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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.

of ADP to the chloroplasts in darkness resulted in the disappearance of ADP from the membrane-bound CF_1 and the appearance of soluble [γ-^{32}P]ATP. This synthesis of ATP did not require additional energy, whether ADP was added to the chloroplasts immediately or up to at least 80 s after the end of the illumination period (Roy and Moudrianakis, 1971b; Tiefert and Moudrianakis, unpublished work). However, synthesis of free ATP did require energy when substrate ADP was not added to the chloroplasts until after they were washed several times (by centrifugation) after the initial formation of the CF_1–ADP complex (Moudrianakis and Tiefert, 1973). Boyer et al. (1973) obtained evidence with mitochondria which they interpreted as suggesting that, although energy was required to release ATP from its site of synthesis, it was not required for ATP synthesis per se.

Thus, it seems probable that the transphosphorylation of bound ADP by the isolated coupling factor is an expression of an activity the enzyme also carries out while bound to its membrane. The characteristics of this synthesis of bound ATP and AMP from bound ADP by the isolated CF_1 are described further in this communication. ATP also was found to be converted by CF_1 into a mixture of bound AMP, ADP, and ATP (Table III). The distribution of bound nucleotides was not directly related to that of the free nucleotides. Most notably, the same relative amount of AMP could be bound to CF_1 whether or not any free AMP was present by the end of the incubation period. AMP was recovered from CF_1 only when CF_1-bound ADP or ATP was converted in situ to bound AMP (Roy and Moudrianakis, 1971a; Tiefert et al., 1977). This bound AMP could be released from CF_1, but free AMP could not bind to the enzyme. Thus, the bound and free AMP were not in equilibrium. Since both ADP and ATP were partially converted into AMP, the composition of the reaction mixture was changing with time. Therefore, in a strict sense, none of the bound and free nucleotides in the reaction mixture was in equilibrium when CF_1 was incubated with added ADP or ATP. The results of this study will show that the binding of adenine nucleotides to CF_1 can no longer be interpreted according to equilibrium binding theories of enzyme regulation. In addition, the distribution of CF_1-bound nucleotides cannot be estimated by analyzing the composition either of only the free nucleotides or of the free and bound nucleotides together. The CF_1–nucleotide complex must be isolated to determine the amounts of each nucleotide bound to the enzyme. The results presented in this paper were obtained by directly analyzing the CF_1–nucleotide complex after its separation from the remainder of the reaction mixture, and underscore the need for such analyses in studies of nucleotide binding by the coupling factor.

Methods and Materials

CF_1, both "EDTA enzyme" and "acetone enzyme", was isolated from spinach chloroplasts as described in the preceding paper of this issue (Tiefert et al., 1977). Samples of CF_1 were incubated with ADP or ATP for assay of nucleotide binding also as described in the preceding paper of this issue. The enzyme–nucleotide complexes were then separated from the remainder of the assay mixtures by exclusion chromatography on Sephadex G-50m columns. In earlier experiments of this study, the isolated enzyme–nucleotide complexes were lyophilized and then were dissolved in 10 M urea to release the bound nucleotides. The previously bound nucleotides were resolved by paper electrophoresis. The electrophoretograms were analyzed with a gas-flow radiochromatogram scanner equipped with a graphical integrating device. The relative

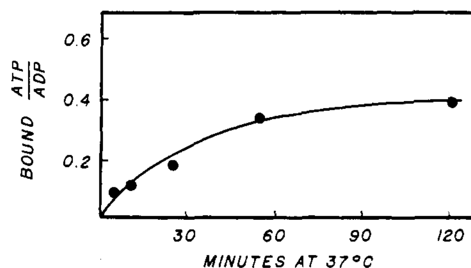

FIGURE 1: Kinetics of the conversion of bound ADP to ATP by CF_1. Aliquots of the acetone enzyme were incubated for various lengths of time at 37 °C with 60 μM [^{14}C]ADP. Then the enzyme-nucleotide complexes were isolated. The enzyme was denatured in 7% perchloric acid to release the bound nucleotides, and carrier nucleotides were added. The perchloric acid was removed by neutralization with KOH and removal of the insoluble $KClO_4$ by centrifugation, instead of by the charcoal column procedure usually used. The nucleotides in the supernate were then separated on a Dowex AG1-X4 column.

proportions of each of the three adenine nucleotides were determined graphically or by cutting out the radioactive spots on the electrophoretograms and counting them by liquid scintillation spectrometry. In later experiments, the bound radionucleotides were extracted from the enzyme with 5% perchloric acid and were separated by a charcoal–Dowex chromatography procedure (Adolfsen and Moudrianakis, 1972). The same results were obtained by either method, but the chromatographic method was faster than the electrophoretic analysis. These same methods were used to analyze the "included" fractions that were collected from the Sephadex columns. These fractions contained the nucleotides that were not enzyme bound. Dowex AG1-X4 was obtained from Bio-Rad Laboratories.

