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Characterization of Nucleotide Binding Sites on Chloroplast Coupling Factor 1†

Lewis C. Cantley, Jr.‡ and Gordon G. Hammes*

ABSTRACT: A study of the equilibrium binding of ADP, 1,N^6-ethenoadenosine diphosphate, adenylyl imidodiphosphate, and 1,N^6-ethenoadenylyl imidodiphosphate to solubilized spinach chloroplast coupling factor 1 (CF_1) has been carried out. All four nucleotides were found to bind to two apparently identical "tight" sites, with characteristic dissociation contants generally less than 10 μM. The binding to these "tight" sites is similar in the presence of Mg^{2+} and Ca^{2+}, is stronger in 0.1 M NaCl than in 20 mM Tris-Cl, and is only slightly altered by heat activation. The slow rate of association of ADP and 1,N^6-ethenoadenosine diphosphate at these sites rules out the possibility that they are catalytic sites for ATPase activity on the solubilized enzyme. A third tight site for adenylyl imidodiphosphate was found on the heat-activated enzyme. The dissociation constant for this interaction (7.6 μM) is similar to the adenylyl imidodiphosphate competitive inhibition constant for ATPase activity (4 μM). ADP, which inhibits ATPase activity but is not a strong competitive inhibitor, binds only weakly at a third site (dissociation constant >70 μM). One mole of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole reacted per mole of CF_1 prevents ADP and adenylyl imidodiphosphate binding at the "catalytic" site and abolishes the ATPase activity. A model is proposed in which the "tight" nucleotide binding sites act as allosteric conformational switches for the ATPase activity of solubilized CF_1.

The coupling factor from spinach chloroplasts (CF_1)[1] is believed to be directly involved in photophosphorylation. The solubilized purified enzyme has no ATPase activity unless activated by trypsin (Vambutas and Racker, 1965), heat (Farron and Racker, 1970), or dithiothreitol (McCarty and Racker, 1968). While photophosphorylation and light induced ATPase activities in chloroplasts are dependent on Mg^{2+} (Petrack et al., 1961, 1965; McCarty and Racker, 1966), the activated CF_1 has a Ca^{2+} dependent ATPase, although a Mg^{2+} dependent ATPase may be induced by carboxylic acids (Nelson et al., 1972). The ATPase activity of CF_1 can be abolished by reacting NBD-Cl with one or two tyrosine groups on the β subunit (Deters et al., 1975), and the inhibition is completely reversed by dithiothreitol which releases the bound NBD.

In this work, equilibrium binding measurements are used to investigate the effects of heat activation, of Ca^{2+} and Mg^{2+}, and of NBD-Cl modification on the nucleotide binding sites of solubilized CF_1. These results are correlated with steady-state inhibition measurements of Ca^{2+}-ATPase activity by ADP and AMP-PNP. The solubilized CF_1 contains two tight binding noncatalytic sites for AMP-PNP, ϵAMP-PNP, ADP, and ϵADP, and the heat-activated enzyme contains an additional tight binding site for AMP-PNP, with the dissociation constant being essentially the same as the competitive inhibition binding constant. A mechanism is proposed for the allosteric control of ATPase activity in heat-activated CF_1.

Experimental Section

Materials. The ATP and ADP were purchased from Sigma Chemical Co. The AMP-PNP was obtained from P. L. Biochemicals and purified on a Dowex 2X-8 column using a 0–1.0 M ammonium formate gradient. The [^{3}H]ADP (5–15 Ci/mmol) and [^{3}H]AMP (5–15 Ci/mmol) were purchased from New England Nuclear. The [^{3}H]AMP-PNP (5–8 Ci/mmol) was obtained from I.C.N. and all the radioactive nucleotides were purified using paper chromatography with the solvent system isobutyric acid–1N ammonia (100:60 v/v). Imidodiphosphate was purchased from Boehringer Mannheim. The NBD-Cl was obtained from Pierce Chemical Company and the [^{3}H]NBD-Cl (8 Ci/mol) was a gift from Dr. D. Deters. All other chemicals were the best available commercial grade, and all solutions were prepared with deionized distilled water.

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‡ National Science Foundation Predoctoral Fellow and National Institutes of Health Predoctoral Trainee (GM 00834).

[1] Abbreviations used are: CF_1, chloroplast coupling factor 1; F_1, mitochondrial coupling factor 1; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; AMP-PNP, adenylyl imidodiphosphate; ϵAMP, 1,N^6-ethenoadenosine monophosphate; ϵADP, 1,N^6-ethenoadenosine diphosphate; ϵAMP-PNP, 1,N^6-ethenoadenylyl imidodiphosphate.

Spinach CF_1 was prepared by known procedures (Lien and Racker, 1971). Fractions with a fluorescence ratio (305:340 nm emission; 280-nm excitation) greater than 1.5 were used. The enzyme was shown to be pure with five subunits of appropriate molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Nelson et al., 1973). Where indicated, the enzyme was heat activated as previously described (Lien and Racker, 1971). The ATPase activity of the heat-activated CF_1 was assayed using the pH-Stat technique. A specific activity of 10–12 μmol/(mg min) was obtained under the following conditions: 5 m*M* ATP, 5 m*M* $CaCl_2$, and *ca.* 6 μg/ml of CF_1, pH 8.0, 23°.

An extinction coefficient of 0.476 ml/(mg cm) at 280 nm (pH 8.0, 0.1 *M* NaCl, 50 m*M* Tris-Cl, and 2 m*M* $MgCl_2$ at 23°), based on a dry weight measurement, was used to determine protein concentrations of CF_1 solutions free of exchangeable nucleotides. A molecular weight of 325,000 (Farron, 1970) was used to determine molar concentrations of enzyme.

