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Binding of Adenine Nucleotides and Pyrophosphate by the Purified Coupling Factor of Photophosphorylation†

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ABSTRACT: The purified, homogeneous coupling factor for photophosphorylation of spinach chloroplasts binds 2 mol each of ATP, adenylyl imidodiphosphate (AMP-PNP), ADP, or pyrophosphate (PP_i) per mol of enzyme, but very little AMP, and no P_i. The saturation curves for the binding of ADP, ATP, and AMP-PNP to the coupling factor exhibited distinct bimodalities when the enzyme was prepared by a procedure that involved extraction of chloroplast membranes with ethylenediaminetetraacetic acid. These bimodalities were barely evident with coupling factor isolated from chloroplasts that had been previously extracted with acetone. This difference in binding profiles indicated that the two isolation and purification procedures may yield coupling factor in somewhat different conformational states. A study of the competition between ADP, ATP (or its analogue AMP-PNP), and PP_i for binding to the coupling factor revealed that both competitive and allosteric mechanisms may operate in the binding of ligands by this enzyme. The similar nucleotides ATP and AMP-PNP behaved differently in these experiments. ADP was completely inhibited from binding to the enzyme by 2 mol of AMP-PNP per mol of enzyme or by only one mole of intact ATP. This may be related to the ability of the coupling factor to hydrolyze ATP but not AMP-PNP. In addition, the results presented here suggest that adenine nucleotides may interact with the chloroplast coupling factor through both their purine base and their terminal pyrophosphoryl group. Bound nucleotides are enzymatically altered subsequent to their association with the coupling factor, and the details of this reaction, as well as its implications, are presented in the following paper of this issue.

The chloroplast coupling factor 1 (CF_1)[1] is generally accepted as the enzyme that catalyzes the terminal steps in the photosynthesis of ATP (Penefsky, 1974a). Studies of the binding of adenine nucleotides to this enzyme should thus be pertinent to the mechanism of photophosphorylation. The isolated, purified CF_1 has two binding sites for ADP, one with a higher affinity for ADP than the other (Roy and Moudrianakis, 1970, 1971a; Girault et al., 1973; Cantley and Hammes, 1975; Vandermeulen and Govindjee, 1975). CF_1 also has two binding sites for ATP (Livne and Racker, 1969) and for PP_i (Girault et al., 1973). The results obtained from a study of

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[1] Abbreviations used are: CF_1, 13S coupling factor of photophosphorylation; acetone enzyme, CF_1 prepared from an aqueous extract of acetone-treated chloroplasts; EDTA enzyme, CF_1 prepared from an EDTA extract of chloroplast membranes; AMP-PNP, adenylyl imidodiphosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DTT, dithiothreitol; DEAE, diethylaminoethyl; ATPase, adenosine triphosphatase; EDTA, ethylenediaminetetraacetic acid; P_i, orthophosphate; PP_i, pyrophosphate; $\bar{\nu}$, moles of ligand bound per mole of enzyme.

ADP binding by CF_1 led to the proposal of a mechanism for energy-dependent ATP synthesis (Roy and Moudrianakis, 1971b; Tiefert et al., 1977).

This communication provides further characterization of the binding of adenine nucleotides and pyrophosphate to the isolated, homogeneous CF_1. Differences in the apparent affinity of the enzyme for binding nucleotides were found, depending on whether the enzyme was isolated from acetone-treated chloroplasts or from an EDTA extract of aqueously prepared chloroplasts. Thus, some properties of isolated CF_1 may be affected by the conditions under which the enzyme is prepared or stored. Pyrophosphate, ADP, ATP, and AMP-PNP competed with each other for binding to CF_1. The patterns of competition that were observed indicated that CF_1 may have more than two types of ligand-binding sites, that this enzyme may be regulated allosterically, and that adenine nucleotides may become attached to CF_1 through both their adenine and terminal pyrophosphoryl groups.

Little further interpretation of the results of these binding studies is presented, however, because whenever CF_1 was incubated with ADP or ATP a mixture of CF_1-bound AMP, ADP, and ATP and free (unbound) ADP, AMP, and P_i was then recovered from the reaction mixture (Roy and Moudrianakis, 1971a; Tiefert et al., 1977). An analysis of the conversions of bound adenine nucleotides which were catalyzed by the enzyme under various experimental conditions is presented in the following paper of this issue (Tiefert et al., 1977). Because of the isolated CF_1's intrinsic ability to interconvert bound nucleotides, the nucleotide-coupling factor reaction system as commonly studied never reaches binding equilibrium.

Methods and Materials

Preparation of CF_1 by EDTA Extraction of Chloroplasts. Chloroplasts were prepared from spinach leaves in Tris–sucrose–NaCl as previously described (Howell and Moudrianakis, 1967), except that the first low-speed centrifugation was omitted, and the chloroplast pellet was washed only twice by suspension in the Tris–sucrose–NaCl and centrifugation. The chloroplast membranes were then washed sequentially with distilled water (once) and with 20 mM NaCl (twice). They were then extracted twice with 1 mM EDTA (pH 8.0) for 15–20 min with illumination and stirring (Karu and Moudrianakis, 1969). The membranes were removed by centrifugation, and the EDTA supernates were pooled and were made 10 mM in Tris-SO_4 (pH 8.0) and, usually, 5 mM in DTT. Solid ammonium sulfate was slowly added to this buffered extract until it was 50% saturated with $(NH_4)_2SO_4$. The precipitated material was recovered by centrifugation, and then was gently resuspended in 10 mM Tris-SO_4 (pH 8.0) with or without 5 mM DTT as indicated, and was dialyzed for 2–4 h against this buffer. Undissolved material was removed by centrifugation; the supernate was layered on a 5–20% (w/v) linear sucrose gradient containing the same buffer (Tris-SO_4 ± DTT) and then was centrifuged in a Spinco ultracentrifuge, in a SW-25.1 rotor at 25 000 rpm for 36 h, or in a SW-50.1 rotor at 37 000 rpm for 10 h. The position of the coupling factor in the gradient was monitored by assay of the Ca^{2+}-dependent ATPase activity and the protein concentration. The isolated coupling factor was homogeneous by the criterion of polyacrylamide gel electrophoresis. The coupling factor was dialyzed to remove sucrose and was stored at 4 °C in 10 mM Tris-SO_4 (pH 8.0), with or without DTT, as indicated, under which conditions it was stable for at least 10 days. The enzyme preparations used in this study contained roughly equal amounts of each of the two major electrophoretic allomorphs of CF_1 (Adolfsen et al., 1975). Incubation of CF_1 with ADP, ATP, or DTT did not noticeably affect the relative proportions of the two allomorphs (Tiefert and Moudrianakis, unpublished work). Unless otherwise specified, most experiments were done with the "EDTA enzyme".

