Interaction of Nucleotides with Chloroplast Coupling Factor 1†

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ABSTRACT: The interactions of ADP, adenylyl imidodiphosphate (AMP-PNP), and ATP with solubilized chloroplast coupling factor (CF_1), a latent ATPase, have been studied with equilibrium and kinetic methods. After extensive Sephadex chromatography, the purified CF_1 contains 1 mol of ADP/mol of CF_1; no other tightly bound nucleotides are found. Radioactively labeled ADP and AMP-PNP can be exchanged for the tightly bound ADP on CF_1 and on 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD) modified CF_1 with a characteristic half-time of ~4 h at 25 °C, pH 8.0. This ADP exchange reaction requires binding of ADP at one or more sites on CF_1 and proceeds ~4 times faster when heat-activated or NBD-modified heat-activated CF_1 is used. The exchange of AMP-PNP for tightly bound ADP requires Mg^{2+} or Ca^{2+}. At 25 °C the incorporation of 1 mol of [γ-^{32}P,^{3}H]ATP/mol of latent enzyme and hydrolysis into tightly bound [^{3}H]ADP and $^{32}P_i$ occur within 10 min in the presence of 5 mM Mg^{2+}; ADP strongly inhibits ATP incorporation. The dissociation of P_i is slow (half-time ~24 h), and replacement of the tightly bound ADP by ATP is prevented by bound P_i. In the presence of 2 mM EDTA, incorporation of ATP is very slow and requires the binding of ATP at one or more sites on CF_1. Binding studies with forced dialysis indicate the presence of one tight binding site per CF_1 for ATP for both the latent and NBD-modified latent enzyme in the presence of 5 mM Mg^{2+}. With 2 mM EDTA, approximately three relatively weak binding sites are found. Incorporation of 1 mol of [γ-^{32}P,^{3}H]ATP/mol of heat-activated CF_1 occurs at 37 °C within 30 s in the presence of 5 mM Ca^{2+}, where the ATPase activity is large; the P_i is completely dissociated, and net catalysis of ATP hydrolysis occurs. Under conditions where the enzyme has a very weak ATPase activity, the P_i is considerably less dissociated. These results suggest a minimum of three nucleotide binding sites on the enzyme. Possible mechanisms for ATP hydrolysis include a single catalytic site with two regulatory nucleotide sites, alternating catalytic sites, and various combinations of these two mechanisms involving three or more nucleotide binding sites.

The coupling factor CF_1[1] from spinach chloroplasts catalyzes the synthesis of ATP in chloroplasts. When isolated, the CF_1 is unable to synthesize ATP but is a latent ATPase which can be activated by a variety of methods (Farron & Racker, 1970). It has a molecular weight of 325 000 (Farron, 1970) and contains five different types of polypeptide chains, probably in the ratio 2:2:1:1:2 (Binder et al., 1978; Baird & Hammes, 1976). The overall structure has been examined with electron microscopy and chemical cross-linking, and a structural map of specific sites on the enzyme has been obtained with resonance energy transfer measurements [cf. Baird & Hammes (1979)].

The interaction of nucleotides with CF_1 has been studied in many laboratories [cf. Roy & Moudrianakis (1971), Cantley & Hammes (1975), Tiefert et al. (1977), VanderMeulen & Govindjee (1977), and Girault & Galmiche (1977)], but a complete understanding of the interplay between nucleotides and the coupling factor still is lacking. In this laboratory, we have shown previously that ADP and AMP-PNP bind tightly to two sites on the enzyme. The steady-state kinetics of ATP hydrolysis can be explained by assuming these are regulatory sites and a third nucleotide site is the catalytic site (Cantley & Hammes, 1975).

In the work reported here, the interaction of nucleotides with CF_1 is examined further. One molecule of ADP per CF_1 molecule remains bound after extensive Sephadex chromatography. Radioactive ATP, AMP-PNP, and ADP can be incorporated into the enzyme in place of the tightly bound ADP in both the latent and heat-activated enzymes, providing at least one additional nucleotide is bound to the enzyme. Incorporation of ATP is accompanied by hydrolysis into ADP and P_i. The products remain tightly bound to the latent enzyme, but the P_i is removed rapidly from the heat-activated enzyme in the presence of Ca^{2+}, which permits rapid displacement of the ADP by ATP. Incorporation of nucleotides into the tight binding site occurs slowly with ADP and AMP-PNP and rapidly with ATP when Mg^{2+} or Ca^{2+} is present. Binding at the other nucleotide site(s) shows a preference for ADP over ATP. These results permit some speculation about the mechanism for ATP hydrolysis by the coupling factor.

Materials and Methods

Chemicals. The ADP and ATP (vanadium-free) and the NBD-Cl were purchased from Sigma Chemical Co. The AMP-PNP from PL Biochemicals was purified on a DEAE-cellulose column (2.5 cm i.d. × 60 cm) by using a triethylamine carbonate gradient (5–350 mM) at pH 8.6. Radioactive nucleotides, ^{3}H- and ^{32}P-labeled, were obtained from New England Nuclear and purified by paper chromatography by using 3MM Whatman paper and isobutyric acid–1 N ammonia (100:60, v/v). Standard chemicals were analytical grade, and solutions were made with deionized distilled water.

