Further Characterization of Nucleotide Binding Sites on Chloroplast Coupling Factor One†

Michael F. Bruist‡ and Gordon G. Hammes*

ABSTRACT: The solubilized coupling factor from spinach chloroplasts (CF_1) contains one nondissociable ADP/CF_1 which exchanges slowly with medium ADP in the presence of Ca^{2+}, Mg^{2+}, or EDTA; medium ATP also exchanges in the presence of Ca^{2+} or EDTA, but it is hydrolyzed, and only ADP is found bound to CF_1. The rate of ATP exchange with heat-activated CF_1 is approximately 1000 times slower than the rate of ATP hydrolysis. In the presence of Mg^{2+}, both latent CF_1 and heat-activated CF_1 bind one ATP/CF_1, in addition to the ADP. This MgATP is not removed by dialysis, by gel filtration, or by the substrate CaATP during catalytic turnover; however, it is released when the enzyme is stored several days as an ammonium sulfate precipitate. The photoaffinity label 3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl]-ATP binds to the MgATP site, and photolysis results in labeling of the β subunit of CF_1. Equilibrium binding measurements indicate that CF_1 has two identical binding sites for ADP with a dissociation constant of 3.9 μM (in addition to the nondissociable ADP site). When MgATP is bound to CF_1, one ADP binding site with a dissociation constant of 2.9 μM is found. One ATP binding site is found in addition to the MgATP site with a dissociation constant of 2.9 μM. Reaction of CF_1 with the photoaffinity label 3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl]-ADP indicates that the ADP binding site which is not blocked by MgATP is located near the interface of α and β subunits. No additional binding sites with dissociation constants less than 200 μM are observed for MgATP with latent CF_1 and for CaADP with heat-activated CF_1. Thus, three distinct nucleotide binding sites can be identified on CF_1, and the tightly bound ADP and MgATP are not at the catalytic site. The active site is either the third ADP and ATP binding site or a site not yet detected.

Coupling factor 1 (CF_1)[1] is a soluble portion of the ATP synthesizing complex from chloroplasts and contains the catalytic site. The synthesis of ATP requires a pH gradient or potential across the membrane, and Mg^{2+} is the normal cofactor. While isolated CF_1 cannot synthesize ATP, it hydrolyzes ATP when activated by various methods (Vambutas & Racker, 1965). In contrast to the synthetic reaction, Ca^{2+} is a much more effective cofactor than Mg^{2+} for this ATPase activity. Five different types of subunits are found in CF_1, with a probable stoichiometry of $\alpha_2\beta_2\gamma\delta\epsilon_2$ (Binder et al., 1978; Baird & Hammes, 1976). Only the α and β subunits are required for ATPase activity (Deters et al., 1975), but the γ subunit is also involved in photophosphorylation (Nelson et al., 1972). The structural relationships of the subunits have been studied extensively (cf. Baird & Hammes, 1979).

In this work, the interaction of CF_1 with ADP and ATP has been studied. Previous studies have shown that CF_1 has several nucleotide binding sites. These sites are responsible for catalysis, for ADP and ATP inhibition of the ATPase activity (Cantley & Hammes, 1975), for modulation of the proton permeability of CF_1 on the membrane, and for control of susceptibility to inhibitors and uncouplers (McCarty, 1979). A tightly bound ADP of unknown function also has been observed (Harris & Slater, 1975; Magnusson & McCarty, 1976; Carlier & Hammes, 1979). Earlier work from this laboratory demonstrated that CF_1 reversibly binds two ADP molecules (Cantley & Hammes, 1975) and that the additional tightly bound ADP is nondissociable but exchanges with solution ADP and ATP (Carlier & Hammes, 1979). This study describes a site which binds MgATP very tightly and is distinct from the site which binds ADP tightly. In all, three types of nucleotide binding sites are characterized and can be designated as follows: the *nondissociable ADP site* contains the tightly bound ADP which can be exchanged but not removed without denaturing CF_1, the *MgATP site* binds ATP essentially irreversibly in the presence of Mg^{2+}, and the *dissociable nucleotide sites* bind ADP reversibly in the presence or absence of Mg^{2+} or Ca^{2+}. The relationship of these sites to the catalytic site has been investigated.

Materials and Methods

Chemicals. The ADP and ATP (vanadium free) were from Sigma Chemical Co. The ^3H-labeled ADP and ATP were from New England Nuclear, and the ^{14}C- and ^{32}P-labeled nucleotides from Amersham Corp. The radioactive ADP was purified by paper chromatography using isobutyric acid–1 N ammonia (100:60 v/v). Both radioactive and nonradioactive ATP were purified by column chromatography on Bio-Rad AG-1-X4 Cl^- (0.4 × 4 cm). The ATP on the column was washed with 50 mM NaCl–10 mM HCl and eluted with 500 mM NaCl–20 mM HCl. The eluant was neutralized with 1 M Tris base, and the ATP was concentrated and desalted by precipitating with 0.2 mL of 2 M barium acetate per 1 mL of solution, washing the precipitate with water, and solubilizing the ATP by the addition of Bio-Rad AG-50W-X8 Li^+ cation-exchange resin. The 4-fluoro-3-nitroaniline was from Aldrich Chemical Co., and the chemicals for electrophoresis were from Bio-Rad Laboratories. All other chemicals were analytical grade, and all solutions were made with deionized distilled water. Unless indicated, experiments were performed at room temperature, 19 ± 2 °C.

Enzyme. The CF_1 was prepared from fresh market spinach (Lien & Racker, 1971; Binder et al., 1978). Enzyme having a 305:340 nm fluorescence ratio (excitation at 280 nm) greater than 1.5 was collected and stored as an ammonium sulfate precipitate in 2 M ammonium sulfate, 10 mM Tris-HCl (pH

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[1] Abbreviations used: CF_1, chloroplast coupling factor 1; EDTA, ethylenediaminetetraacetic acid; arylazido-ADP or -ATP, 3′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl]-ADP or -ATP; Tris, tris(hydroxymethyl)aminomethane.

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7.2), 1 mM EDTA, and 0.5 mM ATP. Its purity was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The CF_1 was activated by heating at 60 °C for 4 min in 40 mM ATP, 30 mM Tris-HCl (pH 8.0), 7 mM dithiothreitol, and 2 mM EDTA (Farron & Racker, 1970), and the ATPase activity was measured on a Radiometer pH stat (Cantley & Hammes, 1975) or by determination of $[^{32}P]P_i$ liberated from [γ-^{32}P]ATP (Avron, 1960). The specific activity immediately after activation was 8–12 μmol of P_i/(mg·min) in 50 mM NaCl, 6 mM $CaCl_2$, 1 mM EDTA, and 0.5 mM ATP at pH 8.0, 20 °C. The extinction coefficient of CF_1 was determined by dry weight measurements (Kupke & Dorrier, 1978). A value of 0.483 cm^2/mg at 277 nm in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA was obtained and used for determining concentrations. Turbid solutions were clarified by centrifuging at 17000*g* for 20 min at room temperature before the absorbance was measured. A molecular weight of 325 000 was used for determining molar concentrations of CF_1 (Farron, 1970).