Results

Effect of Incubation Time, pH, and Enzyme Concentration on the Conversion of CF_1-Bound ADP. The basic nature of the transphosphorylation reaction by which CF_1-bound AMP and ATP are produced from two molecules of ADP bound to CF_1 was documented previously (Roy and Moudrianakis, 1971a). What follows in this paper is further characterization of this reaction. The dependence of the ratio of enzyme-bound ATP to ADP upon the incubation time is illustrated in Figure 1. The amount of bound ATP product gradually increased relative to the bound ADP substrate until a maximum ATP/ADP ratio was reached between 1 and 2 h of incubation. Since ADP binding by CF_1 also required about 1–2 h to reach a maximum (Roy and Moudrianakis, 1971a), ADP must be transphosphorylated quickly once it becomes bound to the enzyme. Transphosphorylation appears to be slow merely because ADP must (slowly) bind to CF_1 before it can be converted to other bound nucleotides.

The pH of the incubation medium had little if any effect on the transphosphorylation of bound ADP by CF_1 (Table I). Thus, perhaps the conversion of bound ADP to bound ATP and AMP occurs at an enzyme site that is protected from the external environment. Changing the concentration of CF_1 did not affect the amount of bound ATP per mole of CF_1; i.e., the total amount of bound ATP was linearly related to the enzyme concentration (data not shown).

Effects of Various Added Reagents on the Conversion of CF_1-Bound ADP. Various substances were added to reaction mixtures containing CF_1 and ADP to determine the conditions under which ADP binding and transphosphorylation by CF_1 might be enhanced or inhibited and to obtain information that might be relevant to the mechanism of transphosphorylation.

TABLE I: Effect of pH on Transphosphorylation of CF_1-Bound ADP.[a]

pH	Enzyme-Bound ATP/ADP
6.0	0.57
7.0	0.54
7.5	0.63
8.0	0.67
9.0	0.46

[a] CF_1 was incubated with [^{14}C]ADP at 37 °C for 2 h. Tricine was used as the buffer at each pH indicated. The CF_1–nucleotide complexes were isolated by Sephadex chromatography and were analyzed by paper electrophoresis as described under Methods.

The binding and transphosphorylation of ADP were affected as follows when CF_1 was incubated with [^{3}H]ADP together with unlabeled AMP, ATP, or P_i.

(1) AMP at concentrations up to 200 μM had no measurable effect on the binding and transphosphorylation of ADP by CF_1. At higher concentrations (2–6 mM), added AMP had no effect on the total amount of [^{3}H]ADP that bound to CF_1 and little if any effect on the amount of [^{3}H]AMP (produced from the bound [^{3}H]ADP) that was recovered from the enzyme. However, added AMP at these high concentrations (10 to 60 times the [^{3}H]ADP concentration) inhibited the synthesis of ATP from ADP by CF_1. The ratio of bound [^{3}H]ATP to bound [^{3}H]ADP was decreased to between 15 and 80% of its uninhibited value (Moudrianakis and Tiefert, 1976). The strength of this inhibition depended on the ratio of unlabeled AMP to [^{3}H]ADP added to the assay mixture. The inhibition of ADP conversion but not of ADP binding by AMP was not changed by adding $MgCl_2$ to the assay mixtures. The effects of AMP were the same whether or not CF_1 had been reduced with DTT. At the high AMP concentrations used in these inhibition experiments (2–6 mM), some externally added AMP bound to CF_1 ($\bar{\nu}$ = 0.2–0.4) but was not converted to any other nucleotide. Perhaps the finding that the added AMP did not affect the amount of bound [^{3}H]AMP indicates that the site where bound ADP is converted to bound AMP is not the same as the site where AMP binds to CF_1 when very high concentrations of this nucleotide are added to solutions containing the enzyme. The results also indicate that the conventional chloroplast adenylate kinase does not take part in the transphosphorylation of ADP by isolated CF_1, since adenylate kinase is inhibited by low amounts of AMP and stimulated by added $MgCl_2$ (Moudrianakis and Tiefert, 1976).