Preparation of CF_1 from Acetone-Treated Chloroplasts. Spinach chloroplasts were treated with acetone as described by Vambutas and Racker (1965). The coupling factor then was extracted from these chloroplasts and was purified as described by Bennun and Racker (1969), except that excess pigmented material was removed from the crude enzyme by passing it through a DEAE-Sephadex column at high ionic strength. The eluent, containing CF_1, was concentrated by ammonium sulfate precipitation in the presence of ATP. After the crude enzyme was redissolved, it was applied to another DEAE-Sephadex column. CF_1 was eluted from this column as described by Bennun and Racker (1969). The coupling factor prepared by this method was homogeneous by the criterion of polyacrylamide gel electrophoresis. It was stored as a suspension in 2 M $(NH_4)_2SO_4$–2 mM ATP–10 mM Tris-Cl (pH 7.1). For use in binding studies, a sample of the suspension was centrifuged, the pellet was dissolved in Tris-Cl buffer, and the soluble protein was desalted by passage through a Sephadex G-50m column equilibrated with 10 mM Tris-Cl (pH 8.0) at room temperature. The excluded protein ("acetone enzyme") was treated exactly like the EDTA enzyme in the subsequent binding studies.

Binding Assays. The coupling factor was incubated with the indicated radiolabeled compounds for 2 h at 37 °C, at a concentration of about 1 mg of CF_1/mL, as described previously (Roy and Moudrianakis, 1971a). No salt or buffer other than the 8–9 mM Tris (pH 8.0) and 0–4.5 mM DTT that were derived from the enzyme solution was added to the assay media to avoid possible problems due to this enzyme's lability in the presence of high concentrations of salt under some conditions (McCarty and Racker, 1967; Adolfsen and Moudrianakis, 1976a). In competition studies, both ligands were added to the assay mixtures before CF_1 was added.

In pilot experiments, we found that maximum binding levels for all ligands were obtained within 90 min at 37 °C and that the variation in these values for incubation periods between 90 and 300 min was within experimental error (ca. 5%). The effect of temperature on adenine nucleotide binding to CF_1 was tested at 4, 15, 22, and 37 °C. At lower temperatures, the rate of binding was slower. Longer incubation periods were required to obtain maximum binding values at 22 than at 37 °C, but the levels of binding observed between 22 and 37 °C were similar. At 4 °C, saturation was not reached even after 3 days, making binding studies at this temperature impractical. Similarly, the dissociation of bound ligands from CF_1 was slower at lower temperatures (Tiefert et al., 1977). For all binding studies reported here, CF_1 and ligands were incubated together at 37 °C for 2 h, after which the incubation mixtures were resolved by exclusion chromatography (at 22 °C) on 1.2 × 17 cm columns of Sephadex G-50m. The flow rate of these columns was fast, requiring only 5–6 min to obtain the excluded phase (enzyme–ligand complex) in a symmetrical peak without any trailing. This first peak was well separated from a second symmetrical peak containing the free ligand(s). The excluded fractions were assayed for protein concentration and for radioactivity to determine the total moles of ligand(s) bound per mole of CF_1. Because of the characteristic slow release of bound nucleotides from CF_1 (measured in hours), the fast (5–6 min) Sephadex-filtration method used in this study yields the

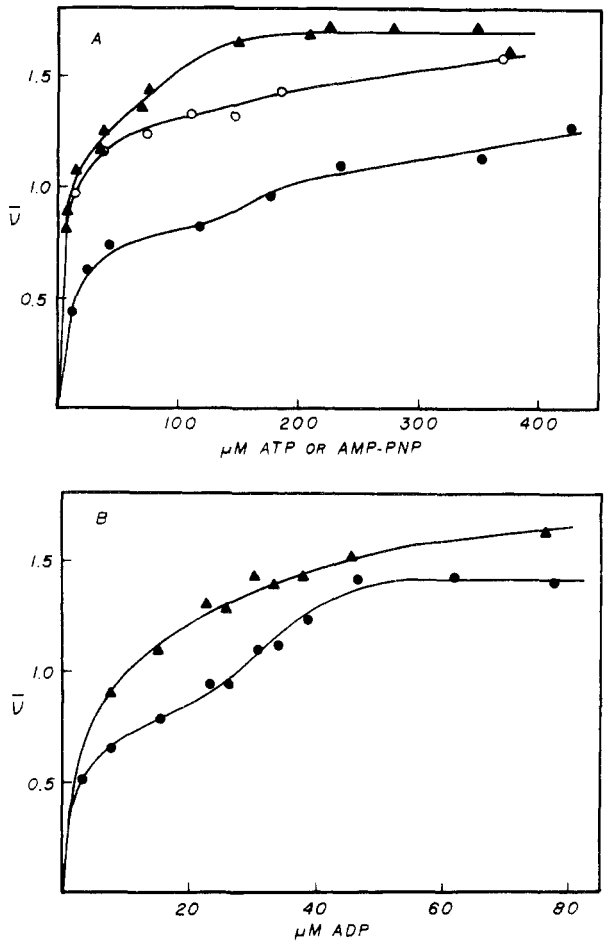

FIGURE 1: Binding of adenine nucleotides to CF_1. Samples of CF_1 were incubated for 2 h at 37 °C with various concentrations of the indicated nucleotide. Then the enzyme–nucleotide complexes were isolated and were assayed for protein and for bound radioactivity as described under Methods. (A) Binding of [γ-^{32}P]ATP or [^{3}H]AMP-PNP to CF_1. (●) Binding of [γ-^{32}P]ATP to the EDTA enzyme (enzyme prepared with 5 mM DTT); (○) binding of [^{3}H]AMP-PNP to the EDTA enzyme (5 mM DTT); (▲) binding of [γ-^{32}P]ATP to the acetone enzyme. (B) Binding of [^{14}C]ADP to CF_1. (●) EDTA enzyme (5 mM DTT); (▲) acetone enzyme.