Enzyme. Solubilized CF_1 was prepared from fresh spinach leaves (Lien & Racker, 1971). Protein having a fluorescence intensity ratio, 305 nm/340 nm (excitation at 280 nm), higher than 1.2 in 20 mM Tris-HCl (pH 7.2), 2 mM EDTA, and 1 mM ATP was collected and stored as a precipitate at 4 °C in 2 M ammonium sulfate, 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 0.5 mM ATP. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) of

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[1] Abbreviations used: CF_1, chloroplast coupling factor 1; AMP-PNP, adenylyl imidodiphosphate; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; EDTA, ethylenediaminetetraacetic acid.

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CF_1 gave the normal five bands associated with purified CF_1 [cf. Baird & Hammes (1976)]. Molar concentrations of CF_1 in the absence of dissociable nucleotides were determined spectrophotometrically with a Cary 118 spectrophotometer by using an extinction coefficient at 278 nm of 0.476 cm^2/mg (Cantley & Hammes, 1975) in 10 mM Tris–HCl (pH 8.0) with 2.5 mM Mg^{2+} or 2 mM EDTA, and a molecular weight of 325 000 (Farron, 1970). When required, the CF_1 was heat-activated at 60 °C for 4 min in 40 mM ATP and 5 mM dithiothreitol (Lien & Racker, 1971). The ATPase activity generated was monitored either by phosphate analysis with ammonium molybdate (Taussky & Shorr, 1953), by the pH-stat method (Cantley & Hammes, 1975), or by determination of radioactive $^{32}P_i$ liberated during hydrolysis (Avron, 1960). The heat-activated enzyme had a specific activity of ~12 units (μmol of ADP liberated per min per mg of CF_1) at 20 °C, pH 8.0, in 50 mM NaCl, 1 mM ATP, 1 mM EDTA, and 6 mM $CaCl_2$. The modification of CF_1 with NBD-Cl was carried out according to Cantley & Hammes (1975).

Analysis for Tightly Bound Nucleotides. Latent CF_1 (15–20 nmol in about 0.5 mL) was freed from dissociable nucleotides by column chromatography with Sephadex G25 (medium, 1 cm i.d. × 50 cm) equilibrated with 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 25 mM sucrose (Cantley & Hammes, 1975). The flow rate was 0.5–0.6 mL/min. The effluent was monitored at 280 nm by using an ISCO recording absorbance monitor. The eluted protein then was concentrated, either by precipitation with an equal volume of saturated ammonium sulfate in 10 mM Tris-HCl (pH 7.2) and 2 mM EDTA or within a collodion membrane (Schleicher and Schuell) dialyzing against the column buffer. The extraction of nucleotides was carried out according to the procedure of Rosing & Slater (1972). The perchloric acid (4% w/v) was added to 10–20 nmol of CF_1 in about 1 mL at 0 °C, and the mixture was centrifuged for 10 min at 18000*g* at 0 °C. The supernatant was neutralized with 20% KOH, the insoluble $KClO_4$ salt was eliminated by centrifugation, and the total amount of nucleotide was determined spectrophotometrically by assuming a nucleotide extinction coefficient of 15 400 M^{-1} cm^{-1} at 259 nm, pH 7.0 (Beaven et al., 1955). The solution then was chromatographed on poly(ethylenimine)-cellulose (0.5 cm i.d. × 3 cm) (Sigma; Magnusson & McCarty, 1976). A stepwise elution was peformed with increasing concentrations of LiCl, and the solution absorption was monitored at 260 nm. Again the amount of eluted nucleotide was determined spectrophotometrically. The nucleotide concentration was sufficiently high (10–20 μM) so that coprecipitation of the nucleotide with $KClO_4$ was negligible (Wiener et al., 1974). Potential adenylate kinase activity was inhibited by 2 mM EDTA (Harris & Slater, 1975). In calculating the amount of extracted nucleotides, corrections were made for the loss of solution in the protein and $KClO_4$ precipitates by measurement of the initial volume and the collected volume, which is smaller because of loss of a few microliters on the walls and in the precipitates. ATP is not hydrolyzed by the extraction procedure.

Nucleotide Incorporation into CF_1. The incorporation of [^{3}H]ADP, [^{3}H]AMP-PNP, [^{3}H]ATP, and [γ-^{32}P,^{3}H]ATP into the latent and heat-activated enzymes was studied under a variety of conditions. The CF_1 was freed from dissociable nucleotides by Sephadex gel chromatography and concentrated to 3–10 μM in the desired buffer as described above. The enzyme then was incubated with a radioactive nucleotide for the desired time period, and the exchange reaction was stopped either by precipitation with an equal volume of saturated ammonium sulfate if the nucleotide concentration was greater than 100 μM or by application of the material to a Sephadex G25 column. For the heat-activated enzyme in the presence of Ca^{2+} or Mg^{2+}, the solution first was made 10 mM in EDTA by addition of 200 mM EDTA in order to quench hydrolysis and the incorporation process. In both cases the enzyme was rechromatographed on Sephadex G25 in 10 mM Tris–HCl (pH 8.0), 2 mM EDTA, and 25 mM sucrose, and the eluted CF_1 was concentrated to 5–20 μM. The amount of enzyme present was determined spectrophotometrically, and the amount of radioactive nucleotide bound to CF_1 was determined by scintillation counting of an aliquot in 10 mL of ACS aqueous scintillation fluid (Amersham) for ^{3}H and in 10 mL of 4.3% NH_4OH for ^{32}P. When both ^{3}H and ^{32}P were present, appropriate corrections were made for the influence of ^{32}P radioactivity on the observed radioactivity of ^{3}H.