(2) To test the effect of added ATP on ADP binding and transphosphorylation by CF_1, the enzyme was incubated with 75 μM [^{3}H]ADP and 24 μM unlabeled ATP. The bound [^{3}H]ATP that was derived from the [^{3}H]ADP was inhibited the most (54.5%). The amount of bound [^{3}H]AMP was 34.4% less than in the control. The unchanged bound [^{3}H]ADP was inhibited the least (24.9%) by the added unlabeled ATP. Results presented in Table II show that added ATP was converted to other nucleotides which were also bound to CF_1. This conversion took place even when CF_1 was prepared in the absence of DTT and was incubated with ATP in the absence of added divalent metal ions and in the presence of ADP, as in this experiment. Under these conditions CF_1's ATPase activity is very low (Vambutas and Racker, 1965; Tiefert and Moudrianakis, unpublished work). Thus, the inhibition of ADP conversion observed here was probably due to both the unlabeled ATP and the unlabeled ADP and AMP that were derived from it.

TABLE II: Effect of Various Reagents on ADP Binding and Transphosphorylation by CF_1.

Additions	Total cpm bound	Enzyme-bound ATP/ADP
Expt A[a]		
None	7969	0.88
1 mM $MgCl_2$	15985	0.96
1 mM $CaCl_2$	16153	0.46
5% (w/v) sucrose	8965	0.75
1 mM phlorizin	9268	1.07
1 mM fluorescein mercuric acetate	3960	0.09
Expt B[b]		
None	8875	0.61
18.3% (v/v) ethanol	2093	0.07

[a] CF_1 (EDTA enzyme prepared with 5 mM DTT) was incubated with [^{14}C]ADP and the indicated additions for 1 h at 37 °C. The enzyme–nucleotide complexes were then isolated and denatured with urea. The nucleotides were analyzed by paper electrophoresis as described under Methods. [b] A different preparation of CF_1 (EDTA enzyme, no DTT) was incubated with [^{3}H]ADP, with or without ethanol as indicated, for 2 h before passage over a Sephadex column. The enzyme-bound nucleotides were analyzed by the charcoal–Dowex procedure described under Methods.

(3) ADP binding to CF_1 was inhibited in the presence of very high concentrations of inorganic orthophosphate (Girault et al., 1973). We found that P_i also interfered somewhat with the transphosphorylation of bound ADP when CF_1 was incubated with 31 mM P_i and 78 μM [^{3}H]ADP. In the presence of this vast excess of P_i, ADP binding was inhibited by 8%, the amount of bound AMP recovered from the enzyme–nucleotide complex was unaffected, and the ratio of bound ATP to bound ADP was inhibited by 22%. Since so much P_i was required to obtain this degree of inhibition, the inhibition may have been a nonspecific ionic effect rather than an effect specific to P_i.

The synthesis of CF_1-bound [^{3}H]ATP was more sensitive to inhibition than was the production of bound [^{3}H]AMP from the bound [^{3}H]ADP in each of the above cases where CF_1 was incubated with [^{3}H]ADP and unlabeled AMP, ATP, or P_i. This suggested that the transphosphorylation of CF_1-bound ADP might take place by a sequential rather than a concerted mechanism. In addition, the conditions required for the synthetic portion of the reaction might be more stringent than those needed for the hydrolytic phase.

The effects of adding various other substances to reaction mixtures containing CF_1 and ADP are shown in Table II. $MgCl_2$ and $CaCl_2$ both increased the extent of ADP binding to CF_1. $MgCl_2$ had little effect on the transphosphorylation of the bound ADP. However, $CaCl_2$ decreased the ratio of bound ATP to ADP, perhaps because ATP hydrolysis by CF_1 is specifically stimulated by Ca^{2+} ions under these conditions (Vambutas and Racker, 1965). It was clear from these results that the transphosphorylation of ADP by CF_1 took place both in the presence and absence of added Mg^{2+} or Ca^{2+} ions. Sucrose and phlorizin had moderate effects on ADP binding and transphosphorylation. Fluorescein mercuric acetate formed a soluble, colored complex with CF_1. It inhibited ADP binding and strongly inhibited transphosphorylation. Ethanol inhibited both ADP binding and transphosphorylation. The inhibition of ADP binding by ethanol varied somewhat with different preparations of CF_1 (Moudrianakis and Tiefert, 1976, and unpublished work). However, the inhibition of transphosphorylation by ethanol was always nearly complete. It should

TABLE III: Binding and Conversion of $[^3H]ATP$ by CF_1.