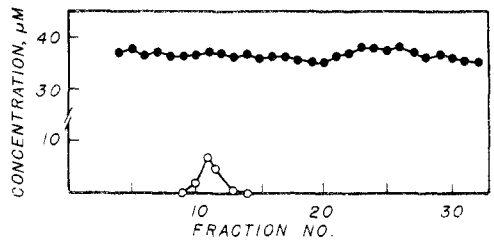

FIGURE 2: Lack of a detectable AMP binding site on isolated CF_1. A Sephadex G-50m column was equilibrated at 37 °C with 10 mM Tris-SO_4 (pH 8.0) containing 35 μM [^{3}H]AMP. Three milligrams of CF_1 (EDTA enzyme, prepared in 5 mM DTT) were incubated with 35 μM [^{3}H]AMP for 2 h at 37 °C. The incubated enzyme was applied to the Sephadex column and was eluted with the equilibration buffer. One-milliliter fractions were collected and were assayed for protein concentration and for radioactivity. (●) Molar concentration of [^{3}H]AMP; (○) molar concentration of CF_1.

true distribution of the ligand present at the end of the incubation period (Adolfsen and Moudrianakis, 1976b). The nature of the particular physical method used to assess the amounts of free and bound ligand is immaterial as long as the distribution of ligand between the free and bound states remains unchanged (within experimental error) during the course of the procedure.

For all calculations, the molecular weight of CF_1 was considered to be 3.5×10^5. The concentration of CF_1 in the fractions collected from the Sephadex columns was determined by the Lowry method, using bovine serum albumin as a standard and the correction factor reported by Farron and Racker (1970). The Lowry method was calibrated with reference to the absorbance of CF_1 at 215 nm minus its absorbance at 225 nm, which is due to the peptide bonds of the protein. This indicated that the correction factor of Farron and Racker was correct also for CF_1 as prepared in this laboratory. Alternatively, the concentration of CF_1 in the fractions collected from the Sephadex columns was determined by their absorbance at 230 nm, after calibration of this absorbance with the Lowry method. Double-reciprocal plots of $\bar{\nu}$ vs. substrate concentration were used to determine the maximum amounts of ligands bound per molecule of CF_1, the substrate concentrations at which half-maximal saturation of enzyme sites occurred, and the maximum inhibition of the binding of one ligand by another. The double-reciprocal plots were used merely as a mathematical tool to linearize the hyperbolic direct plots. Their use *does not imply conformity* to Michaelis–Menten-type considerations used in enzyme kinetics. That is, here the y intercept represents the asymptote of the direct plot ($\bar{\nu}$ that would be observed at infinite ligand concentration), and the x intercept represents not K_m or K_s, but simply the ligand concentration where $\bar{\nu}$ equals one-half the value of the asymptote. However, most biochemists associate double-reciprocal plots with the assumptions made in the Michaelis–Menten treatment of enzyme kinetics. To avoid this, we have illustrated the data of this paper with direct plots because the CF_1–nucleotide system studied here is not a true equilibrium system.

Materials. [γ-^{32}P]ATP was synthesized enzymatically by the method of Weiss et al. (1968). [^{32}P]Orthophosphate, [^{32}P]pyrophosphate, [^{3}H]AMP, [^{3}H]ADP, and [^{3}H]ATP were obtained from New England Nuclear; AMP-PNP and [^{3}H]AMP-PNP from International Chemical & Nuclear Corp.; [^{14}C]nucleotides from Schwarz/Mann; and all other unlabeled nucleotides (sodium salts) from Sigma Chemical Co. "Ultrapure" ammonium sulfate was obtained from Schwarz/Mann.

Results

Binding of Adenine Nucleotides to Isolated, Homogeneous CF_1 Prepared by Various Methods. Saturation curves for the binding of [γ-^{32}P]ATP, [^{3}H]AMP-PNP, or [^{14}C]ADP to CF_1 are shown in Figure 1. A maximum of 2 mol of each nucleotide tested could bind per mol of EDTA enzyme (prepared with 5 mM DTT) or acetone enzyme. The saturation curves for the binding of nucleotides to the EDTA enzyme were distinctly bimodal. The curves for the binding of ADP or ATP to the acetone enzyme were more monophasic. Double-reciprocal plots of most of the results shown in Figure 1 took the form of two intersecting straight lines, concave downward. This may indicate either negative cooperativity or polymorphism of binding sites (Koshland, 1970). The nucleotide concentrations required for half-maximal saturation of CF_1's binding sites were determined by extrapolation of the linear portions of the double-reciprocal plots. These values, as well as the maximum binding values, are listed in Table I for reference and for ready comparison with results in the literature.

It is not likely that the bimodality observed in these saturation curves was artificially introduced by either the assay

TABLE I: Binding Parameters.[a]

Ligands[b]		Varied ligand		Unvaried ligand	Results presented
Varied	Unvaried	Half-max satn (μM)[c]	$\bar{\nu}_{max}$	$\bar{\nu}_{min}$[d]	(Figures)
EDTA enzyme, prepared with 5 mM DTT[e]					
[γ-^{32}P]ATP	—	16, 125	1.70	—	1A
[γ-^{32}P]ATP	66 μM [^{14}C]ADP	11[f]	0.89	<0.14	3
[^{3}H]AMP-PNP	—	5.5, 35	1.68	—	1A
[^{3}H]AMP-PNP	75 μM [^{3}H]ADP	25, 83	$\geqq$1.54	<0.18	6
[^{3}H]AMP-PNP	61 μM $^{32}PP_i$	1, 37	1.43	<0.16	8B
[^{14}C]ADP	—	2.4, 28	2.08	—	1B
ADP	5.5 μM [γ-^{32}P]ATP	—		0.26	5
[^{3}H]ADP	75 μM $^{32}PP_i$	5.7[f]	0.83	<0.25	7B
$^{32}PP_i$	—	7.3, 21	1.25	—	ns[h]
$^{32}PP_i$	173 μM [^{3}H]AMP-PNP	$\leqq$17, 220[g]	1.05	0.83	8A
$^{32}PP_i$	83 μM [^{3}H]ADP	100[f]	1.11	0.58	7A
EDTA enzyme, prepared in absence of DTT[e]					
[^{3}H]AMP-PNP	—	4.7, 50	1.26	—	ns[h]
[^{3}H]ADP	—	10, 83	1.36	—	ns[h]
Acetone enzyme					
[γ-^{32}P]ATP	—	3.7, 21	1.90	—	1A
[^{14}C]ADP	—	9.2[f]	1.78	—	1B

[a] The binding parameters were obtained by extrapolation of double-reciprocal plots. The corresponding direct plots are contained in the Figures listed in the far-right column. [b] The enzyme was incubated with various concentrations of one ligand in the presence or absence of unvaried concentrations of another ligand. [c] The concentration(s) of the varied ligand at which half-maximal saturation of the binding site(s) was obtained. Unless otherwise noted, the double-reciprocal plots were in the form of two intersecting straight lines, and the concentrations for half-maximal saturation were obtained by simple extrapolation of the linear portions of the double-reciprocal plots. [d] Amount of the unvaried ligand that bound at infinite concentration of the varied ligand. [e] It was impossible to perform all these experiments with the same enzyme preparation on the same day, so the binding parameters obtained should not be regarded as absolute numbers. [f] The double-reciprocal plot of these results consisted of a single straight line. [g] The double-reciprocal plot of these results was continuously curved at the lower concentrations of the varied ligand. [h] Not shown.