Binding Measurements. The binding of ATP to latent CF_1 was studied at 20 °C by using the forced dialysis technique (Cantley & Hammes, 1973). After removal of nucleotides by column chromatography on Sephadex G25 as described above, the concentration of CF_1 was adjusted to 15–20 μM in the desired buffer and the protein was incubated with radioactive ATP for 30–120 min. The radioactivity of 10-μL aliquots of effluent (free nucleotide) and equilibrated solution (total nucleotide) was determined in 10 mL of ACS scintillation fluid.

Results

Tightly Bound Nucleotides. Extraction of latent or heat-activated CF_1 with perchloric acid after Sephadex gel chromatography indicated ~1 mol of nucleotide/mol of CF_1, but the spectroscopic determination of the nucleotide concentration was somewhat uncertain due to light scattering from unknown materials. Chromatography on poly(ethylenimine)-cellulose resolved this solution into two fractions containing material absorbing at 260 nm. The first fraction was eluted with 0.1 N LiCl and represented 10–15% of the total absorbance at 260 nm. The amount of this material increased as the CF_1 aged and had a maximum absorbance at 275 nm; this is very likely proteolyzed protein. The second fraction was eluted with 1.0 M LiCl, which is characteristic of ADP, and exhibited an absorption maximum at 260 nm. Repeated determinations of the stoichiometry with freshly prepared CF_1 gave 0.9 ± 0.1 ADP/CF_1. A two-month old preparation of enzyme had a stoichiometry of 0.7 ADP/CF_1. Neither ATP nor AMP was found to be present; they would be detectable if present as more than 5% of the nucleotide content.

Incorporation of ADP and AMP-PNP. When 150 μM [^{3}H]ADP was incubated with latent CF_1 at 25 °C in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA for 18 h, 0.98 [^{3}H]-ADP/CF_1 was found to be tightly bound, and only a single ADP per CF_1 was found. The radioactive ADP could not be removed by dialysis for 18 h against the same Tris-HCl–EDTA buffer (200 times the sample volume). However, if the dialysis buffer contained 1 mM nonradioactive ADP or AMP-PNP, the radioactive nucleotide was removed. The exchange of ADP into the tight nucleotide binding site was characterized further by determining the extent of incorporation of [^{3}H]ADP at 18 h with varying concentrations of radioactive nucleotide (3–100 μM) and 6.8 μM CF_1. The results obtained are shown in Figure 1 as a plot of the moles of radioactive ADP per mole of CF_1, r, vs. the ADP concentration. The values of r were calculated from the observed values, r_0, using the relationship $r = r_0\{([^3H]ADP)/[([^3H]ADP) - r_0(E_0)]\}$, which corrects for isotopic dilution due to the release of unlabeled ADP from the

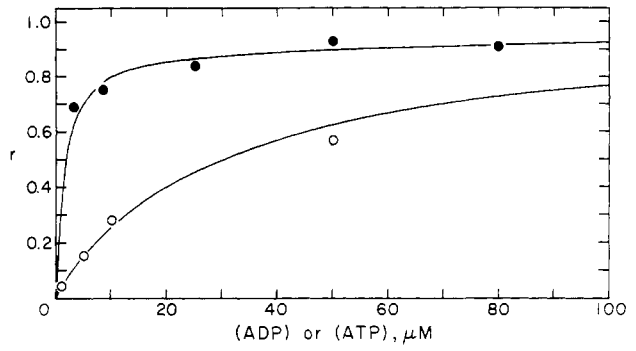

FIGURE 1: A plot of the moles of tightly bound [^{3}H]ADP per mole of CF_1, r, vs. the initial concentration of [^{3}H]ADP (●) or [^{3}H]ATP (○). The CF_1 (5–10 μM) was incubated for 18 h in the presence of various concentrations of radioactive nucleotide in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA at 20 °C and then analyzed for tightly bound nucleotide. Correction for isotopic dilution was made as described in the text.

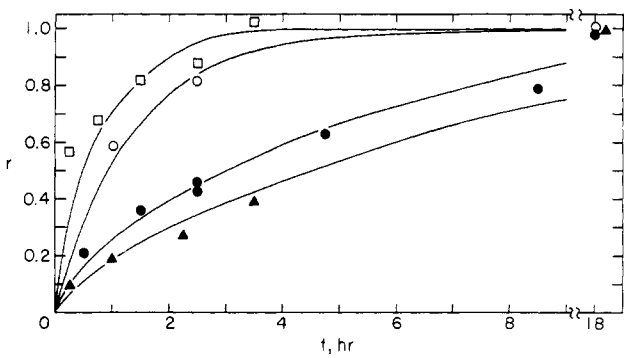

FIGURE 2: Typical time courses of the incorporation of ADP and AMP-PNP into the tight site of CF_1; r is the moles of radioactive nucleotide incorporated per mole of CF_1. The CF_1 (5–10 μM) was incubated with tritiated nucleotides in 40 mM Tris-HCl (pH 8.0) and analyzed for tightly bound nucleotide at the times indicated. The solid symbols designate latent CF_1 and the open symbols heat-activated CF_1: (▲) [^{3}H]AMP-PNP, 5 mM $CaCl_2$; (●) [^{3}H]ADP, 2 mM $MgCl_2$; (○) [^{3}H]ADP, 2 mM EDTA; (□) [^{3}H]ADP, 5 mM $CaCl_2$. The curves have been calculated with the first-order rate constants given in Table I.