NBD Modification of CF_1. The CF_1 (10–20 μM) was incubated with NBD-Cl (400 μM) for 1.5 hr at 23°, pH 7.0 (50 m*M* NaH_2PO_4, 1 m*M* EDTA, and 5 m*M* ATP). After this time period, an equal volume of saturated ammonium sulfate was added; the solution then was cooled on ice and centrifuged at 18,000*g*, at 4° for 10 min. The pellet was warmed to room temperature and dissolved in a small volume of 0.025 *M* sucrose, 10 m*M* Tris sulfate (pH 8.0), and 2 m*M* EDTA, and eluted through a Sephadex G-25 (medium) column (1 cm i.d. × 50 cm) equilibrated with the same buffer. The effluent was monitored by passing it through a microflow cell (Helma Cell, Inc., Type 178-QS, 10-mm path length) in a Cary 14 recording spectrophotometer and measuring the absorbance at 280 nm. After the protein was collected, the effluent was monitored at 260 nm to check for nucleotides. The protein fraction then was precipitated with ammonium sulfate, centrifugated as above, and eluted through a second Sephadex G-25 (medium) column (0.7 cm i.d. × 25 cm) equilibrated with the buffer used in the binding experiment. This procedure gave an enzyme solution with an absorbance peak at 390–400 nm, as previously reported for NBD modified CF_1 (Deters et al., 1974) and F_1 (Ferguson et al., 1974). This absorbance peak is due to the reaction of NBD-Cl with tyrosine. Using [^{3}H]NBD-Cl to measure the moles of NBD present, a difference spectrum extinction coefficient of $10.7 \times 10^3\ M^{-1}\ cm^{-1}$ at 400 nm was determined for NBD covalently bound to CF_1. With this extinction coefficient, the number of moles of NBD reacted per mole of CF_1 under the above conditions varied between 1.0 and 1.6. The NBD-modified CF_1 was always used within a few hours after preparation.

Preparation of [^{3}H]ϵAMP, [^{3}H]ϵADP, and [^{3}H]ϵAMP-PNP. The [^{3}H]ϵAMP and [^{3}H]ϵADP were prepared from labeled AMP and ADP by the method of Secrist et al. (1972). The final product was lyophilized and purified by paper chromatography with the solvent system 1-propanol–ammonia–water (6:3:1 v/v). The [^{3}H]ϵAMP-PNP was prepared from [^{3}H]ϵAMP by the method of Yount et al. (1971) on a reduced scale. The yield was approximately 20% starting with 1 μmol of [^{3}H]ϵAMP. The major impurity was [^{3}H]ϵAMP (70%). The final product was purified by paper chromatography using the solvent system 1-propanol–ammonia–water (6:3:1 v/v). In this system, ϵAMP has an R_f of 0.30, ϵAMP-PNP an R_f of 0.14, AMP an R_f of 0.26, and AMP-PNP an R_f of 0.10. The ϵAMP-PNP had an ultraviolet absorption spectrum and fluorescence spectrum identical with that of ϵADP. An extinction coefficient of $5.6 \times 10^3\ M^{-1}\ cm^{-1}$ at pH 7.0, 275 nm was used to determine the concentration of the fluorescent nucleotides (Secrist et al., 1972).

Fluorescence Measurements. Fluorescence quenching measurements involving ϵADP and ϵAMP-PNP were made with a Perkin-Elmer Model MPF-3 fluorescence spectrophotometer equipped with a cell holder thermostated at 23°. A square fluorescence microcell (light path 0.3 cm × 0.3 cm) was used to decrease inner filter effects. The fluorescence intensity was measured at 400 nm using an excitation wavelength of 320 nm. A typical fluorescence quenching measurement was made as follows: 200 μl of a concentrated CF_1 solution (~20 μM) was placed in a fluorescence microcell and a suitable amount of ϵADP or ϵAMP-PNP (5–10 μl) was added. The solution was stirred by repeated extraction with a Pasteur pipet, and the slits and sensitivity were adjusted to give full scale deflection. The change in fluorescence with time was measured, with the excitation shutter being closed between measurements.

Binding Measurements. All binding measurements were done at 23° using the forced dialysis technique (Cantley and Hammes, 1973). Solutions of varying ligand and enzyme concentration were mixed in volumes of 0.15–0.25 ml. Because of a slow ADP turnover to AMP catalyzed by CF_1 preparations (Roy and Moudrianakis, 1971), it was not possible to incubate ADP or ϵADP with CF_1 for long periods of time. Thus, in direct binding measurements involving these ligands, the solutions were incubated for 30 min. This procedure gave reproducible data with significant ADP hydrolysis occurring only at very low ADP concentrations. In direct binding measurements involving AMP-PNP or ϵAMP-PNP, the solutions were allowed to incubate for 2 hr. As judged by paper chromatography of the incubation mixture in 1-propanol–ammonia–water (6:3:1 v/v), no decomposition of the nucleotide occurred during this time period. In competitive binding experiments, the unlabeled inhibitor was added to the enzyme solution shortly (<5 min) before adding the radioactive ligand. The error in determination of the total or free ligand concentration in the binding experiments is about ±3%. Control experiments showed that the XM 50 membranes used retained all of the CF_1 and did not bind the ligands studied. The radioactivity was measured in 10 ml of scintillation fluid (Bray, 1960) using a Beckman LS-255 liquid scintillation counter. The enzyme used in the binding experiments was passed through two Sephadex columns as described above within 2 hr before the binding experiment. The second Sephadex column was equilibrated with the buffer used in the binding experiments.