		% distributions of labeled nucleotides					
		Bound			Free		
Additions	$\bar{\nu}$	AMP	ADP	ATP	AMP	ADP	ATP
Expt A[a]							
None	1.32	11	75	14	85	15	0
175 μM ADP	0.39	9	34	57	26	73	1
Expt B[b]							
None	1.14	18	43	39	3	54	43
840 μM ADP	0.56	19	29	52	0	26	74
+540 μM AMP	0.56	14	26	60	0	31	69
Expt C[c]							
720 μM ADP	0.73	23	29	48	0	24	76
+17% (v/v) ethanol	0.40	22	44	34	0	79	21

[a] CF_1 (EDTA enzyme, no DTT) was incubated at a concentration of 1.08 mg of protein/mL with 8.9 μM $[^3H]ATP$ for 2 h at 37 °C, with or without added unlabeled ADP. The enzyme-nucleotide complexes were then separated from the remainder of the reaction mixtures by Sephadex gel chromatography. The total moles of $[^3H]$nucleotide bound per mole of CF_1 ($\bar{\nu}$) were determined, and the nucleotide compositions of both the macro- and micromolecular phases of the reaction mixtures were analyzed by the charcoal-Dowex procedure referred to under Methods. [b] A different preparation of CF_1 (EDTA enzyme, no DTT) was incubated at 0.80 mg of protein/mL for 1 h with 53 μM $[^3H]ATP$, with additions as indicated. The bound and free nucleotides were analyzed as for experiment A. [c] The same preparation of CF_1 as used in experiment B was incubated on a different day at 0.68 mg of protein/mL for 1 h with 45 μM $[^3H]ATP$ and the indicated additions. The bound and free nucleotides were analyzed as in experiment A.

perhaps be noted that we always evaporated the ethanol from the commercial radiolabeled nucleotides before diluting them with unlabeled nucleotides to a specific radioactivity appropriate for use in the binding assays.

Release from CF_1 of Nucleotides Derived from Bound ADP. Earlier experiments showed that the enzyme-nucleotide complex formed from ADP and CF_1 was remarkably stable. However, the AMP formed on the enzyme from the bound ADP was less strongly bound than the ADP or ATP (Roy and Moudrianakis, 1971a). In the present study, the stability of the nucleotides' association with CF_1 was tested by incubating aliquots of CF_1 with labeled ADP, isolating the CF_1-nucleotide complexes thus formed by exclusion chromatography on Sephadex columns, and then storing the isolated complexes at 0 °C (in 10 mM Tris, pH 8.0). After various periods of time, the complexes were passed over a second Sephadex column, and the nucleotides that remained associated with the enzyme were determined. AMP dissociated from the enzyme-nucleotide complex with a half-life of about 15 min under these conditions. Bound ADP and ATP were retained for up to 20 h at 0 °C with no detectable loss. If, however, the isolated CF_1-nucleotide complex was incubated at a higher temperature before it was passed over the second Sephadex column, both AMP and some ADP were lost from the complex. Most of the bound ATP was retained. For instance, when the isolated CF_1-nucleotide complex was incubated for 10 min at 37 °C (in 10 mM Tris, pH 8.0), 34.7% of the AMP, 6.7% of the ADP, and 4.8% of the ATP (24.8, 12.1, and 2.1 pmol, respectively) were released from CF_1.

ATP Conversion by CF_1 Prepared in the Absence of DTT. When CF_1 was incubated with ATP under several different conditions, a mixture of AMP, ADP, and ATP could be recovered from the isolated enzyme-nucleotide complex (Table III). This occurred even though the enzyme used in these experiments was not treated with DTT, which would have greatly increased its ATPase activity (Howell and Moudrianakis, 1967; McCarty and Racker, 1968).