Dissociable nucleotides were removed from CF_1 by passage through a Sephadex G-25 medium column (1 × 60 cm) equilibrated with 25 mM sucrose, 10 mM Tris-HCl (pH 8.0), and 2 mM EDTA (Cantley & Hammes, 1975). The eluted enzyme was concentrated either by dialyzing within a collodion membrane against the appropriate buffer or by precipitating the enzyme with an equal volume of column buffer saturated with ammonium sulfate. The precipitated enzyme was cooled on ice and centrifuged at 12000*g* for 10 min at 2 °C. The pellet was dissolved in a minimal amount of 50% glycerol and 50% column buffer (v/v) Holowka & Hammes, 1977) and then passed through a 3.5-mL centrifuge column (Penefsky, 1977) of Sephadex G-50 fine equilibrated with appropriate buffer.

Analysis of Tightly Bound Nucleotides. Bound nucleotides were extracted from CF_1 freed of dissociable nucleotides in 10 mM Tris-HCl (pH 8.0) by perchloric acid denaturation followed by potassium hydroxide neutralization (Rosing & Slater, 1972; Carlier & Hammes, 1979). Correction for the concentration decrease during the extraction was made by determining the change in nucleotide radioactivity per unit volume or by the change in weight when no radioactivity was present. The nucleotide-containing extract was analyzed on poly(ethylenimine)–cellulose columns (ICN Nutritional Biochemicals, 0.5 × 7 cm; Magnusson et al., 1976; Randerath & Randerath, 1967). The poly(ethylenimine)–cellulose was washed with 1.5 M LiCl and 0.1 N HCl before column preparation, and the columns were rinsed with 3 mL of 0.05 M H_3PO_4 raised to pH 3.0 with LiOH (H_3PO_4–LiOH) before addition of the nucleotide sample (1–2 mL). Hydrolyzed protein and other interfering substances were washed off with 3 mL of H_3PO_4–LiOH, AMP was eluted with 3 mL of 0.05 M LiCl in H_3PO_4–LiOH, ADP was eluted with 5 mL of 0.3 M LiCl in H_3PO_4–LiOH, and ATP was eluted with 7 mL of 1.2 M LiCl in H_3PO_4–LiOH. The eluant was monitored at 254 nm, and 1-mL fractions were collected. Each set of columns was calibrated with a standard mixture of [^{3}H]ADP and [γ-^{32}P]ATP. The amount of nucleotide in each fraction was determined spectrophotometrically by using an extinction coefficient of 15.0 × 10^3 M^{-1} cm^{-1} at 256 nm for adenine nucleotides at pH 3.0.

Measurement of Nucleotide Incorporation into CF_1. The nondissociable ADP site of both latent CF_1 and heat-activated CF_1 was labeled by incubating the enzyme with radioactive ADP or ATP as described by Carlier & Hammes (1979). The nondissociable site was prelabeled by incubation of heat-activated CF_1 with 200 μM [^{3}H]ADP in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol, 1 mM EDTA for 2 h to measure the rate of exchange of ATP into the nondissociable ADP site of heat-activated CF_1. The CF_1 again was freed of dissociable nucleotides and put into 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 6 mM $CaCl_2$, and 1 mM EDTA. The exchange of ATP into the nondissociable ADP site of CF_1 in a 0.5-mL aliquot was initiated by the addition of nonradioactive ATP (final concentration 0.5 mM), and it was quenched by the addition of 50 μL of 200 mM EDTA in 20 mM Tris-HCl (pH 8.0). The quenched solution was immediately pipetted onto a freshly prepared 3.5-mL centrifuge column equilibrated with quench buffer. The eluant then was passed through a second centrifuge column equilibrated with 20 mM Tris-HCl (pH 8.0). The addition of 50 μL of buffer to the centrifuge columns after the preliminary centrifugation increased the amount of CF_1 recovered. The amount of radioactive nucleotide in the enzyme was determined by scintillation counting in 10 mL of Ready-Solv GP (Beckman).

For determination of whether or not nucleotide in the MgATP site dissociated during ATP hydrolysis, the MgATP site of heat-activated CF_1 was labeled with [γ-^{32}P,^{3}H]ATP by incubation of 5 μM enzyme with 50 μM [γ-^{32}P,^{3}H]ATP in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, and 1 mM EDTA for 5 min. The dissociable nucleotides were removed, and the labeled enzyme was put into 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. Nonradioactive ATP (final concentration 0.5 mM) in the presence of 6 mM $CaCl_2$ was incubated with the labeled enzyme for 2 min, and the reaction was quenched by the addition of EDTA (final concentration 10 mM). The solution then was pipetted onto a 5-mL centrifuge column equilibrated with 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA. The eluant was put through a second 5-mL centrifuge column equilibrated with the same buffer. The amount of ^{3}H and ^{32}P bound to the enzyme was determined by scintillation counting.

Equilibrium Binding Measurements. The binding of ADP and ATP to CF_1 was studied by the forced dialysis technique (Cantley & Hammes, 1973). Ligand was incubated with 1–35 μM CF_1 (freed of dissociable nucleotides) for 30 min in 0.3–0.5 mL before the forced dialysis was started. When the binding of high concentrations of [^{3}H]ADP to heat-activated CF_1 was studied, the nondissociable ADP site was prelabeled with [^{3}H]ADP of the same specific radioactivity as that used in the forced dialysis experiment.