Steady-State Kinetics. Initial velocities were measured with the pH-Stat technique in 0.1 *M* NaCl, pH 8.0 and 23°. When the ATP plus inhibitor concentration was less than 5 m*M*, the $CaCl_2$ concentration was maintained at 5 m*M*; in all other cases, the $CaCl_2$ concentration was equal to the sum of the ATP and inhibitor concentrations. Some of the initial velocity measurements were complicated by an apparent slow association and dissociation of the inhibitor AMP-PNP with CF_1. This phenomena also has been observed with mitochondrial ATPase, F_1 (Penefsky, 1974). In order to reach an apparent steady state without significant turnover of ATP, the enzyme was incubated in the appropriate concentration of AMP-PNP for at least 1 hr before adding it to the assay mixture. This procedure resulted in a slowly increasing velocity which reached apparent linearity

after about 10 min. During this period of time, less than 5% of the ATP was hydrolyzed. The ADP inhibition was measured in an analogous manner. Before measuring ATPase activities, the CF_1 was heat activated. An equal volume of saturated ammonium sulfate was added to the heat-activated enzyme; this suspension was cooled on ice and centrifugated at 4° for 10 min at 18,000*g*. The protein then was passed through two Sephadex columns as described above; the second column was equilibrated with 20 m*M* Tris sulfate and 5 m*M* Ca^{2+} (pH 8.0). This procedure resulted in a 50–60% loss of activity. However, the specific activity gradually increased to the original specific activity after a period of about 24 hr.

Results

Before presenting the binding isotherms for nucleotide–CF_1 interactions several difficulties in obtaining the equilibrium binding isotherms should be considered: the reproducibility and extent of nucleotide removal by the two Sephadex columns used to treat the enzyme, the rate of equibration of nucleotides and CF_1, and the decomposition of ADP catalyzed by the enzyme.

If the latent enzyme (~30 μM) is incubated with [^{3}H]ADP (~200 μM) for several hours, precipitated with ammonium sulfate, and passed through the two Sephadex G-25 columns as described in the Experimental Section, less than 0.1 mol of labeled nucleotide/mol of enzyme is found. If heat-activated enzyme (equilibrated with 40 m*M* ATP before ammonium sulfate precipitation) is similarly passed through a Sephadex column, a separation between enzyme and released adenine nucleotide can be seen by monitoring the column effluent at 280 and 260 nm. The released adenine nucleotides (closely following the protein) consist of ATP and ADP as judged by thin-layer chromatography in 95% ethanol–1 *M* ammonium acetate (pH 7.0) (70:30 v/v) and the ratio of released adenine nucleotide to enzyme varies between 3 and 5 (mol to mol) as measured by the area under the effluent absorbance curve. Similar experiments with latent enzyme showed no clear separation between released adenine nucleotides and protein. These results suggest the presence of tightly bound adenine nucleotides on the enzyme which are not completely released by the Sephadex treatment of the latent enzyme, but are more readily released by the heat-activated enzyme. Because of the reproducible stoichiometry in forced dialysis experiments (±10%) with six different enzyme preparations, it can be concluded that the passage of the enzyme through two Sephadex columns reproducibly removes essentially all of the "exchangeable" nucleotides.

The rate of association of the fluorescent nucleotides ϵADP and ϵAMP-PNP with the enzyme was measured by following the fluorescence quenching as described in the Experimental Section. The pseudo-first-order rate constants (i.e., first order in fluorescent nucleotide) obtained were as follows: 0.16 min^{-1} for ϵADP reacting with 16.6 μM latent enzyme, 0.18 min^{-1} for ϵADP reacting with 4.6 μM heat activated enzyme, and ~0.02 min^{-1} for ϵAMP-PNP reacting with 20 μM latent enzyme. If the nonfluorescent nucleotides equilibrate at similar rates, the incubation time of 30 min and 2 hr used for the enzyme with ADP or ϵADP and AMP-PNP or ϵAMP-PNP, respectively, should be sufficient for essentially complete equilibration at all but the lowest ligand concentrations.

As previously reported (Roy and Moudrianakis, 1971), ADP is slowly converted to AMP by the enzyme. We have estimated the turnover number to be less than 15×10^{-6} μmol/(mg min) by measurement of the amount of [^{3}H]ADP and [^{3}H]AMP by paper chromatography in 95% ethanol–1 *M* ammonium acetate (pH 7.0) (70:30 v/v) after 30-min incubation of [^{3}H]ADP with enzyme. The decomposition of ADP has an appreciable effect on the equilibrium binding experiments only at very low ligand concentrations. A tightly bound ATP molecule also has been postulated to be formed from bound ADP simultaneously with AMP. The turnover number indicates a maximum of 15% of the enzyme would contain bound ATP after the incubation period used in binding experiments. Moreover the following results indicate the ADP decomposition is not seriously altering the results of the binding experiments: very similar binding stoichiometry and binding constants are observed with all nucleotides; the same results are obtained with incubation times of up to 1.5 hr, except at low ADP concentrations, and the same results are obtained if the enzyme is recycled after a binding experiment by passing it through two Sephadex columns, indicating that very tightly bound nucleotides are not present.

The above considerations indicate that the binding experiments can be interpreted as *equilibrium* experiments. The binding isotherms for the interaction of the enzyme with AMP-PNP, ϵAMP-PNP, ADP, and ϵADP were determined for the latent and heat-activated enzyme in the presence of both Ca^{2+} and Mg^{2+} and for the enzyme modified with NBD. The NBD-modified enzyme contained 1.0–1.6 mol of NBD/mol of enzyme. In agreement with other work (Ferguson et al., 1974; Deters et al., 1974), we have found that 1 mol of NBD bound to the enzyme is sufficient to eliminate essentially all of the ATPase activity. An analysis of the time course of the reaction of [^{3}H]NBD-Cl with CF_1 at pH 7.0 (23°) indicates the kinetics can be quantitatively described by a mechanism in which 1 mol of NBD-Cl reacts with a rate constant of 50 M^{-1} min^{-1} and a second mole reacts with a rate constant of 6 M^{-1} min^{-1}.

