{14}C]ADP were obtained from Schwarz BioResearch. [γ-^{32}P]ATP was synthesized by the method of Glynn and Chappel as modified by Dr. Bernard Weiss (12), who kindly provided the necessary enzymes. [β-^{32}P]ADP was synthesized from [γ-^{32}P]ATP and AMP with the use of adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) (Sigma LDH) supplied by Dr. M. C. Bessman, and was purified by ion-exchange chromatography on Dowex 1. The homogeneity of the radionucleotides was routinely verified by paper electrophoresis as described previously (5). All other materials were reagent grade.

Binding assays

All experiments were performed with enzyme preparations that were homogeneous by several criteria (2, 5), including polyacrylamide gel electrophoresis, in which the enzyme migrated as a single band even when large amounts (20–200 μg) of sample were loaded onto the gel. The coupling factor was incubated at 1 mg protein/ml with appropriate concentrations of radionucleotides in 0.01 M Tris·H_2SO_4 (pH 8.0)–0.005 M dithiothreitol at 37°C. Incubation in 0.1 M Tricine, 0.01 M Tris·HCl, or omission of dithiothreitol gave the same results. In preliminary experiments it was found that Ca^{++} and Mg^{++} did not significantly accelerate the rate of binding of [^{14}C]-ADP, and since it was desirable to avoid possible interference from the cation-dependent ATPase activity of the enzyme, *no* divalent cations were added in these experiments. After a 2-hr incubation with the nucleotide, the solution was chilled, layered on a Sephadex G-50 M column, 1 × 16 cm, and eluted

with 0.01 M Tris·H_2SO_4, pH 8.0, at a flow rate of 1 ml/min, in order to separate any radionucleotide-enzyme complex from free radionucleotides. The rapid flow rate helped to minimize loss of a small portion of the ^{14}C radioactivity. The 13S radionucleotide·enzyme complex, which was eluted in the void volume (5–10 ml), was assayed for protein by the method of Lowry *et al.* (8), and for radioactivity by liquid scintillation in Bray's fluor (9), in a Packard Tri-Carb 2002 counter. From these data and the known or measured specific radioactivity of the radionucleotide and the counting efficiency, the amount (in nanomoles) of isotopically labeled nucleotide per mg of protein was calculated.

Characterization of radionucleotide·enzyme complexes

After isolation by means of Sephadex G-50, the radionucleotide·enzyme complexes, up to a total of 2 mg of protein, were lyophilized and then dissolved in 10–100 μl of 10 M urea at room temperature for 5 min. This technique denatures the enzyme and efficiently releases almost all of the bound radionucleotides into solution. Samples of the urea solutions were then electrophoresed as described previously (5), except that the dried sample was extracted with methanol, on the paper in order to remove the urea without removing any of the radioactive material. The distribution of radionucleotides on the electrophoretograms was determined as described previously (5).

Metabolic experiments

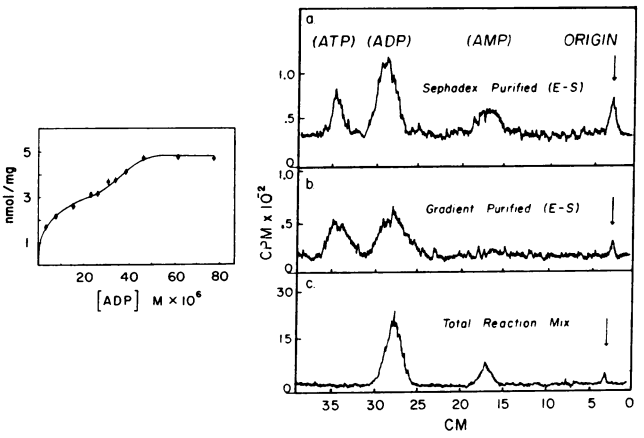
Chloroplasts prepared in subdued light, as previously described (1), from 600 g of commercial spinach were broken in unbuffered 0.02 M NaCl and centrifuged at 4°C. The pelleted membranes were suspended in 150 ml (final volume) of 0.05 M Tricine·NaOH, pH 7.0, containing 0.02 M NaCl, and rendered 2×10^{-5} M in pyocyanine. Aliquots, each 25 ml, were illuminated by a Sylvania "Sun Gun," 650 W tungsten iodide lamp, mounted 45 cm from the thin layer of the sample. A solution of $CuSO_4$ was placed in between to absorb heat, and the reaction mixture was kept at 22°C. The illumination was carried out for 1 min to deplete endogenous intermediates, if any. This step was omitted without effect in later experiments. After several minutes in the dark, AMP and [^{32}P]P_i, each in less than 0.001 M concentration, were added, and the system was again illuminated for 1 min. The sample was then chilled, and the coupling factor was isolated from the membranes at 4°C as described (2, 5), but without dithiothreitol. The specific radioactivity of the sucrose-gradient-purified 13S protein was determined as in the *in vitro* binding experiments.