Photoaffinity Labeling and Gel Electrophoresis. Arylazidoadenine nucleotides were synthesized with nonradioactive and ^{14}C-labeled ATP and ADP (Jeng & Guillory, 1975). High pressure liquid chromatography on a Whatman Partisil PXS 10/25 SAX anion-exchange column with 0.8 M potassium phosphate (pH 3.4) indicated the products were better than 95% pure. Prior to irradiation, the arylazido nucleotides were handled with only a 25-W red safe light for illumination. Samples were photolyzed by irradiating them in 50 mM NaCl and 0.5 mM Tris-HCl (pH 8.0) for 15 min in a 1-cm water-jacketed cuvette at 20 °C with a focused 200-W Hanovia Xe–Hg arc lamp 10 cm away from the sample. Ultraviolet radiation below 350 nm was eliminated with a Corning CSO-51 filter. No heating of the sample occurred. The extent of covalent labeling was determined by denaturing an aliquot of the photolyzed CF_1–arylazido nucleotide complex by the addition of perchloric acid to 4% (w/v) and filtering the precipitated protein on a Whatman glass-fiber C filter. The filters were washed sequentially with 5 mL of 4% perchloric acid, 5 mL of water, and 5 mL of 95% ethanol. The

Table I: Incorporation of Nucleotides into CF_1

nucleotide (μM)	addition	$^3H/CF_1$	$^{32}P/CF_1$	ADP/CF_1[a]	ATP/CF_1
[^{3}H]ADP (74)[b]	2 mM EDTA	0.70		0.90 (0.75)	<0.05
[^{3}H]ATP (170)[b]	2 mM EDTA	0.64		0.93 (0.68)	<0.05
[γ-^{32}P,^{3}H] ATP (49)[b]	5 mM $CaCl_2$	0.88	0.33	1.43 (0.41)	0.18
[γ-^{32}P,^{3}H]ATP (59)[c]	6 mM $CaCl_2$	0.31	0.15	1.21 (0.13)	~0.1
[γ-^{32}P,^{3}H]ATP (50)[d]	6 mM $CaCl_2$	0.60	0.03	0.97 (0.59)	<0.05
[^{3}H]ADP (43)[e]	2 mM EDTA	1.26			
[γ-^{32}P,^{3}H]ATP (49)[f]	5 mM $MgCl_2$	1.16	1.08	1.19 (0.10)	1.07
[^{3}H]ADP (61)[g]	6 mM $MgCl_2$	0.10		1.39 (0.06)	<0.05
[γ-^{32}P,^{3}H]ATP (50)[e]	6 mM $MgCl_2$	1.08	0.83	1.31 (0.25)	0.93

[a] The number in parentheses is the specific radioactivity of the isolated nucleotide divided by the specific radioactivity of the nucleotide incubated with CF_1. [b] Latent CF_1 (8–12 μM) in 40 mM Tris-HCl (pH 8.0); incubated approximately 20 h. [c] Latent CF_1 (13 μM) in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA; incubated 10 min. Extraction of phosphate from this sample indicated <0.01 [^{32}P]P_i/CF_1. [d] Heat-activated CF_1 (4.5 μM) in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA; incubated 5 min. [e] Heat-activated CF_1 (6.6 μM) in 10 mM Tris-HCl (pH 8.0); incubated 2.5 h. [f] Latent CF_1 (12 μM) in 40 mM Tris-HCl (pH 8.0); incubated 10 min. [g] Latent CF_1 (11 μM) in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA; incubated 10 min.

amount of radioactive protein on the dry filters was determined in 10 mL of ACS scintillation fluid (Amersham).

The MgATP site was labeled by incubating CF_1 and arylazido-ATP in the presence of Mg^{2+} and then photolyzing after the dissociable nucleotides had been removed (Carlier et al., 1979). The dissociable ADP sites were labeled as follows: CF_1 or CF_1 with [^{3}H]ATP in the MgATP site was freed of dissociable nucleotides and incubated with various concentrations of arylazido-[^{14}C]ADP in 50 mM NaCl, 0.5 mM Tris-HCl (pH 8.0), and 5 mM $MgCl_2$. After irradiation, the enzyme was precipitated by the addition of an equal volume of saturated ammonium sulfate. The labeled protein was passed through a 1-mL centrifuge column equilibrated with 10 mM sodium phosphate (pH 6.8). Then the protein concentration and the amount of bound [^{3}H]ATP (if present) and arylazido-[^{14}C]ADP were determined.

The subunit location of the covalently bound arylazido nucleotides was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969). Samples were prepared by passing the photolyzed arylazido nucleotide–CF_1 adduct through a 1-mL centrifuge column equilibrated with 10 mM sodium phosphate (pH 6.8) and then adding an equal volume of 10 mM sodium phosphate (pH 6.8), 2% (w/v) sodium dodecyl sulfate, and 0.2% (v/v) β-mercaptoethanol. The samples were heated for 5 min in boiling water, and 20% of their volume of glycerol containing bromthymol blue was added. Approximately 20–50 μg of labeled CF_1 was put onto a 10% (w/v) acrylamide with 0.3% (w/v) *N,N'*-methylenebis(acrylamide) gel (0.6 × 11 cm). The gels were thermostated at 17 °C and electrophoresed for 15 min at 30 V and then for 4–5 h or for 10–12 h at 100 V in 10 mM sodium phosphate (pH 6.8) and 0.1% sodium dodecyl sulfate. The gels were fixed, strained, and spectrophotometrically scanned, and the distribution of radioactivity was determined by slicing the gel as previously described (Baird & Hammes, 1976).

Results

Characterization of the Nondissociable ADP Site. The presence of one nondissociable ADP on latent CF_1 and its ability to exchange with solution [^{3}H]ADP or [^{3}H]ATP in the absence of divalent metal ions was confirmed (Carlier & Hammes, 1979) by the results shown in the first two entries

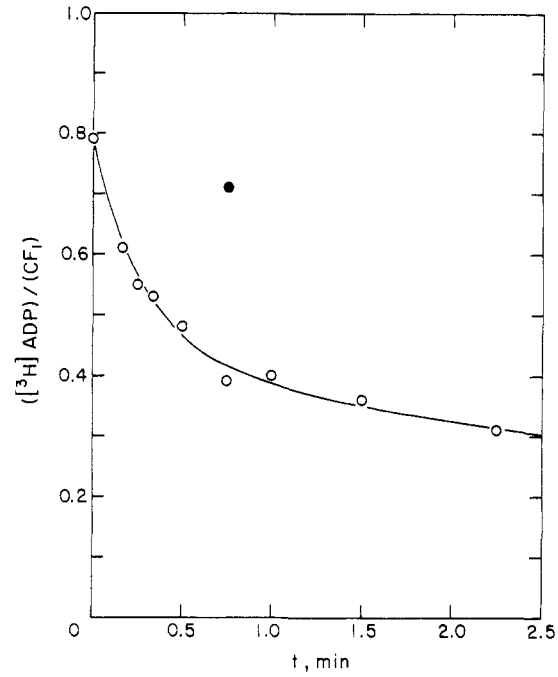

FIGURE 1: Time course of the displacement of [^{3}H]ADP from the nondissociable ADP site of heat-activated CF_1 by ATP. The incubation mixture contained 1.9 μM CF_1 in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 6 mM $CaCl_2$, 1 mM EDTA, and 0.5 mM ATP (○). The reactions were initiated by the addition of ATP, and the zero time point was obtained by adding the ATP after the quenching solution. The closed circle (●) shows the result when Ca^{2+} is omitted.