In Figure 1A a plot of $r/(L)$ vs. r is presented for the binding of AMP-PNP to latent CF_1 in the presence of 2 m*M* Mg^{2+} and for the binding of AMP-PNP to the latent NBD-modified enzyme (1.6 mol/mol) in the presence of 2 m*M* Mg^{2+}. In this figure r is the number of moles of ligand bound per mole of enzyme and (L) is the concentration of free ligand. These experiments were carried out at pH 8.0, in 2 m*M* $MgCl_2$, 50 m*M* Tris-Cl, and 0.1 *M* NaCl. In both cases the binding isotherms can be quantitatively described by eq 1, which assumes n_1 equivalent ligand binding sites

$$r/(L) = n_1/[(L) + K_1] \quad (1)$$

per mole of enzyme. The data were fit to eq 1 by least-squares analysis, and the dissociation constants, K_1, and the values of n_1 (to the nearest integer) obtained are summarized in Table I. The lines in Figure 1A were calculated with the parameters in Table I and eq 1. A binding isotherm for the binding of AMP-PNP to the latent enzyme in the presence of 39 μM ADP also is presented in Figure 1A. The extent of AMP-PNP binding is markedly reduced by ADP, and if the two ligands are assumed to compete for the same site, the binding isotherm can be represented by eq 2.

$$r/(L) = n_1/((L) + K_1[1 + (I)/K_I]) \quad (2)$$

The ADP competitive inhibition constant, K_I, obtained from a least-squares analysis of the data is included in Table I. In Figure 1B, a plot of $r/(L)$ vs. (L) is presented for the binding of ϵAMP-PNP to the latent enzyme in the pres-

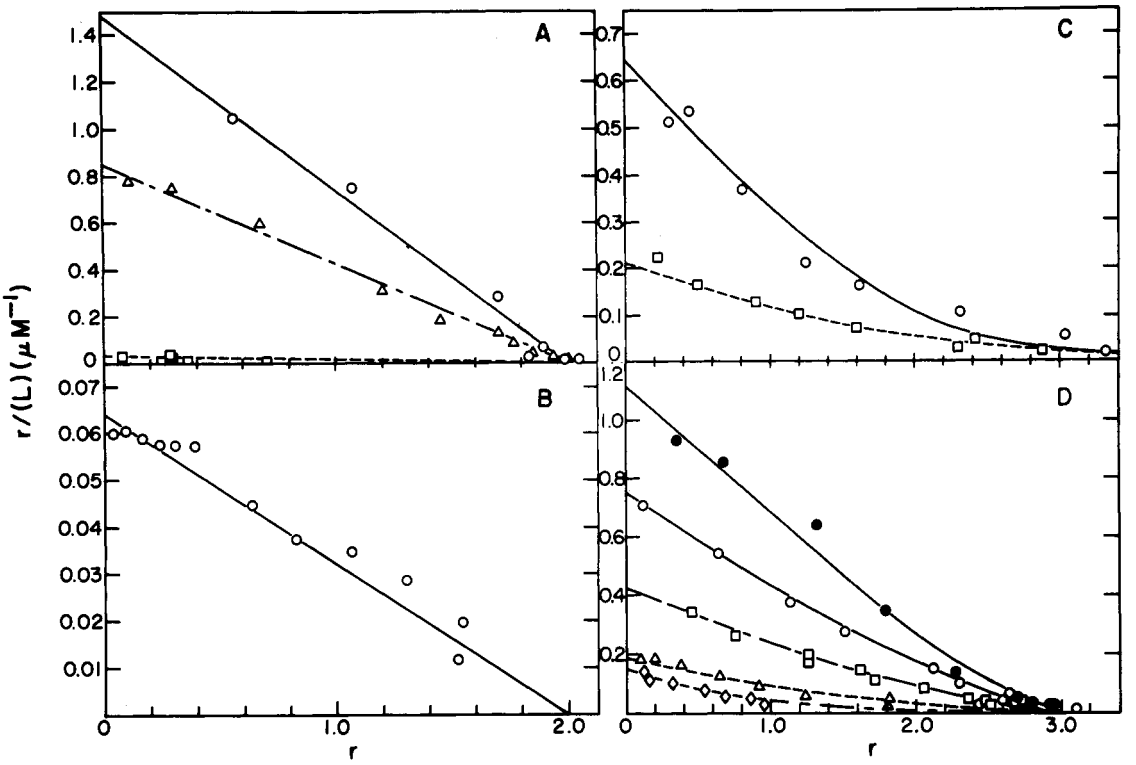


FIGURE 1: Plots of $r/(L)$ vs. r, where r is the number of moles of ligand bound per mole of enzyme and (L) is the concentration of free ligand. (A) The binding of AMP-PNP to latent CF_1 (○), NBD-modified CF_1 (1.60 mol/mol) (△), and latent CF_1 in the presence of 39 μM ADP (□) in 0.1 M NaCl, 50 mM Tris-Cl, and 2 mM Mg^{2+} (pH 8.0), 23°. The curves were calculated with eq 1 and 2 and the parameters in Table I. (B) The binding of ϵAMP-PNP to latent CF_1 under the same experimental conditions as in A. The curve was calculated with eq 1 and the parameters in Table I. (C) The binding of AMP-PNP to latent CF_1 in 5 mM Ca^{2+} and 20 mM Tris-Cl (pH 8.0), 23°; (○) no ADP; (□) 35 μM ADP. The curves were calculated using eq 4 and the parameters in Table I. (D) The binding of AMP-PNP to heat-activated CF_1 in 0.1 M NaCl, 50 mM Tris-Cl, and 5 mM Ca^{2+} (pH 8.0), 23° (●) and in 5 mM Ca^{2+} and 20 mM Tris-Cl (pH 8.0), 23°; (○) no ADP; (□) 25 μM ADP; (△) 91 μM ADP; (◇) 189 μM ADP. The curves were calculated using eq 4 and the parameters in Table I. The enzyme concentration varied from 2 to 10 mg/ml and the total ligand concentrations varied from 1 to 150 μM.