RESULTS

Nucleotide binding

Preliminary experiments showed that the binding of [^{14}C]-ADP to the 13S enzyme is slow, even at 37°C; it reaches a maximum after 1–2 hr of incubation. The low binding velocity is a significant peculiarity of the system, and further details on its characteristics will be published separately. For the present studies, a standard incubation time of 2 hr was selected.

The binding of the ^{14}C label to the 13S enzyme as a function of [^{14}C]ADP concentration is shown in Fig. 1. The values at each point are equilibrium binding values. The biphasic binding profile was always observed with the homogeneous enzyme prepared from EDTA extracts. (The binding of [^{14}C]ADP to the coupling factor was not sensitive—no competition observed—to simultaneous incubation with IDP, CDP, UDP, or GDP at the same molar concentration as ADP.) In spite of complications introduced by the conversion of bound ADP to other nucleotides, as will be shown later, the data suggest the existence of two binding sites for ADP, the necessary concentrations for 50% saturation of each site being about 2×10^{-6} M and 3.5×10^{-5} M, respectively. The details of this saturation profile will be discussed more fully in a subsequent publication. For the present, the properties of the complex formed from [^{14}C]ADP are of paramount significance.



(*Left*) FIG. 1. Saturation of the coupling factor with ^{14}C from [^{14}C]ADP. The coupling factor was incubated with the indicated concentrations of [^{14}C]ADP (15.8 Ci/mol) as described in *Methods* and the amount of ^{14}C bound to protein (specific radioactivity) plotted as a function of the unbound [^{14}C] nucleotide concentration.

(*Right*) FIG. 2. Electrophoresis of enzyme-bound and free nucleotides derived from incubation of the coupling factor and [^{14}C]ADP. The incubation mixture was divided as follows:

a. 500 μl was fractionated on a Sephadex G-50 column. The excluded protein was lyophilized and dissolved in urea to release the nucleotides. An aliquot of this solution was then subjected to paper electrophoresis at pH 5.4.

b. 500 μl was centrifuged for 6 hr at 48,000 rpm in the SW-50 L rotor, through a linear 5–20% sucrose gradient. This was done to test the stability of the enzyme·AMP complex to lengthy purification procedures. The 13S peak was taken from the gradient, passed through a Sephadex G-50 column, lyophilized, dissolved in 10 M urea, and electrophoresed as in *a*. Note that the [^{14}C]-AMP has been lost in the procedure prior to lyophilization. Occasionally, slowly running Sephadex columns alone gave the same results.

c. 5 μl was directly applied to the paper and electrophoresed at pH 5.4. This represents the composition of the free nucleotide population. Note the absence of free [^{14}C]ATP.

Nature of the radionucleotide complex with the coupling factor

The unusually stable nature of the nucleotide·enzyme complex is illustrated by the fact that at least 70% of the ^{14}C bound to the enzyme remained bound even after several hours standing at 4°C and after all free nucleotides had been removed by gel filtration or dialysis. Several different kinds of treatment failed to release nucleotides from the complex. It was finally found that solutions of 7% perchloric acid, 10 M urea, or 5 M formamide could dissociate the complexes to denatured protein and free nucleotides. The denaturation with urea was selected because of its reproducibility and simplicity and the higher recoveries obtained.

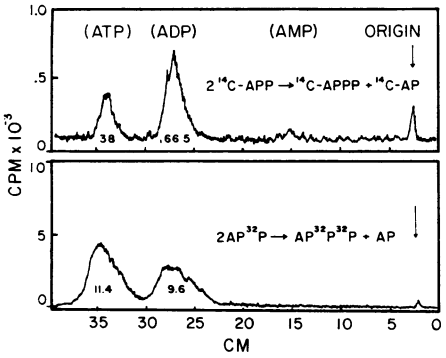


FIG. 3. Distribution of ^{32}P and ^{14}C in bound nucleotides obtained by isolation of the 13S enzyme after incubation with [β-^{32}P]ADP and [8-^{14}C]ADP. Parallel incubations of the 13S enzyme were carried out with 60 μM labeled ADP in Tris-H_2SO_4 buffer. The [^{14}C]ADP was 36.1 Ci/mol and the [β-^{32}P]ADP was 235 Ci/mol. The purified enzyme·nucleotide complexes were denatured and electrophoresed at pH 5.4. Most of the [^{14}C]AMP has been lost during an unusually lengthy purification procedure (see Fig. 2). The numbers under each peak represent integrator tracing units (ISCO model 215) and are proportional to the total cpm of each component (area under each peak).