of Table I. The stoichiometries determined for ADP and ATP labeling of the nondisociable site and the MgATP site are often slightly greater than one. This may arise from nonspecifically bound nucleotides or from an error in the molecular weight or extinction coefficient of CF_1. The extinction coefficient determined in this work differs insignificantly from the value reported by Cantley & Hammes (1975). The addition of Mg^{2+} or Ca^{2+} does not affect the ability of [^{3}H]ADP to exchange into the nondissociable site (data not shown). Line 3 of Table I indicates that incorporation of [γ-^{32}P,^{3}H]ATP occurs in the presence of Ca^{2+}. The small amount of ATP and the ADP in excess of 1 mole per mole of CF_1 can be attributed to Ca^{2+} causing binding to the other nucleotide sites discussed below. No bound [^{32}P]P_i is found, as determined by extraction of phosphate with ammonium molybdate (line 4, Table I). No ^{32}P radioactivity was found in the isolated ADP, and no AMP was detected in any of the experiments, indicating that no appreciable adenylate kinase activity exists. The slight discrepancy between the ratios $^{32}P/CF_1$ and ATP/CF_1 may arise from losses during extraction and chromatography of the nucleotides. The above experiments indicate that when ATP exchanges into the nondissociable ADP site, it is hydrolyzed and the phosphate is not bound by CF_1. This occurs even in the absence of divalent metal ions which are required for catalytic activity. The results are similar for both ATP and ADP when heat-activated enzyme is used, except that their rates of exchange are faster (lines 5 and 6, Table I).

Since the rate of exchange increases significantly upon activation of CF_1, the possibility that the nondissociable ADP site is the active site was considered. The nondissociable ADP site was labeled by incubating heat-activated CF_1 with [^{3}H]ADP, and its rate of exchange was measured by following the loss of radioactivity from the site when the labeled enzyme was incubated with 0.5 mM nonradioactive ATP and 6 mM $CaCl_2$. Figure 1 shows the amount of $^3H/CF_1$ remaining vs.

time. The labeled CF_1 had an ATPase activity of 7.3 μmol of P_i/(min·mg) (turnover number of 2.4×10^3/min) under the exchange conditions. The initial rate of exchange is approximately 0.004 μmol of ADP exchanged/(min·mg) (turnover number of 1.3/min). A similar experiment using [γ-^{32}P]ATP showed that essentially all of the ATP is hydrolyzed in 30 s. The exchange rate slows down when the ATP is depleted because ADP exchanges more slowly than ATP in the presence of Ca^{2+}. Since the rate of exchange is much slower than the rate of ATP hydrolysis, the nondissociable ADP site is not the primary catalytic site.

Characterization of the MgATP Site. When CF_1 is incubated with ATP in the presence of Mg^{2+}, the ATP binds to a site distinct from the nondissociable ADP site. Latent CF_1, prelabeled with [^{3}H]ADP at the nondissociable ADP site, incubated with [α-^{32}P]ATP for 10 min in the presence of 5 mM $MgCl_2$ retains 0.84 of the ^{3}H label and gains 0.94 of the ^{32}P label per mol of CF_1. The results in line 7 of Table I indicate that after incubation with ATP and Mg^{2+} and the subsequent removal of dissociable nucleotides, two nucleotides are bound to CF_1. The MgATP site does not bind [^{3}H]ADP as tightly as ATP in the presence of Mg^{2+} (line 8, Table I). Binding of ATP to the MgATP site is independent of the Mg^{2+} concentration, provided that MgATP is at least stoichiometric with CF_1. Incubation of CF_1 with Mg^{2+}, followed by removal of free Mg^{2+} before the addition of ATP, does not allow ATP to bind tightly. The binding of ATP to the MgATP site occurs within minutes of mixing; however, once bound, it does not readily exchange with solution ATP. If CF_1 containing radioactive ATP at the MgATP site is dialyzed for 20 h against nonradioactive ATP in the presence of Mg^{2+}, only one-third of the label is lost and one ATP/CF_1 is isolated. Heat-activated CF_1 also binds ATP at the MgATP site (line 9, Table I). Solution ATP can still exchange into the nondissociable ADP site when ATP occupies the MgATP site of latent or heat-activated CF_1. This is seen in line 9 of Table I as excess ^{3}H/CF_1 over ^{32}P/CF_1 and as tritium label appearing in the isolated ADP. In another experiment, [^{3}H]ADP was shown to exchange into the nondissociable ADP site when the MgATP site is occupied. However, the rate of exchange appears to be faster if the MgATP site is empty.

Various attempts were made to remove bound ATP from the MgATP site. Calcium ions do not promote the dissociation of ATP from this site. This was shown by transferring CF_1 labeled with [γ-^{32}P,^{3}H]ATP at the MgATP site to a buffer containing 2 mM $CaCl_2$ and1 mM EDTA, dialyzing the enzyme against this buffer for 4 h, and then removing the dissociable nucleotides. After this treatment, the CF_1 still had 0.81 ^{3}H/CF_1 and 0.71 ^{32}P/CF_1. Ammonium sulfate precipitation in the presence of 25% (v/v) glycerol followed by passage through a G-25 fine column (1 × 50 cm) equilibrated with the standard column buffer containing 50% (v/v) glycerol does not remove nucleotides from either the MgATP site or the nondissociable ADP site. The CF_1 treated in this manner still had 1.2 ADP/CF_1 and 0.67 ATP/CF_1. This procedure removes all of the bound nucleotides on the mitochondrial coupling factor F_1 (Garrett & Penefsky, 1975). If CF_1 with 1.13 [^{3}H]ATP/CF_1 at the MgATP site is dialyzed for 50 h against 40 mM Tris-HCl (pH 8.0) and 2 mM EDTA, 0.63 ^{3}H/CF_1 remains bound. If the same labeled enzyme is stored as an ammonium sulfate precipitate in the normal CF_1 storage buffer minus ATP for the same amount of time, only 0.09 ^{3}H/CF_1 is found, and no ATP can be isolated from the enzyme. This CF_1 still has one ADP/CF_1, and if it is incubated with [^{3}H]ATP and Mg^{2+}, one [^{3}H]ATP/CF_1 is bound.

Table II: Amount of [γ-^{32}P,^{3}H]ATP Remaining Bound at the MgATP Site of CF_1 during ATP Hydrolysis[a]

	^{3}H/CF_1	^{32}P/CF_1
initial	1.05	0.82
0.5 mM ATP, 6 mM $CaCl_2$[b]	0.83	0.71
6 mM $CaCl_2$[b]	1.07	0.75

[a] The mixture contained 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 9.4 μM CF_1. The specific activity of the CF_1 was 5.0 μmol of P_i/(min·mg). [b] A 2-min incubation; quenched by the addition of EDTA to a final concentration of 10 mM.