ence of 2 mM Mg^{2+}. The data were fit to eq 1 by a least-squares analysis and the values of K_1 and n_1 (to the nearest integer) are summarized in Table I. Again, two apparently identical sites are observed although the dissociation constant is higher than for AMP-PNP binding. In Figure 1C plots of $r/(L)$ vs. (L) are shown for the binding of AMP-PNP to the latent enzyme in the presence of 5 mM Ca^{2+}–20 mM Tris-Cl (pH 8.0), 23°. Two tight binding sites are present as with Mg^{2+}, but some weaker binding also occurs. These binding isotherms can be interpreted in terms of two mathematically equivalent, but conceptually different, models: interacting binding sites or sets of independent non-interacting binding sites. The second model is arbitrarily utilized. The weak binding is poorly defined in these experiments since the maximum concentration of CF_1 attainable is about 40 μM. Therefore, a more comprehensive analysis was not carried out. The data in the absence of ADP were fit to eq 3, which assumes two different classes of sites, by a

$$\frac{r}{(L)} = \frac{n_1}{[(L) + K_1]} + \frac{n_2}{[(L) + K_2]} \quad (3)$$

least-squares analysis. The values of n_1 and n_2, the number of each type of site, were assumed to be integers. The best fit values of the ligand dissociation constants are $K_1 = 3.26$ μM, and $K_2 = 121$ μM, and the values of n_1 and n_2 are 2 and 3, respectively. In Figure 1D, plots of $r/(L)$ vs. (L) are presented for the binding of AMP-PNP to the heat-activated enzyme in the presence of 5 mM Ca^{2+} and varying concentrations of ADP. The data indicate three binding sites with a similar affinity for AMP-PNP. The data in the presence of 0.1 M NaCl, 50 mM Tris-Cl, and 5 mM Ca^{2+} (pH 8.0) were fit to eq 3 and the values of n_i and K_i are summarized in Table I. High concentrations of ADP (up to 189 μM) markedly reduce binding to two of the sites but have little effect on binding to the other site. Since two tight ligand binding sites are found in all of the analyses and since ADP appears to be competitive with only two of the sites in the concentration range studied, it was assumed that in the presence of ADP the ligand binding isotherms could be described by eq 4, which assumes two binding sites can bind

$$r/(L) = 2/((L) + K_1[1 + (I)/K_I]) + n_2/[(L) + K_2] \quad (4)$$

both ligands and n_2 additional sites can bind AMP-PNP. The sets of data in Figures 1C and D measured in the presence of 20 mM Tris-Cl–5 mM Ca^{2+} (pH 8.0) each were fit simultaneously to eq 4 by a least-squares analysis. Since the concentration of the inhibitor ADP was not directly determined, it was calculated by successive approximations. The concentration of inhibitor, (I), is given in eq 5 in terms of

$$(I) = 0.5\{(I_0) - 2(E_0) - K_I(L)/K_1 - K_I + \sqrt{[K_I + K_I(L)/K_1 + 2(E_0) - (I_0)]^2 + 4(I_0)K_I[1 + (L)/K_1]}\} \quad (5)$$

the total enzyme concentration (E_0), the total inhibitor concentration, (I_0), the free ligand concentration (L), and the dissociation constants K_1 and K_I. The values of K_1, K_2, n_2, and K_I for the data in Figures 1C and D are given in Table I and the curves have been calculated with the parameters

Table 1: Parameters for Binding of Nucleotides to CF_1.[a]

Nucleotide	Enzyme	Metal Ion	"Tight" Sites K (μM) (n)	"Weak" Sites K (μM) (n)	"Catalytic" Sites K (μM) (n)	Method
ADP	Latent	Mg^{2+}, Na^+	1.82 (2)	100 (1)		Direct binding
			0.86 (2)			Competitive binding with AMP-PNP
	Heat activated	Mg^{2+}, Na^+	3.75 (2)	76 (3)		Direct binding
	NBD latent (1.07 mol/mol)	Mg^{2+}, Na^+	2.55 (1), 18 (1)			Direct binding
	NBD heat activated (1.25 mol/mol)	Mg^{2+}, Na^+	2.8 (1), 21 (1)			Direct binding
	Latent	Ca^{2+}	13.2 (2)			Competitive binding with AMP-PNP
	Heat activated	Ca^{2+}	9.6 (2)	125 (3)		Direct binding
			10.0 (2)			Competitive binding with AMP-PNP
		Ca^{2+}, Na^+	1.63 (2)	70 (3)		Direct binding
ϵADP	Latent	Mg^{2+}, Na^+	1.8 (1.9)			Direct binding
	NBD latent (1.15 mol/mol)	Mg^{2+}, Na^+	10.1 (1), 71 (1)			Direct binding
AMP-PNP	Latent	Mg^{2+}, Na^+	1.35 (2)			Direct binding
			1.22 (2)			Competitive binding with ADP
	NBD latent (1.6 mol/mol)	Mg^{2+}, Na^+	2.34 (2)			Direct binding
	Latent	Ca^{2+}	3.26 (2)	185 (3)		Direct binding
	Heat activated	Ca^{2+}	3.4 (2)		7.6 (0.9)	Direct binding
		Ca^{2+}, Na^+	2.0 (2)		7.6 (1)	Direct binding
					4	Steady-state kinetics
	NBD heat activated (0.6 mol/mol)	Ca^{2+}	7.0 (2)		7.0 (0.5)	Direct binding
ϵAMP-PNP	Latent	Mg^{2+}, Na^+	31 (2)			Direct binding
ATP	Heat activated	Ca^{2+}, Na^+	<6.5 (2)		K_m = 1.3 mM	Steady-state kinetics

[a] The buffers used were as follows: Mg^{2+}, Na^+, 0.1 M NaCl, 50 mM Tris-Cl and 2 mM $MgCl_2$ (pH 8.0), 23°; Ca^{2+}, Na^+, 0.1 M NaCl, 50 mM Tris-Cl, and 5 mM Ca^{2+} (pH 8.0), 23°; Ca^{2+}, 20 mM Tris-Cl and 5 mM Ca^{2+} (pH 8.0), 23°. The steady-state kinetics measurements were made in the absence of Tris-Cl as described in the Experimental Section.

in Table I and eq 4.