The free (unbound) ATP also was converted into a mixture of adenine nucleotides. For example, after CF_1 was incubated with a low concentration of $[^3H]ATP$ for 2 h at 37 °C (expt A, line 1, Table III), no free ATP was detected. In addition, little free ADP was left in this reaction mixture. The appearance of unbound AMP in this reaction mixture might have been due to a combination of ATP hydrolysis and ADP transphosphorylation, by which both ADP and ATP would be consumed and only AMP would accumulate. However, it also could be due to a slow, direct hydrolysis of ADP as an alternative substrate of the enzyme's ATPase activity (Vambutas and Racker, 1965).

Effects of Inhibitors on ATP Conversion by CF_1. In experiment A of Table III, CF_1 was incubated with a low concentration of ATP for 2 h. In experiment B, the enzyme was incubated with a higher ATP concentration for only 1 h. As a result, the extent of ATP hydrolysis was less in experiment B than in experiment A. The addition of unlabeled ADP at a concentration 20 times that of the $[^3H]ATP$ decreased the extent of ATP hydrolysis and partially inhibited the binding of labeled nucleotide to CF_1 in both experiments. However, the added unlabeled ADP did not prevent ATP hydrolysis or the appearance of bound $[^3H]AMP$ and $[^3H]ADP$ in either experiment. In an experiment presented in the preceding paper of this issue, CF_1 was incubated with $[^3H,\gamma\text{-}^{32}P]ATP$ (Figure 5 of Tiefert et al., 1977). A 20-fold excess of added ADP was more than enough to decrease the amount of bound tritium to a level equal to the amount of bound ^{32}P in that experiment. However, the experiment discussed above (Table III) showed that this amount of unlabeled ADP did not prevent $[^3H]ATP$ from being converted to other bound nucleotides. Thus, in the earlier experiment (Figure 5 of Tiefert et al., 1977), some $[^3H]ADP$ and $[^3H]AMP$ derived from the doubly labeled ATP must have been bound to the enzyme. Two explanations could then account for the finding that the amount of CF_1-bound ^{32}P equalled the amount of bound tritium in that experiment. One, some $[\beta\text{-}^{32}P]ADP$ (and perhaps $[\beta,\gamma\text{-}^{32}P]ATP$) may have appeared on the enzyme. That is, the transphosphorylation of ADP may be reversible as long as AMP remains bound to the

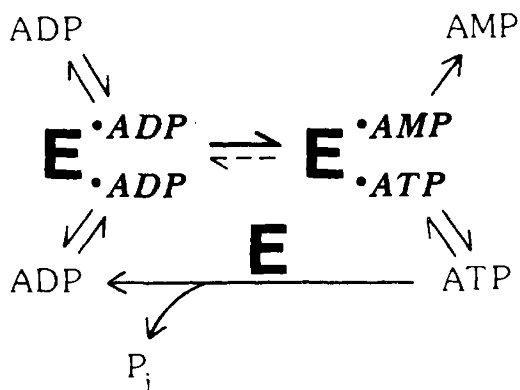

FIGURE 2: Summary of catalytic activities so far discovered in the isolated chloroplast coupling factor. The series of reactions that occur when isolated CF_1 is incubated with ADP or ATP is shown. The bindings of ADP and ATP to CF_1 are reversible steps, but there is as yet no direct evidence on whether the transphosphorylation of bound ADP is reversible. ATP hydrolysis and the release of bound AMP are not detectably reversible with the isolated coupling factor. The italic letters represent bound nucleotides, while the upright letters represent components free in solution. The two free ADP molecules are of course interchangeable; however, it seems likely that the two enzyme sites are not—one site may be specialized for producing AMP, the other, for producing ATP. The intermediate steps in ATP hydrolysis are not shown. This Figure is *not* meant to imply anything about the total number of ligand-binding sites on the enzyme, possible enzyme conformational changes that may take place during binding and conversion of nucleotides, or the mechanism for the biphasic nature of the saturation profiles. It is also not meant to identify which tight-binding site (if any) is involved in ATP hydrolysis or to imply whether ATP hydrolysis and tight binding of nucleotides are functions of the same or different enzyme allomorphs. This Figure is merely a balance sheet of the catalytic activities of isolated CF_1.

enzyme. Two, CF_1 may have initially bound less [^{3}H]ATP than [γ-^{32}P]ATP because of isotope effects. CF_1 would also have bound [^{3}H]ADP that resulted from hydrolysis of the ATP. Thus, the total amount of tritium bound to CF_1 could have been equal to that of ^{32}P, even if there was less [^{3}H]ATP than [γ-^{32}P]ATP bound.