Storing a MgATP·CF_1 complex for 3 h as an ammonium sulfate precipitate results in no loss of bound ATP.

When ATP is bound to heat-activated CF_1 at the MgATP site, the specific activity is not altered. The results in Table II show that the amount of [γ-^{32}P,^{3}H]ATP bound at the MgATP site does not decrease during ATP hydrolysis. The small decrease in ^{3}H/CF_1 after incubation with Ca^{2+} and ATP is due to the slight labeling of the nondissociable ADP site and the exchange of ATP into that site during the 2-min incubation. Therefore, the MgATP site is not the catalytic site.

Characterization of the Dissociable Nucleotide Sites. The dissociable nucleotide binding sites of CF_1 were studied by forced dialysis in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. This method is fast enough to allow an equilibrium binding contstant to be determined before any significant exchange of the binding nucleotide into the nondissociable ADP site occurs. (In the 30 min required for a forced dialysis experiment, less than 0.1 ADP/CF_1 exchanges with 50 μM [^{3}H]ADP or [^{3}H]ATP.) The binding data are presented in Figure 2 as plots of the number of moles of nucleotides bound per mole of CF_1, r, vs. the concentration of unbound nucleotide, L. Nonlinear least-squares analyses were used to fit the data to the equations given below. When one class of binding sites was observed, eq 1 was assumed (n is the number of binding sites per enzyme molecule, and K is the dissociation constant). In cases where two classes of sites were observed, the binding to one class of sites was too tight for the dissociation constant to be determined by the forced dialysis method. Therefore, only binding to the second class of sites was studied, and the first class of sites was assumed to be saturated at all nucleotide concentrations used. For this case

$$r = \frac{nL}{K + L} \quad (1)$$

$$r = n_1 + \frac{n_2 L}{K_2 + L} \quad (2)$$

where n_1 and n_2 are the number of binding sites of classes 1 and 2, respectively, and K_2 is the dissociation constant for the binding of nucleotides to the second class of sites. In analyzing the data, n_1 and n_2 were assumed to be integral multiples of each other. Any exchange with the nondissociable ADP will increase the observed values of n_i and lower the observed value of K or K_2 because the released nonradioactive ADP reduces the specific radioactivity of the unbound nucleotide.

The binding of [^{3}H]ADP to latent CF_1 with and without the MgATP site occupied by nonradioactive ATP is shown in Figure 2A. When the MgATP site is unoccupied by ATP, the results are similar to those observed by Cantley & Hammes (1975) in the presence of Mg^{2+}. When eq 1 is used, the best fit gives $n = 2.1$ and $K = 3.9$ μM. When the MgATP site is occupied by ATP, the number of binding sites is reduced to one, but the dissociation constant remains approximately the same. The best fit gives $n = 1.2$ and $K = 2.9$ μM. Thus either

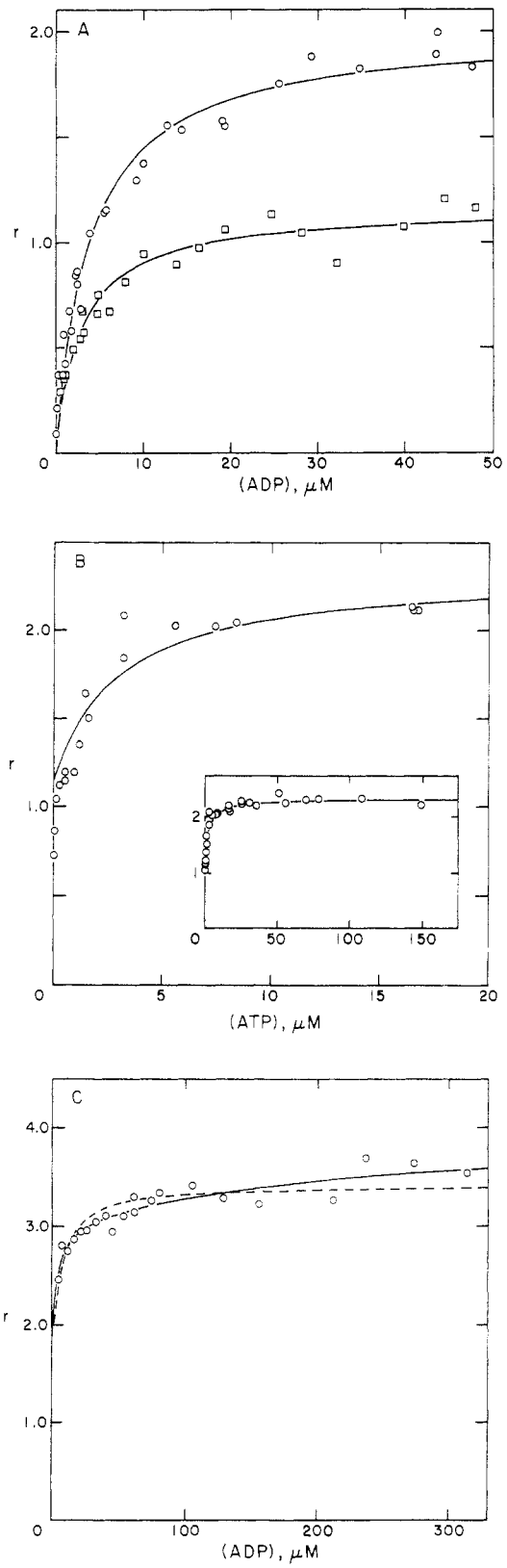

FIGURE 2: Plots of the number of moles of ADP or ATP bound per mol of CF_1, r, vs. the concentration of unbound ADP or ATP. (A) The binding of ADP to latent CF_1 (○) and latent CF_1 with the MgATP site occupied with ATP (□) in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. (B) The binding of ATP to latent CF_1 (○) in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, and 1 mM EDTA. The inset shows the same data plus additional data up to 150 μM ATP. (C) The binding of ADP to heat-activated CF_1 previously labeled with [^{3}H]ADP at the nondissociable ADP site (○) in 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 6 mM $CaCl_2$, and 1 mM EDTA. The CF_1 concentration ranged from 0.9 to 34 μM.

ADP binds directly to the MgATP site or the MgATP site allosterically controls binding to one of the ADP sites. In either case, the two sites are independent and *not* identical; however, the dissociation constants for ADP binding to the two sites are essentially the same.