The binding isotherms for the interaction of ADP and ϵADP with latent CF_1, heat-activated CF_1, and NBD-modified CF_1 are summarized in Figure 2. As noted above the data at low ADP concentrations may be somewhat in error due to the breakdown of ADP to AMP. In Figure 2A, the presence of two tight ADP binding sites and some weaker binding sites can be seen. These data were analyzed according to eq 3, and the binding data in the presence of varying AMP-PNP concentrations (up to 300 μM) were analyzed according to eq 4 as described above. The binding of ADP to the heat-activated enzyme in both the presence of Mg^{2+} and Ca^{2+} (with or without 0.1 M NaCl) (Figure 2B) indicates the presence of at least two different sites, and the data were fit to eq 3 by a least-squares analysis. The binding isotherms for the interaction of ADP with both latent and heat-activated NBD-modified enzyme (Figure 2C) indicate two binding sites, and the data were fit with eq 3. (If two interacting sites are assumed, the binding constants obtained are $K_1K_2/(K_1 + K_2)$and $(K_1 + K_2)$.) The heat-activated NBD-modified enzyme was prepared by first heat activating, and then reacting the enzyme with NBD-Cl as described in the Experimental Section. Under these conditions, NBD-Cl reacts with a sulfhydryl group, as judged by an absorption peak at 420 nm, in addition to the tyrosine group. In an experiment not shown, the NBD-modified latent enzyme was heat activated in the absence of dithiothreitol. The heat activation removed part of the NBD leaving 0.6 mol of NBD/mol of enzyme. The activity was 60% inhibited and could be restored by adding β-mercaptoethanol. A plot of $r/(L)$ vs. r for AMP-PNP binding to this modified enzyme in the presence of Ca^{2+} gave a straight line saturating at 2.5 sites with an intrinsic dissociation constant of 7.0 μM. Figure 2D represents the binding isotherms for the interaction of ϵADP with the latent enzyme and NBD-modified latent enzyme. For the latent enzyme, the data were fit to eq 1, while for the modified enzyme, the data were fit to eq 3. The constants obtained from the least-squares analyses are summarized in Table I, and the curves in Figure 2 have been calculated with these constants and eq 1, 3, and 4.

In the summary of the binding data in Table I, all of the binding sites have been assigned to one of three types of sites: the "tight" sites are two apparently identical sites (except in the case of NBD-modified enzyme where some heterogeneity appears) which bind all of the nucleotides studied tightly (dissociation constant generally less than 10 μM) and competitively. The "catalytic" site is an additional site which appears only in the heat-activated enzyme and only binds AMP-PNP tightly. It is so named because its dissociation constant is near the competitive inhibition constant for AMP-PNP inhibition of the Ca^{2+}-ATPase activity (see below) and because binding to this site is inhibited in heat-activated NBD-modified CF_1. All additional sites have been grouped together under the title "weak" sites. The uncertainty in the binding constants associated with the tight sites is ±20%, while that associated with the binding constants greater than 100 μM is ±40%. The uncertainty in n_1 is ±10% and in n_2 ±30%. With the NBD-modified enzyme, the uncertainty in n_2 is ±10% when ADP and ϵADP are the