The addition of unlabeled AMP to assay mixtures containing CF_1, [^{3}H]ATP, and unlabeled ADP (expt B, line 3, Table III) had little or no effect on the binding and conversion of ATP. In experiment C, the addition of ethanol to the assay system (unlabeled ADP was added to decrease the extent of ATP hydrolysis) inhibited ATP binding and stimulated ATP hydrolysis. The enhanced ATPase activity resulted in an increase in the amount of free ADP and also in the relative amount of bound ADP.

Two reaction mechanisms, which are not necessarily mutually exclusive, could account for the conversion of ATP to a mixture of bound nucleotides. One, CF_1 might convert bound ATP to bound ADP and AMP in situ in a manner that is inhibited by exogenously added ADP. Two, CF_1 might bind both ATP and the free ADP resulting from its hydrolysis and then produce bound AMP and additional bound ATP by transphosphorylation of the ADP.

Relationship between Free and Bound Nucleotides. The distribution of enzyme-bound nucleotides observed after CF_1 was incubated with ATP was apparently unrelated to the distribution of free nucleotides. For instance, in experiment A (first line), AMP was the predominant free nucleotide, but the most abundant bound nucleotide was ADP. The distributions of bound nucleotides in the second lines of experiments A and B were similar, even though the distributions of free nucleotides in these two experiments were very different. These results implied that the nucleotide conversions that take place in the tight-binding sites of CF_1 may be relatively independent of the conversions that occur at the enzyme's hydrolytic site(s). Thus, the bound and free nucleotides may not be in equilibrium with each other.

No free AMP was detected in four of the five assays in experiments B and C of Table III. Yet, in each case significant amounts of AMP were recovered from the CF_1-nucleotide complex. In other experiments where CF_1 was incubated with low concentrations of exogenously added AMP, no AMP bound to CF_1, even in the presence of other adenine nucleotides (Tiefert et al., 1977). Therefore, the AMP that was recovered from the enzyme in experiments B and C of Table III almost certainly must have been generated within the 90-Å domain of the enzyme molecule. This AMP must have been tightly bound to CF_1 without previously having been released into solution. These findings provided further evidence that the CF_1-nucleotide complex was not in equilibrium with the unbound nucleotides in the assay mixture even though the amount of bound nucleotide reached a maximum after 1–2 h of incubation (Roy and Moudrianakis, 1971a) and apparently did not change when the reaction mixtures were kept at 37 °C for several additional hours (Tiefert and Moudrianakis, unpublished work).

Discussion

Analysis of nucleotides recovered from isolated CF_1-nucleotide complexes showed that a mixture of CF_1-bound AMP, ADP, and ATP resulted when CF_1 was incubated with ADP or ATP (Roy and Moudrianakis, 1971a, and present study). In addition, free (unbound) AMP and ADP were produced from the ADP and ATP. However, the bound and free nucleotides did not seem to be in simple equilibrium with each other. Evidence for this was as follows. (1) No quantitative relationship between the bound and free nucleotides was observed when CF_1 was incubated with [^{3}H]ATP under several conditions (Table III). (2) Free ATP usually could not be detected when CF_1 was incubated with ADP, even though bound ATP was detected (Roy and Moudrianakis, 1971a; Tiefert and Moudrianakis, unpublished work). (3) Nucleotide binding, conversion of bound nucleotides, release of nucleotides, and hydrolysis of free nucleotides seemed to occur simultaneously, but at greatly different rates, in reaction systems comprising CF_1 and adenine nucleotides. The reactions so far known to take place under these conditions are illustrated in Figure 2. (4) CF_1 could produce bound AMP from ADP or ATP and could readily release this bound AMP (Roy and Moudrianakis, 1971a, and present study). However, no bound AMP or any other nucleotide could be recovered from CF_1 when low concentrations of AMP were added to solutions containing this enzyme (Tiefert et al., 1977). Thus, nucleotide that bound to CF_1 as ADP or ATP was eventually released as AMP, which could not rebind to the enzyme, even in the presence of P_i, ADP, and/or ATP (Tiefert et al., 1977, and unpublished work). If ADP and ATP were not converted to AMP during their incubation with purified, membrane-free CF_1, their binding with CF_1 would have reached equilibrium. However, their conversion to AMP meant that the composition of the coupling factor–adenine nucleotide incubation system changed with time. The system thus was not at equilibrium when maximum values of CF_1-bound adenine nucleotides were obtained.