enzyme. In this relationship ([^{3}H]ADP) is the initial concentration of labeled ADP and (E_0) is the molar concentration of enzyme. Since these are not equilibrium data, a quantitative analysis is not possible. However, these results, together with the finding that the dissociation of ADP from the enzyme is immeasurably slow in the absence of added ADP, indicate that nucleotide binding at additional sites is required for the exchange reaction.

The time course of the exchange of [^{3}H]ADP and [^{3}H]-AMP-PNP with enzyme-bound ADP was studied at 25 °C, using nucleotide concentrations of 150–200 μM. A variety of different conditions was used with the latent enzyme, the heat-activated enzyme, and NBD-labeled latent and heat-activated enzymes. The experiments were carried out in 40 mM Tris-HCl (pH 8.0) and one of the following: 2 mM EDTA, 2 mM $MgCl_2$, or 5 mM $CaCl_2$. Some typical time courses are presented in Figure 2. The exchange reaction is essentially a first-order process, although in some experiments the first 20–30% of the reaction proceeded faster than the remaining 70–80% of the reaction. The nature of this occasional initial burst was not explored, and the data were analyzed in terms of a first-order rate process. The results obtained are summarized in Table I, and the lines in Figure 2 have been calculated assuming a first-order reaction and the rate constants in Table I. For the latent enzyme, the rate of

Table I: Rate of Incorporation of Nucleotides into CF_1[a]

nucleotide	CF_1	additions	r after 18 h	10^3k (min^{-1})
[^{3}H]ADP	latent	2 mM EDTA	0.98	~1.8[b]
[^{3}H]ADP	latent	2 mM $MgCl_2$	1.0	1.5
[^{3}H]ADP	latent	5 mM $CaCl_2$		1.2
[^{3}H]ADP	latent, 1.4 NBD	2 mM EDTA	1.0	~2
[^{3}H]AMP-PNP	latent	2 mM EDTA	0.05	
[^{3}H]AMP-PNP	latent	5 mM $CaCl_2$		1.0
[^{3}H]ADP	heat-activated	2 mM EDTA	1.0	4.0
[^{3}H]ADP	heat-activated	5 mM $CaCl_2$	1.05	8.0
[^{3}H]AMP-PNP	heat-activated	2 mM EDTA	0.09	
[^{3}H]AMP-PNP	heat-activated	2 mM $MgCl_2$		8.0
[^{3}H]AMP-PNP	heat-activated	5 mM $CaCl_2$	1.08	8.0
[^{3}H]AMP-PNP	heat-activated, 2.5 NBD	5 mM $CaCl_2$		8.0

[a] 40 mM Tris-HCl (pH 8.0), 25 °C. [b] Not strictly first order; initial 20% was ~10 times faster.

exchange of [^{3}H]ADP into the enzyme was not appreciably different in the presence of EDTA, Mg^{2+}, or Ca^{2+}. However, the exchange of AMP-PNP into the enzyme appears to require a divalent metal ion. A similar pattern is found for the heat-activated enzyme, although the rate is about 4 times faster than with the latent enzyme, and the presence of Ca^{2+} or Mg^{2+} accelerates ADP incorporation somewhat. The modification of either the latent or heat-activated enzyme with NBD had no effect on the exchange rate. In preliminary experiments, the presence of 0.5 mM dinitrophenol or 10 mM methylamine, uncouplers of oxidative phosphorylation, had little effect on the exchange rate with the latent enzyme. Perchloric acid extraction and nucleotide analyses of the enzyme after incubation with AMP-PNP showed that AMP-PNP was tightly bound to the enzyme and was not hydrolyzed to AMP-PNH_2.

Incorporation of ATP. The latent enzyme (15–20 μM), freed from dissociable nucleotides, was incubated with 40–55 μM [^{3}H]ATP or [γ-^{32}P,^{3}H]ATP in 40 mM Tris-HCl (pH 8.0) containing either 5 mM $MgCl_2$ or 2 mM EDTA at 25 °C. After 10–30 min, the amount of tightly bound ^{3}H, ^{32}P, and nucleotide was determined. In all cases the only nucleotide found bound to the enzyme was ADP, with 1 mol of ADP/mol of CF_1. The results obtained are summarized by the first six entries in Table II. In the presence of Mg^{2+}, one [^{3}H]ADP and one $^{32}P_i$ are found bound to the enzyme after a 10-min incubation, whereas in the presence of EDTA the amount of ATP incorporated is greatly reduced (first three entries in Table II). The dependence of the ATP incorporation on free ATP was examined in the presence of EDTA as previously described for ADP incorporation. The incorporation was found to be dependent on ATP binding to the enzyme, similar to the behavior observed for the ADP exchange reaction; the relevant data are included in Figure 1. Two control experiments were done to show that the finding of only ADP on the enzyme is not an artifact of the experimental procedure. The latent enzyme (20 μM) was incubated with [^{3}H]ATP (20 μM) in the presence of 5 mM $MgCl_2$ as above; after 10 min, 4% $HClO_4$ was added at 0 °C and the mixture was analyzed for nucleotides: only ADP was found. A similar experiment was carried out by incubating 27 μM CF_1 with 50.6 μM [^{3}H]ATP for 30 min. The ratio of ADP/ATP after nucleotide extraction and poly(ethylenimine)-cellulose separation of nucleotides was 2.25, and the specific radioactivity of ADP was one-half that of the original [^{3}H]ATP radioactivity. If the amount of ATP hydrolyzed to ADP and the amount of ADP initially present are each assumed to be equal to the enzyme concentration, a final ratio, (ADP)/(ATP), of 2.29 would be expected [(27 × 2)/(50.6 − 27)]. Thus, one ATP molecule is hydrolyzed,