method (exclusion chromatography on Sephadex) or the graphical analysis, for the following reasons. First, the saturation curve for the binding of ADP to the acetone enzyme was monophasic, while the corresponding curve for the EDTA enzyme was biphasic (Figure 1, Table I). Since these enzymes were assayed under *identical* conditions and analyzed by the same methods, the bimodality evident for the EDTA enzyme represents an intrinsic property of that enzyme. Second, several of these saturation curves were also determined by the method of equilibrium dialysis (Roy and Moudrianakis, unpublished work). Identical results were obtained with both assay methods. This is because the enzyme–nucleotide complex, once formed, is stable enough so that quickly passing the complex over a Sephadex column does not alter the amount of nucleotides bound. Since the Sephadex chromatography was quicker, simpler, and more accurate than equilibrium dialysis, especially at high ligand concentrations, we used exclusion chromatography for all the experiments presented here (except for the special case shown in Figure 2).

When DTT was omitted during preparation of the EDTA enzyme, the maximum possible binding of adenine nucleotides was less than when DTT was included, and the nucleotide concentrations at which half-maximum binding occurred were higher (Table I). The double-reciprocal plots were again biphasic (not shown), indicating at least two different types of binding sites. In addition, the EDTA enzyme prepared in the continuous presence of 5 mM DTT seems to have a third type of nucleotide binding site. When this enzyme was isoelectrically precipitated by lowering the pH (Knowles and Penefsky, 1972), it then could bind 2.4 mol of ADP per mol of CF_1 at pH 8.0 (200 μM ADP).

Inability of AMP and P_i to Bind to CF_1. No AMP binding occurred when CF_1 was incubated with 30 μM AMP, whether binding was assayed by our standard procedure of exclusion chromatography (see Methods) or by equilibrium chromatography (Figure 2) on Sephadex G-50m columns. The inclusion of 40 μM ADP or 200 μM ATP with the labeled AMP failed to promote AMP binding (data not shown). This low AMP concentration (30 μM) was approximately the amount present after CF_1 was incubated with ADP in routine assays of ADP binding and transphosphorylation (Roy and Moudrianakis, 1971a; Moudrianakis and Tiefert, 1975). Thus, these results indicate that the AMP recovered from CF_1 after its incubation with ADP is not simply bound from the pool of nucleotides free in solution, but is generated within the enzyme from bound ADP.

When CF_1 was incubated with tenfold higher concentrations of AMP for longer periods of time, for example, with 0.21 mM AMP for 3 h at 37 °C, a very low level of AMP binding ($\bar{\nu}$ = 0.094) could be detected. The binding increased to 0.112 mol of AMP per mol of CF_1 after a total of 6 h of incubation. Addition of $MgCl_2$ to the assay mixture did not affect the binding of AMP to CF_1.

The detection of similarly low levels of apparent P_i binding required that CF_1 be incubated with extremely high concentrations of P_i, on the order of 10 mM or higher. P_i at micromolar concentrations did not bind to isolated, soluble CF_1 at all, even in the presence of added nucleotides (data not shown). The results were the same whether P_i binding was assayed by exclusion or equilibrium chromatography.

Binding of Pyrophosphate to CF_1. In contrast to orthophosphate, which did not bind to CF_1, pyrophosphate did. PP_i

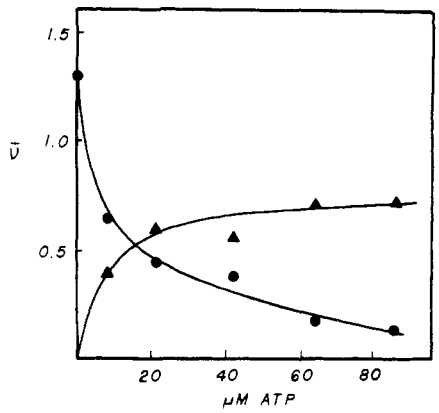

FIGURE 3: Competition between [^{14}C]ADP and [γ-^{32}P]ATP for binding to CF_1. CF_1 (EDTA enzyme, 5 mM DTT) was incubated for 2 h at 37 °C with 65.5 μM [^{14}C]ADP and various concentrations of [γ-^{32}P]ATP. The enzyme-nucleotide complexes were isolated on Sephadex G-50m columns. Radioactivity was determined by dual-channel liquid scintillation spectrometry, with corrections made for spillover of ^{32}P into the ^{14}C channel. (●) ^{14}C; (▲) ^{32}P.

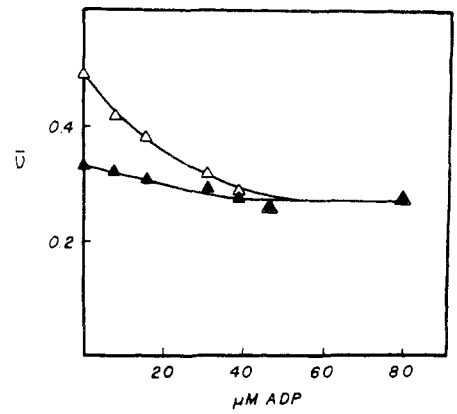

FIGURE 5: Elimination of the "extra" ^{3}H on CF_1 after incubation with [^{3}H,γ-^{32}P]ATP. CF_1 (EDTA enzyme, prepared with 5 mM DTT) was incubated for 2 h with 5.5 μM ATP labeled with both [γ-^{32}P]ATP and [^{3}H]ATP, in the presence of the indicated concentrations of unlabeled ADP. The enzyme-nucleotide complexes were isolated and the protein concentration and bound radioactivity were determined. (△) ^{3}H; (▲) ^{32}P.