Existing theories on allosteric enzyme regulation require that the enzyme–ligand complex be an equilibrium state (Wold, 1971; Hammes and Wu, 1974). Thus, we feel we ought to caution against interpreting the results from binding studies

of nucleotide and CF_1 or related enzymes according to these theories. It appears inappropriate to illustrate the results of such studies with Hill or Scatchard plots or to use mathematical formulas that depend on the attainment of binding equilibrium, unless it can be determined directly that the ligand used was not converted to a new chemical entity during the course of the binding assay, as is the case for AMP-PNP.

In the study of the binding of nucleotides to coupling factors, the mere use of a conventional *equilibrium method* of assay (e.g., equilibrium dialysis) will not necessarily yield an *equilibrium state.* For a system to be analyzed as an equilibrium system, it must be demonstrated that the proposed equilibrium state can be approached from both directions. In the case of CF_1 and ADP this is not so. Because of the combined transphosphorylation and ATPase activities of CF_1, the bound ADP is converted into bound AMP and P_i, which after their release *do not* rebind to CF_1 to form bound ADP. This is very similar to the case of ATP binding to isolated mitochondrial coupling factor (F_1), yielding F_1-bound ATP, which is then converted into F_1, ADP, and P_i. However, incubation of pure F_1 with ADP and P_i does not yield F_1-bound ATP.

The method for preparing the chloroplast coupling factor and the biological source of this enzyme did not seem to affect CF_1's ability to interconvert adenine nucleotides. Three different CF_1 preparations were tested and were found to catalyze the interconversion of bound adenine nucleotides. One was the enzyme prepared by EDTA extraction of spinach chloroplasts (Howell and Moudrianakis, 1967; Tiefert et al., 1977). Another was CF_1 prepared in the same manner from Swiss chard chloroplasts (Roy and Moudrianakis, 1971a). The third was CF_1 prepared from acetone-treated chloroplasts by the entirely different procedure of Vambutas and Racker (1965) (Figure 1, this paper). The interconversion of bound adenine nucleotides appeared to be a reaction intrinsic to CF_1. A comparison under several conditions of the activities of CF_1 and the conventional adenylate kinase from spinach chloroplasts indicated that the transphosphorylation of bound ADP by CF_1 could not be attributed to contamination of CF_1 with the conventional adenylate kinase (Moudrianakis and Tiefert, 1976). On the basis of these results, it therefore seems that any homogeneous preparation of the chloroplast coupling factor might have the ability to convert both ADP and ATP into CF_1-bound ATP, ADP, and AMP. We would like to suggest that it seems not only prudent but essential to directly analyze the compositions of both the free and the bound nucleotides in studies of adenine nucleotide binding by CF_1. This precaution should probably also be applied to studies of nucleotide binding to any other related coupling factor–ATPase enzyme.

Because of the known ATPase activity of coupling factors, many investigators analyze the composition of bound nucleotides after incubating one of these enzymes with ATP. However, the possibility that bound ADP might also be converted by these enzymes has not received equal consideration. To our knowledge, the only previously published studies in which the composition of bound nucleotides was analyzed after incubating a purified coupling factor or related ATPase with ADP are those by Roy and Moudrianakis (1971a), Catterall and Pedersen (1972), Moudrianakis et al. (1973), and Abrams et al. (1973). Abrams et al. incubated ADP with the isolated ion transporter-active ATPase from *Streptococcus faecalis* and found that ADP was bound and not converted to any other nucleotides. Moudrianakis et al. found that bound ADP was converted to bound AMP and ATP by the coupling factor–latent ATPase from *Alcaligenes faecalis.* In the study by Catterall and Pedersen, after the mitochondrial coupling factor F_1 (isolated as an active ATPase) was incubated with radiolabeled ADP, the chemical form of the bound radioactivity was identified exclusively as ADP. We have confirmed their observations using *their* F_1 preparation (unpublished). Thus, this particular type of coupling factor preparation does not convert bound ADP, and ADP binding can be treated as an equilibrium phenomenon in this case. Coupling factors are polymorphic, and changes in the isolation procedure can affect the isolated enzyme's distribution among its allomorphs (Moudrianakis and Adolfsen, 1975). It is reasonable to expect that certain reactions may be catalyzed by one enzyme allomorph but not by another. That this indeed can explain why some F_1 preparations do not convert bound ADP will be the subject of a forthcoming publication.

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