Table II: Incorporation of ATP into CF_1[a]

nucleotide (μM)	CF_1 (μM)	addition	[^{3}H]ADP/CF_1	$^{32}P_i/CF_1$
[^{3}H]ATP (40.5)	15.6 (l)	5 mM $MgCl_2$	0.93	
[^{3}H]ATP (40.5)	15.6 (l)	2 mM EDTA	0.22	
[γ-^{32}P,^{3}H]ATP (53)	18.8 (l)	5 mM $MgCl_2$	1.10	1.10
[γ-^{32}P,^{3}H]ATP (53)[b]	18.8 (l)	5 mM $MgCl_2$	0.97	0.97
ATP (42)[c]	19.4 (l)	5 mM $MgCl_2$	0.98	0.82
[γ-^{32}P,^{3}H]ATP (50)[d]	15.8 (l)	1 mM $MgCl_2$, 10 mM P_i	0.79	0.61
[^{3}H]ATP (4600)	10 (ha)	5 mM $CaCl_2$	0.99	
[γ-^{32}P,^{3}H]ATP (470)	43 (ha)	5 mM $CaCl_2$	0.97	0.03
[γ-^{32}P,^{3}H]ATP (462)	13.4 (ha)	5 mM $CaCl_2$	0.79	0.05
[γ-^{32}P,^{3}H]ATP (462)[e]	13.4 (ha)	5 mM $CaCl_2$	0.28	0.02
[γ-^{32}P,^{3}H]ATP (460)	8.5 (ha)	5 mM $MgCl_2$	0.67	0.46
[γ-^{32}P,^{3}H]ATP (460)[e]	8.5 (ha)	5 mM $MgCl_2$	0.40	0.32
[γ-^{32}P,^{3}H]ATP (462)[f]	13 (ha)	5 mM $CaCl_2$	0.66	0.12
[γ-^{32}P,^{3}H]ATP (462)[e,f]	13 (ha)	5 mM $CaCl_2$	0.24	0.013
[γ-^{32}P,^{3}H]ATP (470)	10.6 (ha)	2 mM EDTA	0.27	0.03
[γ-^{32}P,^{3}H]ATP (470)[e]	10.6 (ha)	2 mM EDTA	0.20	0.04

[a] 40 mM Tris-HCl (pH 8.0); 20 °C for latent enzyme (l); 37 °C for heat-activated enzyme (ha). Incubation times: 30 min for entries 1 and 2, 20 min for entries 3–6, and 30 s for all other entries. [b] Initial incubation was followed by addition of 475 μM unlabeled ATP for 20 min. [c] CF_1 initially had 1.0 [^{3}H]ADP/CF_1 and 0.97 $^{32}P_i/CF_1$. [d] CF_1 was incubated with 10 mM P_i and 1 mM $MgCl_2$ for 21 h before adding labeled ATP. [e] The initial incubation was followed by addition of unlabeled ATP (10 times the concentration of labeled ATP) for 30 s. [f] The enzyme was modified with NBD-Cl (3.5–3.8 NBD/CF_1).

one ADP molecule previously on the enzyme is displaced, and ADP and P_i remain tightly bound to the enzyme.

Attempts were made to displace the tightly bound [^{3}H]ADP and $^{32}P_i$ from the enzyme. If the enzyme was incubated for 20 min with [γ-^{32}P,^{3}H]ATP in the presence of 5 mM $MgCl_2$, followed by addition of a 10-fold excess of unlabeled ATP for 20 min, the radioactivity was not displaced from the enzyme (entry 4, Table II). If the radioactively labeled enzyme was isolated by Sephadex chromatography, incubated with 50 μM ATP in the presence of 5 mM $MgCl_2$, and rechromatographed on Sephadex G25, the amount of [^{3}H]ADP was unchanged and the amount of $^{32}P_i$ was reduced ~10% (entry 5, Table II). The incorporation of radioactive ATP was not greatly inhibited by preincubation of the enzyme in 10 mM phosphate (entry 6, Table II). When the radioactively labeled CF_1 is dialyzed for 3 h against 40 mM Tris-HCl (pH 8.0) with either 2 mM EDTA or 5 mM $MgCl_2$, no loss in [^{3}H]ADP occurs and the amount of $^{32}P_i$ is reduced 20%. If the labeled enzyme is rechromatographed on Sephadex G25 18 h after its isolation, the amount of [^{3}H]ADP present is unchanged and the amount of $^{32}P_i$ is reduced 80%. When the latent enzyme is incubated with 1 mM [γ-^{32}P,^{3}H]ATP for 7 h in the presence of 5 mM $MgCl_2$, precipitated with an equal volume of saturated ammonium sulfate in 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA, and stored in 2 M ammonium sulfate, 40 mM Tris-HCl (pH 8.0), and 1 mM EDTA at 4 °C for 3 days, it is found to contain 0.05 $^{32}P_i/CF_1$ and 0.81 [^{3}H]ADP/CF_1 after analysis in the usual manner. Since this is the normal procedure used in the preparation of the enzyme, this result indicates that the enzyme as isolated contains tightly bound ADP, but not tightly bound P_i. Furthermore, release of tightly bound P_i from the enzyme is a prerequisite for incorporation into the enzyme of ATP (with subsequent hydrolysis to ADP and P_i).