For the experiment shown in Fig. 2, the complex derived from incubation of the homogeneous enzyme with [^{14}C]ADP was purified from unbound nucleotides by passage through a Sephadex G-50 column, lyophilized, and then denatured in 10 M urea. The resulting solution was then applied to chromatographic paper and co-electrophoresed with standard nonradioactive nucleotide markers. The radioactivity profile shows three distinct peaks (Fig. 2*a*), which coincide with the ultraviolet-absorbing spots of the three nucleotide markers. It can be seen that most of the radioactivity was recovered as [^{14}C]ADP, and a lesser amount was converted to [^{14}C]AMP and [^{14}C]ATP. (The peak at the origin is an artefact due to incomplete solubilization of the protein, a difficulty minimized in later experiments.) When techniques are used that require longer times to isolate the complex, the [^{14}C]AMP is lost to the solution, while the [^{14}C]ADP and [^{14}C]ATP remain tightly bound (Fig. 2*b*). During the incubation of 13S enzyme with [^{14}C]ADP, none of the [^{14}C]ADP is converted to free [^{14}C]ATP (Fig. 2*c*). The only [^{14}C]ATP in the reaction mixture is tightly complexed to the 13S enzyme itself. It follows that the [^{14}C]ATP must have been synthesized there from bound [^{14}C]ADP and could not possibly have been derived from the solution. The same holds for the formation of [^{14}C]-AMP. The observed increase of [^{14}C]AMP in free solution with time of incubation is the sum of the spontaneous hydrolysis of [^{14}C]ADP and the [^{14}C]AMP released from the enzyme. Added AMP in solution cannot be recovered bound to the 13S enzyme, even when high concentrations of AMP are employed (control experiment, data not shown here). Therefore, both the [^{14}C]AMP and the [^{14}C]ATP on the enzyme were formed on the enzyme from [^{14}C]ADP.

To determine the source of the γ-phosphate of the enzyme-bound ATP, we incubated the enzyme with [β-^{32}P]ADP or [^{14}C]ADP in separate but otherwise identical solutions, and measured the distribution of ^{32}P and ^{14}C radioactivity in the resulting ATP and ADP fractions of the isolated radionucleotide·protein complexes. If the source of the γ-phosphate in the product ATP is ADP, the specific radioactivity of [^{32}P]-ATP should be twice that of the [^{32}P]ADP, while the specific radioactivity of the [^{14}C]ATP should be the same as that of the [^{14}C]ADP, so that the ratio of counts at any given time for [^{32}P]ATP/[^{32}P]ADP should be twice that of [^{14}C]ATP/[^{14}C]ADP. Fig. 3 shows that this is indeed so; the ATP:ADP ratio for ^{32}P is 1.2 and for ^{14}C is 0.57. It can be concluded that the product ATP must be β,γ-labeled.

We verified this conclusion by eluting the [^{32}P]ATP from the paper and degrading it with hexokinase in the presence of glucose. Addition of hexokinase to the incubation mixture caused an instant reduction in the charcoal-adsorbable radioactivity from 1790 to 890 cpm/ml. (The product of the hexokinase reaction, glucose 6-phosphate, is not charcoal-adsorbable in 5% trichloroacetic acid (14), the condition used in this experiment.) After the completion of the hexokinase reaction, the entire incubation mixture was freed from some contaminating orthophosphate and then subjected to acid hydrolysis, to degrade the remaining [^{32}P]ADP while leaving the acid-stable glucose [^{32}P]6-phosphate intact (10). In this experiment, 57% of the counts remained extractable as organic phosphate and 43% became extractable as orthophosphate (the isobutanol–benzene, acid molybdate extraction procedure of Berenblum and Chain as modified by Adolfsen and Moudrianakis (14) was used). The results show that the enzyme-bound [^{32}P]ATP was labeled to the same extent in the β and the γ positions.