Before the binding of ATP was studied, the ATPase activity of latent CF_1 was measured. In 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.5 mM [γ-^{32}P]ATP with no metal, 6 mM $MgCl_2$, or 6 mM $CaCl_2$, the specific activities were < 0.1, 0.14, and 6.1 nmol/(mg·min), respectively. If a Michaelis constant of 100 μM is assumed (unpublished experiments) in the presence of 6 mM $MgCl_2$, 25 μM ATP is only 3% hydrolyzed by 3 μM CF_1 after 30 min. Figure 2B shows that 2 mol of ATP bind per mol of CF_1 in the presence of Mg^{2+}. The data in Figure 2B were fit to eq 2. The best fit gives $n_1 = n_2 = 1.2$ and $K_2 = 2.9$ μM.

Since the Michaelis constant for CaATP is 100–200 μM (unpublished experiments), binding at higher nucleotide and enzyme concentrations was studied. These data for MgATP are shown in the inset in Figure 2B. If a third binding site was assumed in the data analysis, the dissociation constant for the additional site derived from the data was greater than 1 mM. At the high protein concentration used, as much as 20% of the ATP may be hydrolyzed to ADP. However, the same results were obtained at different protein concentrations. The binding of ADP to heat-activated CF_1 in the presence of Ca^{2+} also was studied at high concentrations. Since solution [^{3}H]ADP readily exchanges with ADP in the nondissociable ADP site under these conditions, the nondissociable ADP was labeled with [^{3}H]ADP before performing the binding experiment. An additional site would appear as a fourth site. Figure 2C shows the isotherm for ADP binding to heat-activated CF_1 in the presence of Ca^{2+}. The dashed line is the best fit to eq 2, which gives $n_1 = 1.1$, $n_2 = 2.3$, and $K_2 = 5.0$ μM. The solid line is the best fit when an additional binding site is assumed, which corresponds to a third term in eq 2 similar to the second term. This gives $n_1 = 1.0$, $n_2 = 2.0$, $n_3 = 1.0$, $K_2 = 2.0$ μM, and $K_3 = 240$ μM, where n_3 and K_3 are the stoichiometry and dissociation constant of the third site. The accuracy of the data is not sufficient to distinguish between these two models, but if a third site exists, the characteristic dissociation constant is greater than 200 μM. The adenylate kinase activity of CF_1 was insignificant in these experiments. Thin-layer chromatography on poly(ethylenimine)–cellulose plates (Brinkmann) with 1.2 M LiCl resulted in barely detectable [^{3}H]AMP after 74 μM [^{3}H]ADP was incubated with 12 μM CF_1 for 2 h.

Photoaffinity Labeling of CF_1. The detailed characterization of the ATP and ADP binding sites on CF_1 presented here allowed further elaboration of the photoaffinity labeling experiments previously reported (Carlier et al., 1979). The subunit locations of the MgATP and the dissociable nucleotide sites were determined by using the photoaffinity nucleotide analogues arylazido-[^{14}C]ADP and -[^{14}C]ATP and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Arylazido-ATP binds to CF_1 at the MgATP site in the presence of Mg^{2+} and is not removed with the dissociable nucleotides. The binding stoichiometry was assessed by both the incorporation of ^{14}C radioactivity and the absorbance increase at 475 nm (assuming a molar extinction coefficient of 4.2×10^3 M^{-1} cm^{-1}; Jeng & Guillory, 1975). Both methods indicated that 1.0–1.1 arylazido-ATP/CF_1 is bound. Photolysis of the complex caused 0.3 arylazido-ATP/CF_1 to be covalently attached. Figure 3A shows a spectrophotometric scan of an electrophoresis gel of the labeled protein and the distribution of ^{14}C radioactivity in the gel. Only the α and β subunits are labeled.

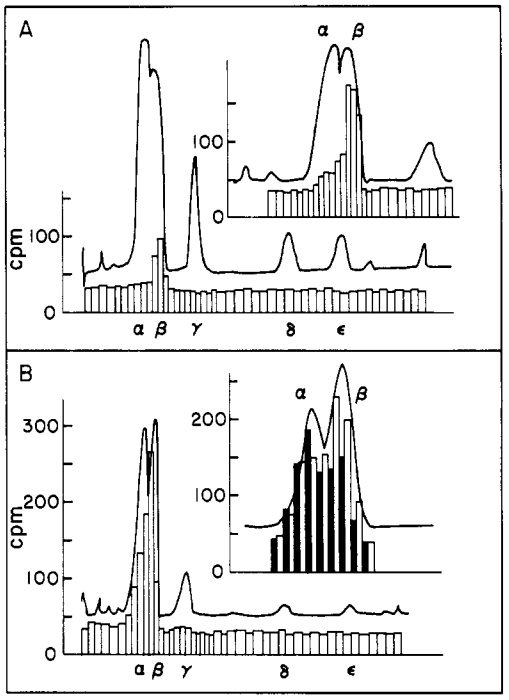

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of latent CF_1 labeled with arylazido-[^{14}C]ATP or arylazido-[^{14}C]ADP. The solid line indicates the absorbance of the stained gels at 560 nm, and the bars indicate the amount of radioactivity in the slices. The subunits are designated α, β, γ, δ, and ϵ. The insets show gels which were electrophoresed longer in order to separate the α and β subunits better. (A) CF_1 with 0.3 arylazido-[^{14}C]ATP covalently bound to the MgATP site. (B) CF_1 with 0.8 arylazido-[^{14}C]ADP covalently bound to the dissociable ADP sites. (Inset) (Open bars) CF_1 with 1.7 arylazido-[^{14}C]ADP covalently bound; (closed bars) CF_1 with the MgATP site blocked during photolysis with 1.1 arylazido-[^{14}C]ADP/CF_1 covalently bound.

The inset shows a separate gel which was electrophoresed longer to separate the α and β subunits better. Approximately 85% of the radioactivity was found in the β subunit, and 15% was found in the α subunit. This result is the same as observed by Carlier et al. (1979), except that the MgATP site is now known to be labeled.

The dissociable nucleotide sites were labeled by incubating and photolyzing CF_1 with or without the MgATP site occupied by [^{3}H]ATP with various concentrations of arylazido-[^{14}C]-ADP in 50 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM Tris-HCl (pH 8.0). After photolysis and removal of unreacted nucleotides, an average of 0.86 [^{3}H]ATP/CF_1 remained bound. Figure 4 shows the number of arylazido-ADP/CF_1 that are covalently bound vs. the total concentration of arylazido-ADP added; the distribution of nucleotides and radioactivity in the gels is shown in Figure 3B. When the MgATP site is occupied, a maximum of 1.2 arylazido-ADP/CF_1 react, and the label is distributed 57% in α, 37% in β, and 8% in γ (average of three gels). When the MgATP site is unoccupied, a maximum of 1.8 arylazido-ADP/CF_1 react, and the label is distributed 47% in α, 49% in β, and 4% in γ (average of three gels). The arylazido-ADP labeling of the dissociable nucleotide sites is consistant with the following model: one dissociable nucleotide site is located near the interface of the α and β subunits and reacts with arylazido-ADP with 100% efficiency; a second site, found in the absence of MgATP, incorporates radioactivity 15% in the α subunit and 85% in the β subunit and reacts with only 50% efficiency.