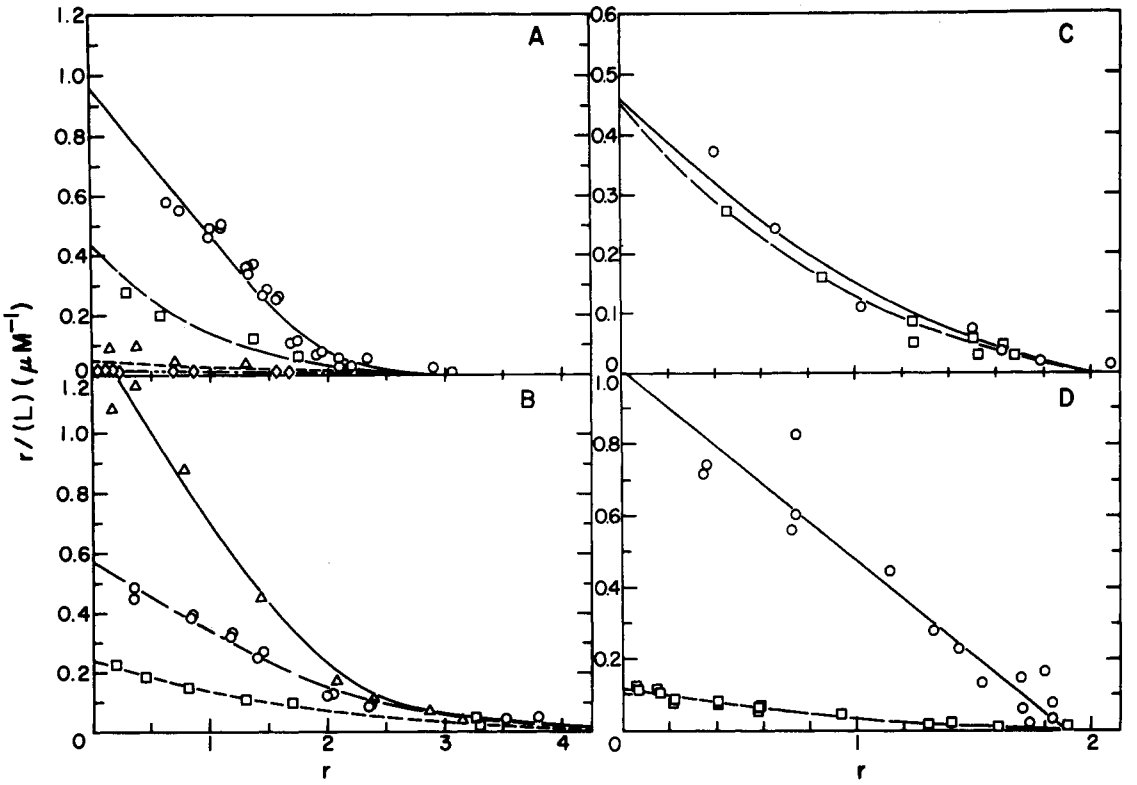


FIGURE 2: Plots of $r/(L)$ vs. r where r is the number of moles of ligand bound per mole of enzyme and (L) is the free ligand concentration. (A) ADP binding to latent CF_1 in 0.1 *M* NaCl, 50 m*M* Tris-Cl, and 2 m*M* Mg^{2+} (pH 8.0), 23°; (○) no AMP-PNP; (□) 9.8 μM AMP-PNP; (△) 49 μM AMP-PNP; (◇) 300 μM AMP-PNP. The curves were calculated using eq 4 and the parameters in Table I. (B) ADP binding to heat-activated CF_1 in 0.1 *M* NaCl, 50 m*M* Tris-Cl, and 5 m*M* Ca^{2+} (pH 8.0), 23° (△); in 0.1 *M* NaCl, 50 m*M* Tris-Cl, and 2 m*M* Mg^{2+} (pH 8.0), 23° (○), and in 5 m*M* Ca^{2+} and 20 m*M* Tris-Cl (pH 8.0), 23° (□). The curves were calculated using eq 3 and the parameters in Table I. (C) ADP binding to NBD-modified latent CF_1 (1.07 mol/mol) (○), and NBD-modified heat-activated CF_1 (1.25 mol/mol) (□). Measurements were made in 0.1 *M* NaCl, 50 m*M* Tris-Cl, and 2 m*M* Mg^{2+} (pH 8.0), 23°. The solid curve is the fit to the data for the latent enzyme and the dashed curve is the fit to the data for the heat-activated enzyme. The curves were calculated using eq 3 and the parameters in Table I. (D) ϵADP binding to latent CF_1 (○), and NBD-modified CF_1 (1.15 mol/mol) (□) in 0.1 *M* NaCl, 50 m*M* Tris-Cl, and 2 m*M* Mg^{2+} at pH 8.0, 23°. The curves were calculated using eq 1 and 3 and the parameters in Table I. The enzyme concentration varied from 2 to 15 mg/ml and the total ligand concentrations varied from 1 to 400 μM.

ligands.

The nucleotide sites of the enzyme were further characterized by investigating the effect of AMP-PNP and ADP on the steady state initial velocity of the Ca^{2+}-ATPase activity of the heat-activated enzyme. As shown in Figure 3, the AMP-PNP was found to be a competitive inhibitor of the ATPase activity over an ATP concentration range of 0.23–7 m*M* and an AMP-PNP concentration range of 0–38.4 μM at pH 8.0, 0.1 *M* NaCl and 5.0–10.0 m*M* Ca^{2+}. The inhibition constant was found to be 4 μM by a weighted least-squares analysis of plots of the reciprocal initial velocity vs. the reciprocal ATP concentration at varying concentrations of AMP-PNP. The turnover number of the enzyme is increased by approximately a factor of 2 if the NaCl is removed from the assay mixture, although the Michaelis and inhibition constant are not significantly altered by this change in NaCl concentration.

The effect of ADP on the steady-state initial velocity is complex. Plots of the steady-state initial velocity vs. the ATP concentration at varying ADP concentrations are presented in Figure 4. The ADP is clearly not a simple competitive inhibitor at the concentrations utilized but inhibits the enzyme to a considerable extent. A similar inhibition pattern has been previously reported (Nelson et al., 1972; Datta et al., 1974). A simple mechanism consistent with the data is as follows: the binding of ADP to either one or both of the two tight sites switches the enzyme to a state of little or no activity, while the binding of ATP to the two tight sites switches the enzyme to an active conformation. As shown in Figure 4, very high concentrations of ATP inhibit the ATPase activity; the proposed mechnism does not include an explanation of this observation. A detailed analysis of the proposed mechanism is given in the Appendix, and the data were fit to eq A1 by a least-squares analysis. In order to reduce the number of parameters, the ADP dissociation constant at the tight sites was assumed to be 1.63 μM, as calculated from the binding data. The following parameters were obtained from the least-squares analysis: the ATP Michaelis constant (K_m) and turnover number (k_s) in the active state are 1.3 m*M* (±5%) and 4.5 μmol/(mg min) (±5%), the corresponding parameters for the less active state (K_m' and k_s') are 0.82 m*M* (±10%) and 0.003 μmol/(mg min) (±200%) and the ATP dissociation constant for the control site is 6.5 μM (±20%). The curves in Figure 4 have been calculated with the above parameters and eq A1, and they span the range of ATP concentrations used in the least-squares analysis.