These conclusions were confirmed in an experiment in which CF_1 with 1 mol of [^{3}H]ADP and 1 mol of $^{32}P_i$ incorporated was dialyzed against 40 mM Tris-HCl (pH 8.0) containing 1 mM unlabeled ATP and 5 mM $MgCl_2$. Aliquots were taken at different times, and the amount of ^{32}P and ^{3}H present on the enzyme was determined. The radioactivity of both substances decreases at the same rate (Figure 3) and follows first-order kinetics with a first-order rate constant of 0.025 h^{-1}. (If 2 mM EDTA is substituted for the 5 mM $MgCl_2$, a similar rate constant is found.) Since this rate is much slower than incorporation of ATP into enzyme without tightly bound P_i, phosphate dissociation must be rate limiting in the incorporation of unlabeled ATP.

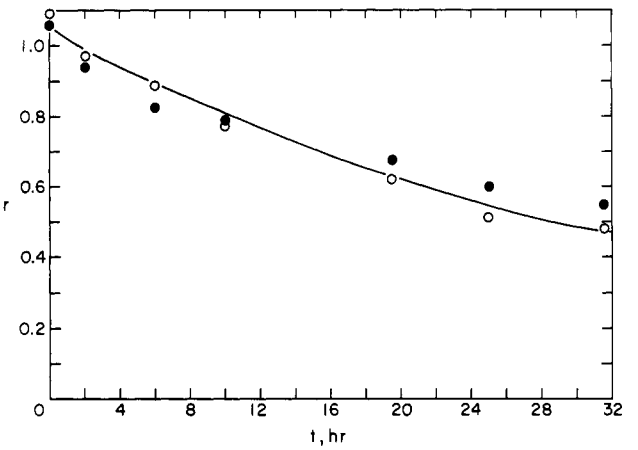

FIGURE 3: Time course of the dissociation of [^{3}H]ADP (○) and $^{32}P_i$ (●) from the latent enzyme (10.3 μM) during dialysis against 1 mM ATP, 40 mM Tris-HCl (pH 8.0), and 5 mM Mg^{2+} at 20 °C. r is the moles of radioactive nucleotide or P_i per mole of CF_1, and the curve has been calculated assuming a first-order rate constant for dissociation of 0.025 h^{-1}.

The incorporation of ATP into the heat-activated enzyme also was investigated. The heat-activated enzyme was incubated with radioactive ATP for 30–60 s in 40 mM Tris-HCl (pH 8.0) with 5 mM Ca^{2+}, 5 mM Mg^{2+}, or 2 mM EDTA at 37 °C. The ATPase reaction was quenched by addition of EDTA when Ca^{2+} or Mg^{2+} was present and then was processed as usual. The results obtained are summarized in Table II (entries 7–16). In the presence of Ca^{2+}, the amount of [^{3}H]ADP found bound to the enzyme is 0.7–1.0/CF_1 (entries 7–9, Table II). This variation is attributed to variable denaturation of CF_1 during heat activation. The ratio of $^{32}P_i$/[^{3}H]ADP is ≤0.06. Moreover, if 5 mM unlabeled ATP is incubated with radioactively labeled enzyme for 30 s, a substantial amount of the labeled nucleotide is displaced (entries 9 and 10). The lack of complete displacement may be due to incomplete activation of the enzyme. Rapid dissociation of P_i and subsequent displacement of labeled nu-

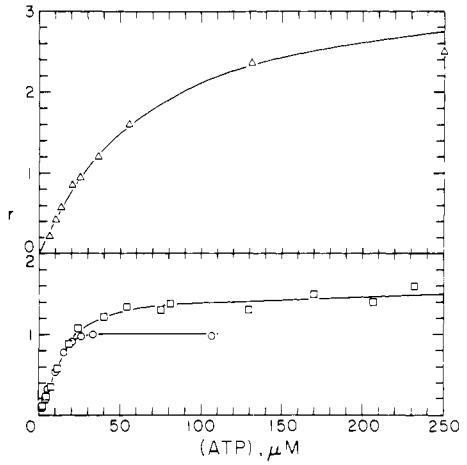

FIGURE 4: Plot of the moles of [^{3}H]ATP per mole of CF_1, r, vs. the total initial ATP concentration as determined by the forced dialysis technique in 40 mM Tris-HCl (pH 8.0): (□) 5 mM $MgCl_2$, 20 μM latent enzyme; (○) 5 mM $MgCl_2$, 17.5 μM NBD-modified latent enzyme (0.92 NBD/CF_1); (△) 2 mM EDTA, 16.5 μM latent enzyme.