The 13S enzyme, then, is able to carry out a stoichiometric transphosphorylation reaction on its surface. The β-phosphate of one ADP molecule is transferred to the β-phosphate of another ADP molecule while both molecules are located on the surface of the enzyme. The products, bound ATP and bound AMP, are recoverable by gel filtration and denatura-

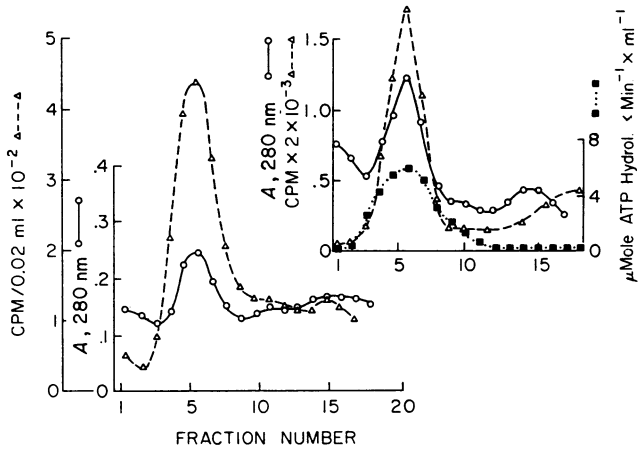


FIG. 4. Sucrose gradients of ^{32}P-labeled 13S enzymes derived from chloroplast membranes of spinach or Swiss chard, illuminated in the presence of [^{32}P]P_i and pyocyanine.

Spinach: Experiment carried out as described in *Methods*, except that Tris buffer at pH 7.95 was used instead of Tricine at pH 7.0, and carrier-free [^{32}P]P_i was employed without *added* AMP.

Swiss chard (*inset*): Experiment employed conditions optimal for spinach, essentially as described under *Methods*. After the radioactivity and A_{280} measurements were made, the gradient fractions were rendered 50 mM in dithiothreitol and stored overnight in the cold. ATPase assays were then performed on the individual fractions, as described earlier (2).

tion of the enzyme, although AMP tends to be released if too much time is spent in isolating the complex.

Isolation of [^{32}P]ADP complex with the coupling factor from metabolizing chloroplast membranes

Since the complexes of nucleotides with this enzyme are very stable, it was considered possible that if the enzyme were labeled under metabolic conditions, the nucleotides interacting with it would remain bound even during the rather lengthy purification time. Preliminary experiments in which chloroplast membranes were illuminated with pyocyanine as electron transport cofactor, and carrier-free [^{32}P]P_i as a tracer, showed that the enzyme could be labeled (Fig. 4). Also shown is a more recent experiment performed with Swiss chard chloroplasts, and giving identical results (Fig. 4, inset). In each case, the ^{32}P radioactivity was released from the enzyme with urea, and was shown to co-electrophorese with ADP at both pH 5.4 and pH 3.5 and to migrate with ADP in paper chromatography in the phosphate-buffered ammonium sulfate–propanol system III of Pabst (11). If [^{3}H]AMP is used as a tracer instead of [^{32}P]P_i, the enzyme again becomes labeled, in a strictly light-dependent step, and all of the bound label is recoverable in the form of [^{3}H]ADP and only [^{3}H]-ADP. No bound [^{3}H]AMP has ever been observed. Fig. 5 shows the electrophoretic profile of the enzyme-bound tritium: exactly identical resolutions were obtained by all three methods of analysis and with both tracers employed, i.e., [^{3}H]-AMP or [^{32}P]P_i. When concentrations of [^{3}H]AMP and [^{32}P]P_i are increased in the presence of illuminated membranes at about 450 μg of chlorophyll per ml, the amount of the enzyme-bound ^{3}H reaches saturation with a limit of one molecule of [^{3}H]ADP bound per molecule of enzyme. The saturating

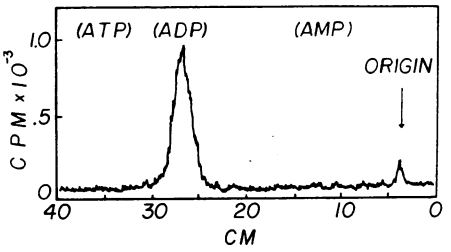


FIG. 5. Electrophoresis of enzyme-bound [^{3}H]ADP derived from chloroplast membranes illuminated in the presence of [^{3}H]AMP and pyocyanine. The experiment was carried out as described in *Methods*, and the gradient-purified, [^{3}H]nucleotide·protein complex was freed of sucrose, denatured, and electrophoresed at pH 3.5.

concentration of [^{3}H]AMP was $<6 \times 10^{-5}$ M, and of phosphate $<10^{-3}$ M. In these experiments, "dark" controls showed low levels of labeling of the enzyme and of the ADP pool in the supernatant. In a typical case, the saturation of the enzyme with the [^{3}H]AMP tracer represents a 60-fold stimulation of the labeling of the enzyme by light (200-fold above background), while the amount of [^{3}H]ADP in the nucleotide pool is stimulated only about 3-fold (Table 1). In view of this, it appears that the newly synthesized ADP does not have to migrate through the nucleotide pool to get onto the enzyme. It is therefore likely that the coupling factor is contiguous with, or identical to, the site of synthesis of the ADP.