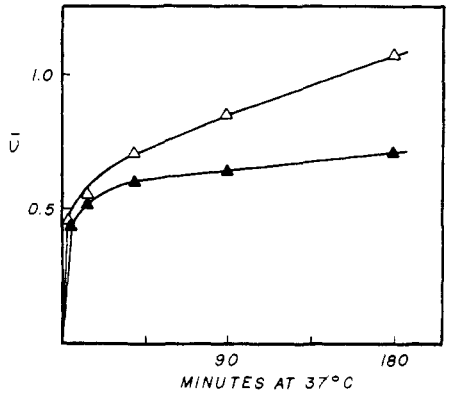

FIGURE 4: Time course for binding of [^{3}H,γ-^{32}P]ATP to CF_1. The EDTA enzyme (prepared in 5 mM DTT) was incubated for various lengths of time with 40 μM ATP labeled with both [^{3}H]ATP and [γ-^{32}P]ATP. After isolating the enzyme-nucleotide complex, the amount of bound nucleotide was determined using dual-channel liquid scintillation spectrometry with appropriate corrections for spillover of ^{32}P into the ^{3}H channel. (△) ^{3}H; (▲) ^{32}P.

bound to CF_1 at two independent, equivalent sites in the presence of relatively high concentrations of $MgCl_2$ and NaCl (Girault et al., 1973). However, the two PP_i binding sites had unequal apparent affinities for PP_i under the low salt conditions used in the present study (Table I). The relatively low maximum binding observed here for PP_i (Table I) could indicate one of the following alternatives. One, if two different ligand binding sites are present on each molecule of CF_1, perhaps PP_i binds less strongly to these sites than do adenine nucleotides in the presence of low concentrations of salt. Two, perhaps these two different binding sites are somehow less accessible to PP_i under low salt conditions than they are to adenine nucleotides. Three, perhaps only one allomorph of CF_1 binds PP_i (at two sites) under these conditions, even though both allomorphs can bind adenine nucleotides.

Competition between Adenine Nucleotides for Binding to CF_1. The results of a series of three experiments on the competition between ADP and ATP for binding to CF_1 emphasize the need for great caution in interpreting experiments involving ATP, because the hydrolysis of this nucleotide by CF_1 cannot be absolutely prevented. A fourth experiment, on the competition between ADP and AMP-PNP for binding, permitted a straightforward interpretation, because the analogue AMP-PNP was not hydrolyzed by CF_1.

[γ-^{32}P]ATP at high concentrations inhibited the binding of [^{14}C]ADP to CF_1 (Figure 3) in a manner that suggested that the binding of only one molecule of ATP to the coupling factor was enough to prevent two molecules of ADP from binding. However, the ATPase activity of CF_1 (Vambutas and Racker, 1965) could convert some of the [γ-^{32}P]ATP into unlabeled ADP. This ADP would not be detected in the analysis of enzyme-bound radioactivity, and would dilute the specific radioactivity of the [^{14}C]ADP to result in a level of ADP binding apparently lower than what actually occurs.

A time course of the binding to CF_1 of ATP labeled with both [^{3}H]ATP and [γ-^{32}P]ATP (Figure 4) showed that the amount of bound tritium exceeded the amount of bound ^{32}P when CF_1 was incubated with doubly labeled ATP at 37 °C for more than 30 min. Thus, some [^{3}H]ADP derived from the hydrolysis of [^{3}H,γ-^{32}P]ATP also was bound to the enzyme.

CF_1's ATPase activity is inhibited by ADP (Vambutas and Racker, 1965). Therefore, various amounts of unlabeled ADP were added to assay mixtures containing CF_1 and [^{3}H,γ-^{32}P]ATP to try to prevent ATP hydrolysis (Figure 5). The added unlabeled ADP would also be expected to decrease the specific radioactivity of any product [^{3}H]ADP and thus diminish its binding to CF_1. Fifty micromolar ADP was enough to eliminate the difference between the amounts of tritium and ^{32}P bound to CF_1, presumably by preventing the appearance of [^{3}H]ADP on the enzyme. Since this concentration of added ADP was less than that used in the experiment of Figure 3, it would seem that the binding of one intact ATP molecule, and nothing more, might prevent all ADP binding. However, this interpretation is valid only if the added ADP absolutely prevented ATP hydrolysis in the experiments of Figures 3 and 5, and if the transphosphorylation of bound ADP is irreversible, so that no unlabeled or β-^{32}P-labeled ADP was present on the enzyme in these experiments. The first of these two assumptions, and possibly also the second, is not true, however, as shown by analysis of the composition of the bound nucleotides derived from ATP under these conditions. This analysis is presented in the following paper of this issue (Tiefert et al., 1977). Because of the slow hydrolysis of ATP by CF_1 in the

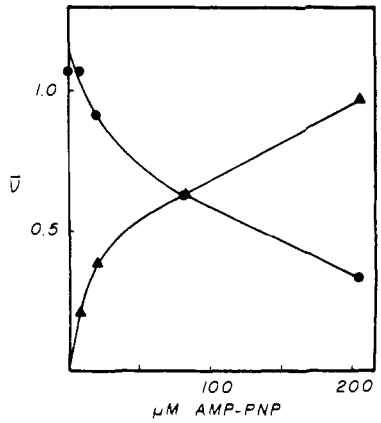

FIGURE 6: Competition between ADP and the nonhydrolyzable ATP analogue, AMP-PNP, for binding to CF_1. CF_1 (EDTA enzyme, prepared in 5 mM DTT) was incubated with 76 μM [^{3}H]ADP and various concentrations of unlabeled AMP-PNP, or with 75 μM unlabeled ADP and various concentrations of [^{3}H]AMP-PNP, at 37 °C for 2 h. The amount of bound nucleotide was determined after chromatography on Sephadex G-50m. (●) ADP; (▲) AMP-PNP.

absence of divalent cations and the necessity for long incubation times in studies of nucleotide binding by CF_1, interpretation of the inhibition of ADP binding by ATP must necessarily remain tentative.

AMP-PNP is a modified adenine nucleotide whose structure is very similar to that of ATP (Yount et al., 1971). The oxygen between the β- and γ-phosphates of ATP is replaced by an NH group in AMP-PNP. This analogue was not hydrolyzed by CF_1, and CF_1-bound AMP-PNP was not converted to any other nucleotide (data not shown). Thus, a study of the effect of AMP-PNP on the binding of ADP by CF_1 would yield a much more certain interpretation than is possible with ATP. When CF_1 was incubated with ADP and various amounts of AMP-PNP, ADP binding was completely inhibited by AMP-PNP at high concentrations (Figure 6). Approximately two molecules of bound AMP-PNP (determined from a double-reciprocal plot of the results) were required to completely inhibit ADP binding. These results with AMP-PNP suggest that one intact [γ-^{32}P]ATP and one hydrolyzed ATP (i.e., unlabeled ADP), when bound to CF_1, prevented [^{14}C]ADP from binding to CF_1 in the experiment presented in Figure 3.