Attempts to label the nondissociable ADP site with arylazido nucleotides were unsuccessful. Conditions could not be found under which arylazido-ADP would exchange into this site. A

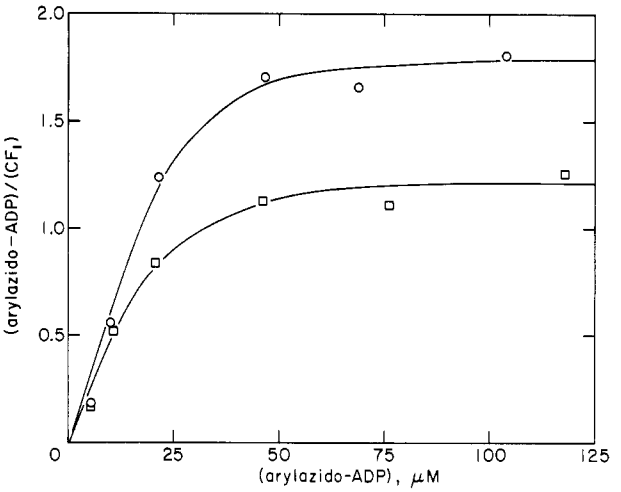

FIGURE 4: Plot of the number of moles of arylazido-[^{14}C]ADP covalently bound to latent CF_1 vs. the total concentration of arylazido-[^{14}C]ADP. The MgATP site of CF_1 was either unoccupied (○) or occupied (□). The CF_1 concentration was 11 μM in both cases. The inset in Figure 3B is an electrophoresis gel of the adducts produced at about 70 μM arylazido-[^{14}C]ADP in this experiment.

small amount of arylazido-ADP and arylazido-ATP ($\sim$ 0.1/CF_1 for each nucleotide) was incorporated into the enzyme, and a corresponding loss of [^{3}H]ADP from the nondissociable ADP site occurred when arylazido-[^{14}C]ATP was incubated with heat-activated enzyme and Ca^{2+} for 30 min. Polyacrylamide gel electrophoresis following photolysis indicated both α and β subunits were labeled. This result cannot be interpreted unambiguously. Attempts to covalently link [^{14}C]ADP in the nondissociable ADP site with CF_1 utilizing intense 250–260-nm light also were unsuccessful.

Discussion

The results presented identify three different types of nucleotide binding sites on CF_1. Isolated CF_1 always has one ADP/CF_1 at the nondissociable ADP site. When CF_1 is heat-activated, this ADP exchanges rapidly with solution ATP in the presence of Ca^{2+}. During the exchange, the ATP is hydrolyzed to ADP, and the phosphate is released into the medium. The rate of exchange is approximately 1000 times slower than the turnover number of the activated Ca^{2+}-ATPase. This difference indicates that exchange at the nondissociable ADP site and catalytic turnover are different events. Either the nondissociable ADP site has a separate ATP hydrolyzing activity or the exchange is loosely coupled to ADP production at the active site. If this site can hydrolyze ATP, it could contribute significantly to the overall rate of hydrolysis at low ATP concentrations. These properties are analogous to those of the tightly bound ADP on CF_1 when it still is bound to chloroplasts. The tightly bound ADP exchanges rapidly with solution ADP or ATP when there is a pH gradient across the thylakoid membrane (Magnusson & McCarty, 1976). The rate of this exchange is slower than the rate of photophosphorylation (Bickel-Sandkötter & Strotmann, 1976; Dunham & Selman, 1981). The mechanistic role of this site is not known. Perhaps regulation similar to the GTPase activity of adenylate cyclase is occurring (Cassel et al., 1977). Regulation of light-induced ATPase activity of CF_1 on the chloroplasts by nondissociable ADP has been proposed (Dunham & Selman, 1981).

In the presence of Mg^{2+}, one ATP/CF_1 binds tightly to the MgATP site. This tight binding of ATP is not found with Ca^{2+}, and neither Ca^{2+} nor EDTA causes the release of bound ATP. The MgATP site remains occupied, and the ATP is not

hydrolyzed while heat-activated CF_1 hydrolyzes ATP. Thus this site also is distinct from the active site. The function of this site is unknown, but experiments are underway to investigate how occupation of this site effects the ATPase steady-state kinetics.

Previously reported results have suggested that in the presence of Mg^{2+}, ATP exchanges rapidly into the nondissociable ADP site with the ATP being hydrolyzed and the phosphate remaining bound to the enzyme (Carlier & Hammes, 1979). The results presented here indicate that this is not the case. The error in the previous work is probably associated with the poly(ethylenimine)–cellulose columns. If the pH is not controlled, ATP sometimes elutes with ADP. Furthermore, each batch of poly(ethylenimine) must be calibrated with standard mixtures of ADP and ATP. No tightly bound phosphate was found in the experiments described here.

The forced dialysis experiments demonstrate the presence of two nucleotide binding sites for ADP. One of these is blocked when the MgATP site is occupied. In the presence of Mg^{2+}, ATP binds to the very tight MgATP site and only one other binding site. A binding experiment done with [^{3}H]adenylyl imidodiphosphate indicated binding similar to ADP; tight binding at the MgATP site was not observed (Cantley & Hammes, 1975). This indicates that the MgATP site distinguishes between ATP and adenylyl imidodiphosphate and shows the danger of using analogues in place of the true ligand. The photoaffinity labeling studies indicate that both one dissociable ADP site and the MgATP site are located primarily on the β subunit and have low efficiencies of photolabeling. The simplest interpretation is that these two sites are one and the same. This also explains the inhibition by ADP of the labeling of the MgATP site with [^{3}H]ATP observed by Carlier & Hammes (1979). The small amount of labeling of the α subunit (15%) may be due to incomplete resolution of the α and β subunits, or the α subunit may be within the range of the arylazido arm ($\sim$1 nm). When arylazido-ADP binds to the dissociable nucleotide site which is not blocked by MgATP, it labels both the α and β subunits of CF_1. Wagenvoord et al. (1981) observed a similar labeling of both α and β subunits using 8-azido-ADP and interpreted this to mean separate sites exist on each subunit. The evidence presented here indicates that the α and β subunits are both labeled when only a single site exists for arylazido-ADP. Therefore, this one site must be at or near the interface of the α and β subunits of CF_1. Ligand binding sites shared by two polypeptide chains have been found in other proteins by X-ray crystallography, e.g., hemoglobin (Arnone, 1972), hexokinase (Anderson & Steitz, 1975), and phosphofructokinase (Evans & Hudson, 1979). Unfortunately, the subunit location of the nondissociable ADP binding site could not be determined.