TABLE 1. *Light-induced stimulation of labeling of the 13S enzyme and of the ADP fraction of the free nucleotide pool*

*A. Stimulation of the labeling of the enzyme with [^{3}H]AMP**

Sample condition	1 "Dark"	2 Light	3 Light	4 Light
[^{3}H]AMP (μM)	50	12.5	25	50
^{3}H incorporated (cpm/mg protein)	1400†	28,000	47,500	85,600
Stimulation (×)	1	20	34	61

B. Stimulation of the labeling of the ADP fraction of the free nucleotide pool‡

Fraction	Sample 1 ("Dark") cpm	Sample 1 ("Dark") %[^{3}H]ADP	Sample 4 (Light) cpm	Sample 4 (Light) %[^{3}H]ADP
AMP (Input)	27,000		13,590	
ADP	497	1.6	573	3.8
ATP	67		79	

* Chloroplast membranes were illuminated with [^{3}H]AMP and phosphate as described in *Methods*. After the membranes were centrifuged, the nucleotides from the supernatants of samples 1 and 4 were collected by adsorption on charcoal and saved for part *B*. The coupling factors were prepared from the membrane pellets as usual, and their specific radioactivity was tabulated.

† 150 cpm/0.05 ml, corrected for background of 30 cpm.

‡ Nucleotides from the supernatants of samples 1 and 4 (part *A*) were eluted from charcoal, and aliquots were electrophoresed at pH 3.5. The spots corresponding to each nucleotide were cut out and counted in liquid scintillation vials.

DISCUSSION

It must be emphasized that in all reactions described here the 13S enzyme is reacting stoichiometrically with the adenine nucleotides. There is no detectable turnover *in vitro*, and probably negligible turnover *in vivo* under the limiting conditions employed in these studies.

It therefore appears that *in vitro* the enzyme uses ADP both as a donor and as an acceptor of the phosphate in the synthesis of ATP. In the *in vitro* experiments the transphosphorylation reaction occurs apparently irreversibly, in the "solid phase." The turnover number is zero, since the product ATP is not released from the enzyme. The reaction is in that sense different from ordinary myokinase reactions, and should not be confused with them. It has been shown previously in this laboratory that the 13S enzyme is free of any adenylate kinase: the true myokinase of spinach chloroplasts has a sedimentation constant of 6 S and has been recovered quantitatively from the other end of the same sucrose gradient (5). In addition, the transphosphorylation reaction carried out by the 13S enzyme is labile under conditions known not to affect the activity of the true myokinase of spinach chloroplasts (data to be published elsewhere); and the 13S enzyme derived from Swiss chard chloroplasts, which are free of myokinase (13), also carries out the transphosphorylation reaction. The reason why the product ATP is not released from the enzyme *in vitro* is not clear at present.

The fact that the isolated homogeneous coupling factor has the enzymatic machinery to transfer the β-phosphate of one (donor) ADP molecule to another (acceptor) ADP molecule, and thus generate ATP, raises the possibility that *in vivo* an enzyme-bound ADP might also serve as the immediate phosphate donor to substrate (in solution) ADP for photosynthetic ATP formation; if so, this enzyme-bound ADP should be

formed photosynthetically. This proposal is supported by the data presented in this paper. When [^{3}H]AMP or [^{32}P]P_i is supplied to membranes undergoing cyclic electron transport, the enzyme becomes labeled in a strictly light-dependent reaction. The label associated with the enzyme is exclusively in the form of [^{3}H]ADP or [^{32}P]ADP. The evidence indicates that this enzyme-bound ADP is not derived from free ADP. It therefore appears that in a light-driven reaction AMP is an early acceptor of incoming phosphate to form enzyme-bound ADP. The fact that the same reaction occurs in the Swiss chard system not only serves to generalize the results, but also underscores the fact that myokinase cannot be invoked to account for them, since it has been shown that Swiss chard chloroplasts do not have myokinase activity (13). It must be concluded that the enzyme-bound, labeled ADP is photosynthetically generated from AMP, phosphate, and the energy-conserving apparatus of the chloroplast membrane. We propose that this enzyme-bound ADP be considered as a stable, high-energy, phosphorylated intermediate of photosynthetic phosphorylation, and that AMP be considered as a coenzyme of photophosphorylation.

We believe that the analysis presented here bears upon the terminal events of photophosphorylation. Additional support of the hypothesis outlined here has been derived from experiments with metabolizing chloroplast membranes, and will be the subject of a subsequent publication.

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