Competition between ADP and PP_i for Binding to CF_1. It has been shown that PP_i and ADP compete for CF_1 binding sites (Girault et al., 1973). However, the assay conditions used in that study differed significantly from those used in the present study in two ways. First, ligand binding to CF_1 was assayed in the presence of $MgCl_2$ and NaCl in media of 10- to 20-fold higher ionic strength than what was routinely used here. In the present work, no $MgCl_2$ or NaCl was added to the binding assay mixtures because we wanted to minimize divalent cation-induced nucleotide hydrolysis and to avoid possibly damaging the enzyme by high concentrations of salt. Second, it was found in this study that the order of adding ligands to the enzyme affected the pattern of competition between the ligands, a subject not mentioned in the earlier publication (Girault et al., 1973). A comparison of the two types of experimental conditions (Table II) revealed that a high concentration of $MgCl_2$ increased ADP binding, and a high concentration of NaCl inhibited PP_i binding, relative to the results obtained in a low-salt experiment. Incubating CF_1 with PP_i for 15 min *before* adding ADP (as in Girault et al., 1973) resulted in a greater level of PP_i binding than when ADP and PP_i were added *simultaneously* to the reaction mixture, as in the present study.

TABLE II: Effect of Reaction Conditions Upon PP_i and ADP Binding to CF_1.[a]

Modifications	PP_i $\bar{\nu}$	ADP $\bar{\nu}$
None	0.547	0.673
+13 mM $MgCl_2$	0.635	1.12
+13 mM $MgCl_2$ + 130 mM NaCl	0.387	1.21
PP_i added before ADP[b]	0.853	0.674

[a] CF_1 (EDTA enzyme, no DTT) was incubated for 2 h with both 13 μM [^{32}P]pyrophosphate and 33 μM [^{3}H]ADP (in 7 mM Tris-SO_4, pH 8.0, plus the additions indicated), and then was separated from the remainder of the reaction mixture by Sephadex chromatography. [b] [^{3}H]ADP was added after CF_1 was incubated with $^{32}PP_i$ for 15 min (in 7 mM Tris-SO_4, pH 8.0).

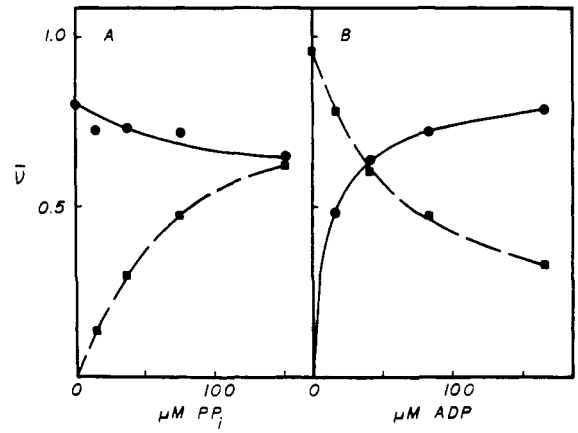

FIGURE 7: Competition between ADP and PP_i for binding to CF_1. (A) CF_1 (EDTA enzyme, prepared in 5 mM DTT) was incubated with 83 μM [^{3}H]ADP and the indicated concentrations of [^{32}P]pyrophosphate for 2 h at 37 °C. (B) CF_1 was incubated with 75 μM $^{32}PP_i$ and various concentrations of [^{3}H]ADP at 37 °C for 2 h. (●) ADP; (■) PP_i.

Competition between ADP and PP_i for binding to CF_1 occurred under the conditions used by Girault et al. (1973) and also under the conditions used throughout the present study, as follows. PP_i was a weak inhibitor of ADP binding, as shown by an experiment in which the concentration of PP_i was varied and that of ADP was kept constant (Figure 7A). However, the amount of PP_i that bound to CF_1 at each PP_i concentration was greater than the amount of ADP that was prevented from binding. A double-reciprocal plot for PP_i of binding vs. concentration, which was derived from the results presented in Figure 7A, was linear (Table I). Thus, the CF_1 sites did not exhibit negative cooperativity for PP_i binding in the presence of ADP, or only one allomorphic form of CF_1 bound PP_i under these conditions.

CF_1 was next incubated with a constant amount of PP_i and various concentrations of ADP. Extrapolation of a double-reciprocal plot of the results presented in Figure 7B showed that ADP at high concentrations almost completely inhibited PP_i binding (Table I). However, the maximum amount of ADP that could bind to the enzyme in the presence of PP_i was slightly less than 1 mol of ADP per mol of CF_1. The total amount of ligands bound to CF_1 was actually lower at ADP concentrations greater than 15 μM. Only the enzyme's high-affinity site for ADP seemed to be functioning (Table I), and no evidence for negative cooperativity or enzyme polymorphism was observed in the double-reciprocal plot.

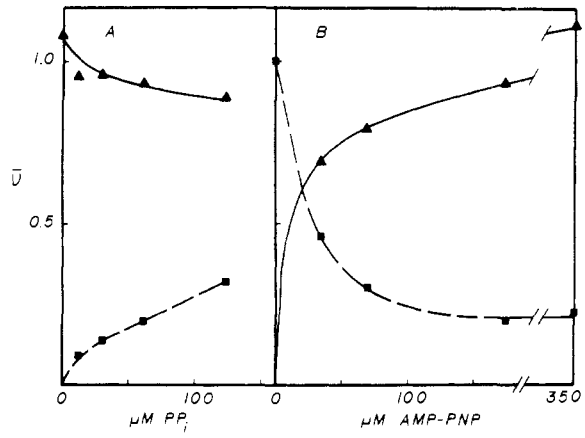

FIGURE 8: Competition between AMP-PNP and PP_i for binding to CF_1. (A) CF_1 (EDTA enzyme, 5 mM DTT) was incubated with 173 μM [^{3}H]AMP-PNP and various concentrations of $^{32}PP_i$ at 37 °C for 2 h. (B) CF_1 was incubated with 61 μM $^{32}PP_i$ and the indicated [^{3}H]AMP-PNP concentrations at 37 °C for 2 h. (▲) AMP-PNP; (■) PP_i.

In the experiments presented in Figure 7, ADP and PP_i were added simultaneously to the reaction mixtures, and no NaCl or $MgCl_2$ was added to the incubation media. In an earlier study (Girault et al., 1973), the incubation media contained 0.2 M NaCl and 20 mM $MgCl_2$, and CF_1 was incubated with PP_i for 15 min before various amounts of ADP were added. Under the latter conditions, ADP at high concentrations inhibited PP_i binding by no more than 50%, and 1 mol each of ADP and PP_i were bound per mol of enzyme at high ADP concentrations. This pattern of competition between PP_i and ADP is quite different from the results presented here in Figure 7B. Thus, the assay conditions seem to affect the apparent affinity and/or specificity of CF_1's ligand binding sites.