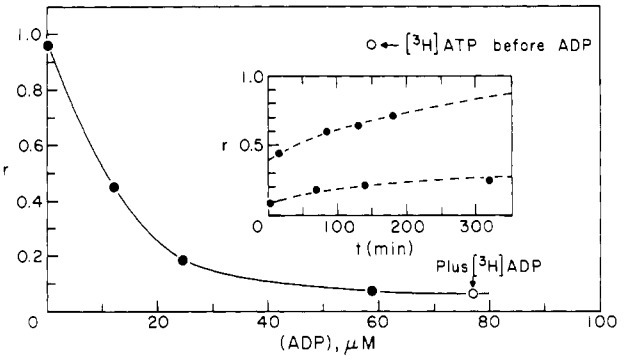

FIGURE 5: Inhibition of the incorporation of [^{3}H]ATP into CF_1 by ADP; r is the moles of tritiated nucleotide per mole of CF_1. Latent enzyme (16.5 μM) was incubated with the indicated concentration of ADP in 40 mM Tris-HCl (pH 8.0) and 5 mM $MgCl_2$; after 10 min, 50 μM [^{3}H]ATP was added; after 10 min more, the amount of tightly bound [^{3}H]ADP was determined. Also shown are the results of control experiments in which [^{3}H]ADP was added rather than [^{3}H]ATP and in which the [^{3}H]ATP was added before the ADP. The inset shows the time dependence of [^{3}H]ATP incorporation after preincubation with ADP as above: upper curve, 28 μM CF_1, 35 μM ADP, 63 μM [^{3}H]ATP; lower curve, 24.7 μM CF_1, 48 μM ADP, 52 μM [^{3}H]ATP.

cleotide by unlabeled nucleotide occur to a lesser extent when 5 mM Mg^{2+} is substituted for Ca^{2+} or when the enzyme is modified with NBD-Cl (3.5–3.8 NBD/CF_1) (entries 11–14, Table II). In the presence of 2 mM EDTA, very little incorporation of the nucleotide occurred in 30 s, and almost no displacement of the radioactive ADP by unlabeled ATP was found (entries 15 and 16, Table II). The activity of the enzyme assayed with 1 mM ATP relative to that of heat-activated enzyme in the presence of 5 mM $CaCl_2$ was 0.35% with 5 mM $MgCl_2$ and <0.1% with 2 mM EDTA. The NBD-modified enzyme was 0.68% active.

Binding Experiments. The binding of ATP to the latent enzyme was studied using the forced dialysis technique in 40 mM Tris-HCl (pH 8.0) and 5 mM $MgCl_2$ or 2 mM EDTA at 20 °C. The results obtained are shown in Figure 4, where the moles of ligand bound per mole of enzyme, r, is plotted vs. the initial ATP concentration. In the presence of Mg^{2+}, a very tight binding of 1 mol of ATP/mol of CF_1 can be seen, with at least one weaker binding site. A tight binding site also was observed with the NBD-modified latent enzyme (Figure 4). Since binding to this site is accompanied by hydrolysis, an equilibrium analysis of this binding curve cannot be made. On the other hand, in 2 mM EDTA this tight binding is absent and over 2 mol of ATP/mol of CF_1 is found, with half-saturation of three sites occurring at about 25 μM *free* ATP. During the time of the dialysis experiment (~30 min) 0.2 mol of ATP/mol of CF_1 is incorporated as tightly bound ADP when the [^{3}H]ATP concentration is 50 μM. If the incubation of the latent enzyme with more than 100 μM [^{3}H]ATP in 2 mM EDTA was prolonged for 18 h, up to 1 mol of tightly bound nucleotide was found. Because partial incorporation of [^{3}H]ATP into the tight binding site occurs during the forced dialysis experiment, an equilibrium analysis of the binding data is not possible.

Interaction between ADP and ATP Binding. The interaction between the ADP and ATP binding to CF_1 was investigated by incubating latent enzyme (16 μM) with varying amounts of ADP (0–60 μM) in 40 mM Tris-HCl (pH 8.0) and 5 mM $MgCl_2$ at 25 °C for 10 min; 50 μM [^{3}H]ATP then was added and the incubation continued for 10 min. The enzyme subsequently was analyzed for bound radioactivity. The results obtained are shown in Figure 5, where the moles of [^{3}H]ADP per mole of CF_1, r, is plotted vs. the ADP concentration in the initial incubation mixture. Clearly ADP strongly inhibits the incorporation of ATP. If the order of incubation with nucleotides is reversed, 0.96 [^{3}H]ADP/CF_1 is found; if [^{3}H]ADP (76 μM) is incubated with CF_1 for 10 min, the ratio [^{3}H]ADP/CF_1 is less than 0.08. As shown in the inset to Figure 5, when the incubation with [^{3}H]ATP is extended to longer times, the amount of [^{3}H]ATP incorporated into the enzyme slowly increases. In view of the irreversible hydrolysis of ATP accompanying incorporation, this result is as expected.