The results of binding studies from other laboratories are consistent with those reported here. Shoshan et al. (1978) reported that lettuce CF_1 binds ATP with a dissociation constant of 0.1 μM in the presence of Mg^{2+}. They also observed three sites for ADP binding (dissociation constant 2 μM), one of which was inhibited competitively by ATP. Harris & Slater (1975) reported finding one ADP and two ATP molecules per CF_1 tightly bound to chloroplasts. The ADP is presumed to be at the nondissociable ADP site, and one of the two ATP molecules may be at the MgATP site. The ATP remains bound at the MgATP site on chloroplasts because they are not stored as ammonium sulfate precipitates. The dicyclohexylcarbodiimide-sensitive ATPase from spinach chloroplasts, of which CF_1 is a part, has approximately one tightly bound ADP and one tightly bound ATP per mole (Cerione & Hammes, 1981). The ADP will exchange with solution ADP and ATP; however, this exchange appears to be a single turnover reaction. The two bound nucleotides are probably in sites which correspond to the nondissociable ADP site and the MgATP site. An additional ADP binding site with a dissociation constant of about 2 μM, analogous to the dissociable nucleotide site, also is observed.

In characterizing these three different types of nucleotide binding sites on CF_1, the active site for ATP hydrolysis still has not been positively identified. Since two of these nucleotide sites have been shown not to be the catalytic site, the remaining dissociable nucleotide site located near the α–β subunit interface may be the active site, or the active site may not have been detected by the methods used in this work. In the latter case, the dissociation constant for MgATP or CaADP binding to the catalytic site must be greater than 200 μM. This problem currently is being pursued further.

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Proton–Adenosinetriphosphatase Complex of Rat Liver Mitochondria: Effect of Energy State on Its Interaction with the Adenosinetriphosphatase Inhibitory Peptide†

Klaus Schwerzmann and Peter L. Pedersen*

ABSTRACT: The effect of energy state on the capacity of the H^+–ATPase of inverted inner membrane vesicles of rat liver mitochondria to interact with a homogeneous inhibitor peptide from the same source [Cintron, N. M., & Pedersen, P. L. (1979) *J. Biol. Chem. 254*, 3439–3443] has been examined in some detail. The study has been conducted by using an assay procedure which allows both ATP synthetic and hydrolytic activities of the H^+–ATPase to be monitored in the same assay system. When the purified inhibitor is incubated with inverted inner membrane vesicles in the presence of MgATP and then sedimented to remove excess inhibitor and MgATP, the ATPase activity of the H^+–ATPase is markedly inhibited. When ATP synthesis is induced in the same assay system by initiation of respiration (addition of succinate), the synthetic rate proceeds with a brief lag phase, in the order of seconds, and then assumes a linear steady-state rate. Under these conditions, most of the peptide inhibitor remains associated with the inner membrane vesicles. It is released into the supernatant only when respiration is allowed to proceed several minutes. Inhibitor release is accompanied by a parallel rise in the capacity of the H^+–ATPase to catalyze ATPase activity under nonenergized conditions (succinate absent). These results emphasize that binding of the peptide inhibitor to the H^+–ATPase complex of rat liver and its release therefrom correlate well with the capacity of the enzyme to catalyze ATP hydrolysis rather than ATP synthesis. The lag phase in ATP synthesis when inhibitor is present is very brief and may reflect the time required for a respiration-induced electrochemical gradient to weaken the binding of the inhibitor to the enzyme surface. It would seem that if the peptide inhibitor is a regulatory molecule, one of its major roles in intact rat liver mitochondria may be to preserve newly synthesized ATP following a burst of "phosphorylating" respiration.

ATPase inhibitor peptides have been isolated from mitochondria (Pullman & Monroy, 1963; Horstman & Racker, 1970; Satre et al., 1975; Chan & Barbour, 1976a; Ebner & Maier, 1977; Cintrón & Pedersen, 1979; Yamada et al., 1980), from chloroplasts, (Nelsen et al., 1972; Younis et al., 1980), and from bacteria (Smith & Sternweis, 1977). In all cases, they have been shown to be potent inhibitors of the ATP hydrolytic activity catalyzed by H^+–ATPases (F_0F_1-ATPases) associated with these systems. These inhibitors have been studied in detail with respect to their physicochemical properties. Without exception, they are small molecules ranging from 5000 to 16000 daltons. They constitute either the smallest of the five types of subunits characteristic of the F_1 moiety or a distinct "sixth" subunit. In chloroplasts, there have been reports of two inhibitor proteins, one constituting the ϵ or the smallest subunit (Nelson et al., 1972) and another which is distinct from the F_1 subunits (Younis et al., 1980).

To date, the role of these various ATPase inhibitor proteins is not at all clear. Although it has become common to refer to this class of proteins as regulatory molecules, it remains to be established what their regulatory role is. It has been suggested that such inhibitors may regulate the direction of energy flux through H^+–ATPases, i.e., toward ATP synthesis by blocking only the ATP-dependent or ATP hydrolytic activities (Asami et al., 1970). However, it has also been suggested that, depending on the conditions, these inhibitors may regulate and/or inhibit ATP synthesis (Harris & Crofts, 1978; Gómez-Puyou et al., 1979; Harris et al., 1979), the ADP–ATP transport system (Chan & Barbour, 1976b), and even Ca^{2+} uptake (Gómez-Puyou et al., 1980).

Several workers have indicated that inhibitor binding may be dependent on the energy state of the mitochondrion (Van De Stadt et al., 1973; Gómez-Puyou et al., 1979; Harris et al., 1979). In the most recent set of studies along these lines, it has been assumed that the inhibitor is associated with the H^+–ATPase complex in such a way as to block both ATP synthesis and ATP hydrolysis (Gómez-Puyou et al., 1979; Harris et al., 1979). Initiation of respiration is suggested to dissociate the inhibitor so that the electrochemical gradient of protons can drive ATP synthesis (Harris & Crofts, 1978; Gómez-Puyou et al., 1979; Harris et al., 1979). Although these studies certainly provide a novel view of the regulatory nature of peptide inhibitors, they provide no direct evidence (i.e., appearance of the released inhibitor in the supernatant) to support the suggested hypothesis. Rather, increase in ATPase activity is used as an indirect index of inhibitor release.

† From the Laboratory for Molecular and Cellular Bioenergetics, Department of Physiological Chemistry, The John Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received April 10, 1981.* This work was supported by a U.S. Public Health Service grant (CA 10951) from the National Institutes of Health and by a Fogarty International fellowship (1-F05 TWO 2755).

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