Competition between PP_i and AMP-PNP for Binding to CF_1. PP_i and AMP-PNP also interfered with each other's binding to CF_1. The results obtained when CF_1 was incubated with various concentrations of PP_i and unvaried amounts of AMP-PNP (Figure 8A) showed that PP_i was only a weak inhibitor of AMP-PNP binding. At each PP_i concentration, the amount of AMP-PNP that was prevented from binding to the enzyme was approximately equal to the amount of PP_i that became bound. Extrapolation of a double-reciprocal plot of the results presented in Figure 8A showed that a maximum of little more than 1 mol of PP_i could bind per mol of CF_1 under these conditions. However, this double-reciprocal plot was concave downward (Table I), so it seemed that PP_i was bound at more than one type of CF_1 site in the presence of AMP-PNP.

AMP-PNP was a strong inhibitor of PP_i binding. This was shown by an experiment in which CF_1 was incubated with a constant amount of PP_i and various concentrations of AMP-PNP (Figure 8B). AMP-PNP at high concentrations could completely inhibit PP_i binding, and two CF_1 sites could bind AMP-PNP in the presence of PP_i (Table I).

Discussion

Effects of Method for Preparing CF_1 on Its Nucleotide Binding Properties. Two different techniques have been used for the isolation of CF_1. One of these involves extraction of chloroplasts with EDTA. The other entails aqueous extraction of acetone-treated chloroplasts. Both methods were shown to yield the same protein in terms of function and antigenic specificity (McCarty and Racker, 1967). However, subtle differences between the EDTA enzyme and the acetone enzyme were found in the present study, which provide the first indication that the enzymes prepared by the two methods are not completely identical. The saturation curves for nucleotide binding to the EDTA enzyme were distinctly bimodal, while those for the acetone enzyme were more nearly monophasic (Figure 1). Thus, one or more of the isolation or purification steps may have affected the coupling factor so as to change the affinity of one of its classes of binding sites for adenine nucleotides. If each CF_1 molecule has two different types of nucleotide binding sites, then one type of site on each molecule of CF_1 may have been altered. Alternatively, if the biphasic binding curves result from enzyme polymorphism, then the distribution of CF_1 among its allomorphic forms may have been changed. The conformation of a similar coupling factor from *Alcaligenes faecalis* was found to change in response to the salt conditions present during specific stages in its preparation (Adolfsen et al., 1975). Thus, it is possible that the method of purification can also affect CF_1's conformation.

Other modifications of the preparative procedure also influenced CF_1's ability to bind adenine nucleotides. Omission of DTT during the preparation of the EDTA enzyme resulted in a lower level of nucleotide binding. Preincubating the EDTA enzyme at a moderately acid pH increased its capacity for binding nucleotides, perhaps by extracting endogenous nucleotides from the enzyme.

After the completion of the experimental phase of this work, a paper by Cantley and Hammes (1975) on nucleotide binding to CF_1 appeared. The results of that study were not quantitatively the same as the results reported here, probably because divalent metal ions and higher salt concentrations were included in the binding assays (see Table II, this paper). The coupling factor was extracted from the chloroplasts by similar procedures in the two laboratories. However, the enzyme was purified and stored under different conditions. It is not yet known what effects, if any, this might have on CF_1's properties. Their results were qualitatively similar to those presented here, since they also found that CF_1 bound nucleotides at sites with different apparent affinities, and that ADP and AMP-PNP competed with each other for binding to CF_1. Our results are also similar to those of Livne and Racker (1969), who found that the acetone enzyme possessed two equivalent or nearly equivalent binding sites for [γ-^{32}P]ATP when binding was assayed at room temperature with a moderate concentration of salt.

Differences between ATP and Its Analogue AMP-PNP. AMP-PNP bound to CF_1 with a greater apparent affinity than did ATP (Figure 1A). Since the angle and length of the P-O-P and P-N-P bonds are almost the same (Yount et al., 1971), one possible explanation for this observation would be the enzyme's ability to hydrolyze ATP but not the analogue. CF_1 may destroy much of the ATP by hydrolysis before it can reach CF_1's tight binding sites. It may thus be necessary to add more ATP than AMP-PNP to obtain the same level of binding.

Another explanation is possible, however. ATP bound to CF_1 might induce conformational changes in the enzyme that are associated with, but occur prior to, the actual hydrolytic event. These conformational changes might make it more difficult for CF_1 to bind additional ATP. AMP-PNP, which is not hydrolyzed by CF_1 (data not shown), may not be able to induce this prehydrolytic conformational change. Thus, more AMP-PNP may readily bind to CF_1. The similar coupling factor from mitochondria was shown to undergo an ATP-induced conformational change that was distinct from the actual hydrolysis of ATP (Penefsky, 1974b). Evidence for this was (a) AMP-PNP had little effect on the conformational

change that ATP induced in the isolated mitochondrial coupling factor; (b) AMP-PNP strongly inhibited the ATPase activity of the mitochondrial coupling factor; and (c) AMP-PNP did not induce these conformational changes in the enzyme. (The conformational changes were monitored by changes in the fluorescence intensity of aurovertin bound to the enzyme.)

Interpretation of Binding Studies. The results presented in this report suggest that isolated CF_1 has at least two different types of ligand binding sites (Figure 1). However, any further interpretation of the ligand binding patterns should be done cautiously, for two major reasons. First, and most important, adenine nucleotides undergo conversion subsequent to their binding to the coupling factor. Some of these conversion steps are apparently irreversible; thus, CF_1 and the bound and free nucleotides are not in a state of equilibrium. Evidence for this is presented and discussed in the following paper of this issue (Tiefert et al., 1977). Second, this enzyme has a far more complex structure than the models used to develop theories on the allosteric regulation of multisubunit enzymes (e.g., Koshland, 1970). The coupling factor is composed of five different types of subunits whose molecular weights range from 13 000 to 59 000 (Nelson et al., 1973). (A consensus on the subunit stoichiometry has not been reached yet.) In addition, CF_1 is a polymorphic enzyme which exists as two major electrophoretic variants, each of which contains the full complement of subunits (Adolfsen et al., 1975; Tiefert and Moudrianakis, unpublished work).

Thus, conventional theories on enzyme regulation may not be strictly applicable to the coupling factors. To avoid overinterpretation of the results through use of these theories, we have avoided the terminology associated with them. The enzyme's apparent affinities for binding ligands have been referred to as half-maximal saturation values rather than as dissociation constants (Table I). In addition, results of the binding studies have been illustrated with direct plots rather than with double-reciprocal, Hill, or Scatchard plots. However, some *tentative* interpretations can be drawn from the results of the binding studies presented here, as follows.