Discussion

The results presented show that one tightly bound ADP is present per CF_1 molecule. Previously, forced dialysis experiments have shown that this enzyme–ADP complex binds two ADP or AMP-PNP molecules at apparently equivalent sites (Cantley & Hammes, 1975). The results presented here indicate that the exchange of ADP and AMP-PNP into the tight binding site of the latent enzyme is sufficiently slow so as to be negligible during the forced dialysis binding experiments. However, with the heat-activated enzyme, the exchange of ADP and AMP-PNP into the site occurs within 2 h; this probably is responsible for the third nucleotide binding site found in forced dialysis experiments with the heat-activated enzyme rather than the occurrence of a "loose" binding site (Cantley & Hammes, 1975). Thus, at least three nucleotide binding sites are present per CF_1. Incorporation of ADP and ATP into the enzyme to give tightly bound ADP requires nucleotide binding at another site (or sites), although the number of these sites is indeterminate. With the latent enzyme, 1 mol of MgATP/mol of CF_1 is rapidly incorporated with hydrolysis of the ATP into ADP and P_i; the ADP and P_i remain tightly bound to CF_1, and P_i prevents displacement of ADP by ATP. Experiments with the heat-activated enzyme are not as easy to interpret because the heat-activated enzyme is not well characterized: enzyme with various specific activities and partially denatured enzyme are probably present. However, the simplest interpretation of the results obtained is that 1 mol of ATP/mol of CF_1 is incorporated into the enzyme and the NBD-modified enzyme in the presence of Ca^{2+} and Mg^{2+}, but only in the presence of Ca^{2+} is the release of phosphate sufficiently rapid to permit displacement of the tightly bound ADP. Unfortunately the NBD-modified enzyme and the heat-activated enzyme in the presence of Mg^{2+} have a small amount of ATPase activity so that they are not ideal

controls. The heat-activated enzyme in the presence of EDTA incorporates very little radioactively labeled nucleotide or P_i.

Nucleotide incorporation into the tight site shows a strong preference for a *metal*-complexed *tri*phosphate in both binding and kinetic experiments, whereas the other two nucleotide sites appear to bind ADP more strongly than ATP. Moreover, the binding of ADP to the enzyme strongly inhibits ATP incorporation. The occurrence of more than two nucleotide binding sites on CF_1 and differential binding of ATP and ADP has been noted by others (Girault & Galmiche, 1977; VanderMeulen & Govindjee, 1977; Nabedrick-Viala et al., 1977; Shoshan et al., 1978). The nonequilibrium behavior noted in titrations of CF_1 with fluorescent analogues of ATP and ADP (VanderMeulen & Govindjee, 1977) can be understood in terms of the ATP hydrolysis reported here. Three nucleotide binding sites also have been reported for coupling factors from beef heart mitochondria (Garrett & Penefsky, 1975) and *Mycobacterium phlei* (Lee et al., 1977).

A functional model of the enzyme incorporating the known information about nucleotide binding is necessarily ambiguous. A *possible* model is that the very tight site is the catalytic site and activity is regulated by nucleotide binding to the other two known nucleotide binding sites. The possibility that very tight nucleotide binding sites participate in catalysis has been suggested by many investigators [cf. Tiefert et al. (1977) and Shoshan et al. (1978)]. The data suggest that phosphate release from the enzyme is rate limiting in ATP hydrolysis for the latent enzyme and partially active forms of the heat-activated enzyme (i.e., NBD-modified and in the presence of Mg^{2+}). Another *possible* mechanism is that some or all of the nucleotide binding sites are equivalent and some type of alternating site mechanism is occurring (Smith & Boyer, 1976; Hackney & Boyer, 1978). For example, binding of ATP to the enzyme might simultaneously create a tight binding site and release ADP from what was previously a tight binding site. For either of these mechanisms to be valid, turnover at the proposed catalytic site would have to be comparable to the turnover number of the enzyme. Kinetic experiments are underway to examine this question. Finally, more than three nucleotide binding sites may exist; the mechanism of action of the enzyme could involve more complex variants of the above with multiple regulatory and/or alternating catalytic sites [cf. Harris et al. (1977)].

The results reported here may be relevant in understanding experimental observations with intact chloroplasts. The incorporation of a single radioactively labeled ADP per CF_1 has been found when chloroplasts are incubated with radioactively labeled AMP and P_i (Roy & Moudrianakis, 1971). Three tight nucleotide binding sites, which can exchange upon energization of the membrane, have been reported (Harris & Slater, 1975); these sites can contain both ATP and ADP. More recent results have suggested that regardless of whether ATP or ADP is present in the medium, illumination of chloroplasts results in one tightly incorporated ADP per CF_1 (Magnusson & McCarty, 1976; Strotmann et al., 1976; Shavit et al., 1977). This site may be identical with the very tight nucleotide binding site on CF_1. The role of this site in phosphorylation is not clear: the rate of nucleotide exchange is slower than that of phosphorylation (Strotmann et al., 1976; R. McCarty, personal communcation), and the nucleotide specificity is different for exchange and phosphorylation (Strotmann & Bickel-Sandkötter, 1977). Further work is needed to clarify the correspondence between the nucleotide binding sites on the chloroplast and on CF_1 and to establish the functions of the various nucleotide binding sites.

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