When CF_1 was incubated with various concentrations of one ligand in the presence of a constant amount of another ligand, for most combinations of ligands the half-maximal saturation values for the varied ligand were affected more than the maximum binding of that ligand (Table I). This suggested that the competition between these pairs of ligands for binding to CF_1 took place in a manner that was primarily (though not purely) "competitive". The saturation curve for ADP binding in the presence of an unvaried PP_i concentration (Figure 7B) suggested that the coupling factor may also be regulated by allosteric mechanisms, since this competition did not take place by a simple one-for-one replacement of one ligand by another. Only 1 mol of ADP bound per mol of CF_1 under these conditions, even though CF_1 has two sites at which ADP can bind (Figure 1). The one bound ADP did not permit any PP_i to bind to the enzyme. The results presented in Figure 3 seem to imply that one bound ATP molecule prevented two ADP molecules from binding to the enzyme. However, as noted before, the probable binding of unlabeled ADP derived from the [γ-^{32}P]ATP means that the results presented in Figure 3 cannot be taken as evidence for allosterism in CF_1.

The experiment in which CF_1 was incubated with 80 μM ADP and various concentrations of PP_i (Figure 7A) suggested that CF_1 may have at least three types of ligand binding sites. The amount of PP_i that bound to CF_1 at higher PP_i concentrations was larger than the amount of ADP that was prevented from binding. Thus, the total amount of bound ligand rose as the PP_i concentration was increased. The presence of at least three types of ligand binding sites was more strongly suggested by the preliminary experiment in which CF_1 was preincubated under mildly acidic conditions and then brought to pH 8.0 where it bound 2.4 mol of ADP per mol of enzyme.

Finally, the results of this binding study indicate that the CF_1 sites may bind adenine nucleotides by both their adenine group and their terminal pyrophosphoryl group. (1) ATP and its analogue AMP-PNP were the strongest inhibitors of the binding of other ligands to CF_1. They could prevent both ADP and PP_i from binding (Table I, Figures 3, 6, 8B). Perhaps, then, ATP and this analogue occupied the CF_1 site more completely than did the other ligands. (2) ADP, which has one less phosphoryl group than ATP, was only a weak inhibitor of ATP binding (Figure 5). However, ADP could prevent PP_i from binding (Table I, Figure 7B). (3) PP_i, which would be unable to interact with the supposed adenine-binding end of the site, was but a weak inhibitor of the binding of ADP or AMP-PNP to CF_1 (Figure 7A, 8A). (4) AMP, which does not have a pyrophosphoryl group, bound to CF_1 only at very high AMP concentrations. It had little ability to inhibit ADP or ATP binding even at such high AMP concentrations (Tiefert et al., 1977). (5) P_i, which lacks both an adenine group and a second phosphate, did not significantly bind to CF_1.

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Conversion of Bound Adenine Nucleotides by the Purified Coupling Factor of Photophosphorylation†

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ABSTRACT: When purified coupling factor of photophosphorylation was incubated with ADP, the bound nucleotide was recovered from the enzyme as a mixture of AMP, ADP, and ATP. This conversion was due to the transphosphorylation reaction discovered previously (Roy, H., and Moudrianakis, E. N. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 464). The addition of divalent metal ions increased the level of ADP binding to the enzyme. Mg^{2+} had little effect on the conversion of bound ADP to bound ATP, while Ca^{2+} decreased the extent of this conversion. However, the conversion of enzyme-bound ADP occurred whether or not divalent cations were added to the assay mixture. The three nucleotides derived from bound ADP were released from the coupling factor at different rates. ATP bound to the coupling factor and also was recovered from the enzyme as a mixture of AMP, ADP, and ATP. Addition of unlabeled ADP inhibited but did not prevent the conversion of bound ATP by the enzyme. Analysis of bound and free nucleotides after incubation of the coupling factor with ADP or ATP showed that, for example, the amount of AMP recovered from the enzyme-nucleotide complex was completely unrelated to the concentration of free (unbound) AMP recovered from the reaction mixture. Thus, the nucleotide-coupling factor system did not represent a case of simple equilibrium binding. The system instead reached a steady state in which free nucleotide became bound and was converted to products that were released. The released product ADP and ATP, but *not* the AMP, could bind again to the enzyme. Studies of the binding of nucleotides to this enzyme thus cannot be interpreted according to equilibrium binding theories. In addition, such studies are not complete unless the compositions of both the enzyme-bound and free (unbound) nucleotide pools are analyzed.

The isolated, purified coupling factor of photophosphorylation (CF_1)[1] can bind 2 mol of ADP per mol of CF_1 (Roy and Moudrianakis, 1971a; Girault et al., 1973; Cantley and Hammes, 1975; Vandermeulen and Govindjee, 1975). Analysis of the bound nucleotides shows that the bound ADP is partially converted into bound AMP and ATP (Roy and Moudrianakis, 1971a). This transphosphorylation of bound ADP is catalyzed by CF_1 itself, not by the conventional adenylate kinase from chloroplasts, which could possibly contaminate preparations of CF_1 (Moudrianakis and Tiefert, 1976). These findings suggest that the transphosphorylation of bound ADP by CF_1 may be important in the mechanism of energy-dependent ATP synthesis. If so, then the following reactions may account for ATP synthesis in the chloroplast (Roy and Moudrianakis, 1971b): first, phosphorylation of a *special,* CF_1-associated AMP to form a CF_1-bound ADP as an intermediate; second, transfer of the terminal phosphoryl group from the CF_1-ADP intermediate to an exogenously added (substrate) ADP to synthesize ATP. An energy input is obligatory for the first of these reactions, but not for the second. Nevertheless, the second reaction may *appear* to require an energy input, depending upon the time and events separating it from the first reaction.

Results that are consistent with the first proposed reaction step are as follows. Incubation of chloroplasts with AMP and $^{32}P_i$ under conditions for light-dependent electron transport or for the artificial generation of a transmembrane pH gradient resulted in the synthesis of a stable CF_1-[β-^{32}P]ADP complex that could be extracted from the membranes and purified (Roy and Moudrianakis, 1971b, and unpublished work). [^{32}P]ADP was formed faster than was [^{32}P]ATP in the first 50 ms during which chloroplasts were illuminated with $^{32}P_i$ (Boyer et al., 1975). Both AMP and arsenate were required to relieve the inhibition by ATP of ferricyanide reduction (Mukohata and Yagi, 1975).

The following results are consistent with the second reaction step proposed by Roy and Moudrianakis (1971b). After the CF_1-ADP complex was formed from AMP plus $^{32}P_i$, addition

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[1] Abbreviations used are: CF_1, 13S coupling factor of photophosphorylation; acetone enzyme, CF_1 prepared from an aqueous extract of acetone-treated chloroplasts; EDTA enzyme, CF_1 prepared from an EDTA extract of chloroplast membranes; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; ATPase, adenosine triphosphatase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $\bar{\nu}$, moles of ligand bound per mole of enzyme.