The above assays were done on heat-activated enzyme which had been stripped of exchangeable nucleotides and then incubated with the appropriate concentration of inhibitor before the asssay. In view of the slow rate at which AMP-PNP and ϵAMP-PNP bind to the control site (approximately 2 hr to reach equilibrium), and the observed lag of approximately 10 min before the initial velocities

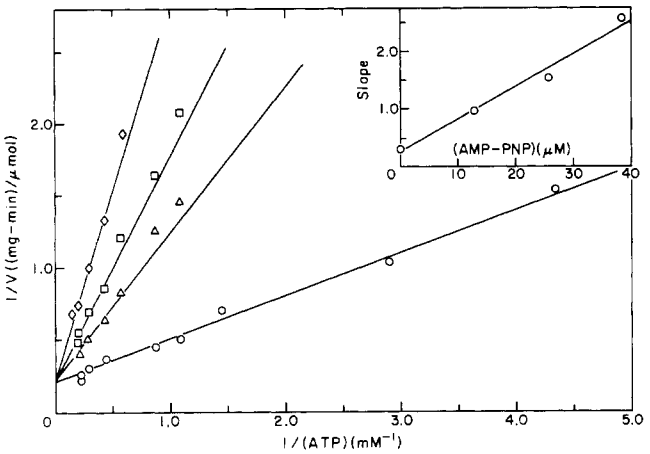


FIGURE 3: A plot of the reciprocal steady state initial velocity vs. the reciprocal ATP concentration at several concentrations of AMP-PNP (pH 8.0). The AMP-PNP concentrations were 0 (○), 12.8 μM (△), 25.6 μM (□), and 38.4 μM (◇). The assays were carried out with heat activated CF_1 in the presence of 0.1 M NaCl and Ca^{2+} at 23° as described in the Experimental Section. The ATP concentrations were varied from 0.23 to 7 mM. The inset is a plot of the slopes from a weighted least-squares fit of the individual lines vs. the AMP-PNP concentration.

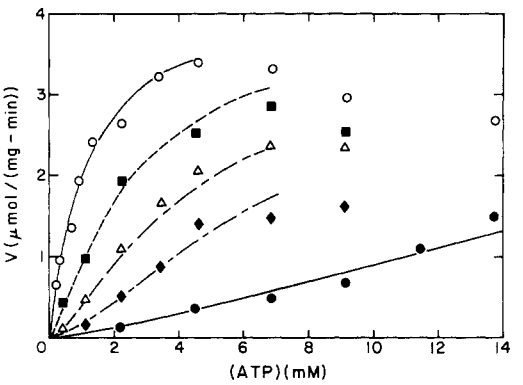


FIGURE 4: A plot of the steady state initial velocity vs. the ATP concentration at several concentrations of ADP (pH 8.0). The ADP concentrations were 0 (○), 115 μM (■), 344 μM (△), 687 μM (♦), and 2360 μM (●). The assays were carried out with the heat-activated CF_1 in the presence of 0.1 M NaCl and Ca^{2+} at 23° as described in the Experimental Section. The curves were calculated using eq A1 and the parameters K_m = 1.3 mM, k_s = 4.5 μmol/(mg min), K_m' = 0.82 mM, k_s' = 0.003 μmol/(mg min), K_I = 6.5 μM, and K_I = 1.63 μM. The curves span the range of ATP concentrations used in the least-squares analysis.

reach a constant value, it seems probable that ATP has not equilibrated at the control site during the course of the assay (10–15 min). Thus, the actual dissociation constant characterizing the interaction of ATP with the control site is probably lower than the apparent constant of 6.5 μM obtained from the fitting of the data. Experiments in which the nucleotides are not removed before measuring the ADP inhibition also suggest a lower ATP dissociation constant for the control sites (Datta et al., 1974).

Discussion

The results summarized in Table I indicate two "tight" nucleotide binding sites on both latent and heat-activated CF_1 which bind AMP-PNP, ϵAMP-PNP, ADP, and ϵADP The binding strength is similar in the presence of Mg^{2+} and Ca^{2+} and is stronger in 0.1 M NaCl than in 20 mM Tris-Cl. Binding to these sites is essentially the same with both the latent and heat-activated proteins. The slow association of ADP and ϵADP with the enzyme rules out any possibility that the "tight" sites are active sites for the ATPase activity. However, a third tight AMP-PNP binding site is found with the heat-activated enzyme. The equilibrium dissociation constant associated with this site is 7.6 μM, and the competitive inhibition constant determined under conditions similar to those used in the binding experiments is 4 μM. Thus, this site is very probably the ATPase active site. Under these same conditions ADP is a very weak competitive inhibitor of the ATPase activity and binds only weakly to a third site ($K > 70$ μM). A single NBD-Cl reacted per mole of CF_1 is sufficient to prevent both AMP-PNP and ADP binding to this third site and removes all ATPase activity (Deters et al., 1974), thus supporting the assignment of this site as the ATPase active site.

Two nonidentical ADP sites (half-saturation at 2 and 35 μM) have been found on CF_1 which exhibit a myokinase activity (Roy and Moudrianakis, 1971). We have observed a similar very slow breakdown of ADP which is much too slow to be of catalytic importance in the solubilized enzyme, although the rate might be quite different in the membrane bound system. Using circular dichroism, two tight ADP binding sites on CF_1 with dissociation constants of 1.25 and 2.5 μM were found (Girault et al., 1973). Binding experiments of the interaction between ADP and mitochondrial ATPase, F_1, have been analyzed in terms of two ADP sites with dissociation constants of 0.28 and 47 μM (Hilborn and Hammes, 1973) based on a protein molecular weight of 285,000 (Forrest and Edelstein, 1970; Penefsky and Warner, 1965). If a molecular weight of 360,000 is assumed (Lambeth et al., 1971), the data also could be fit to two tight ADP binding sites and an additional weak site, although the postulation of two tight sites is not required by the data.

The steady-state kinetics can be quantitatively analyzed in terms of a model in which the tight sites serve as an allosteric conformational switch for the ATPase activity of the solubilized CF_1. However, the function of the tight sites in intact chloroplasts is not known, and this model should only be viewed as a working hypothesis. In an accompanying paper (Cantley and Hammes, 1975), we present evidence indicating the tight sites are quite far from the active site, ~40 Å. Although ϵADP and ϵAMP-PNP associate slowly with both CF_1 and F_1 (Tondre and Hammes, 1973) and both proteins have "tight" sites, an allosteric effect of ADP on the ATPase activity of solubilized F_1 is not observed (Hammes and Hilborn, 1971; Tondre and Hammes, 1973).

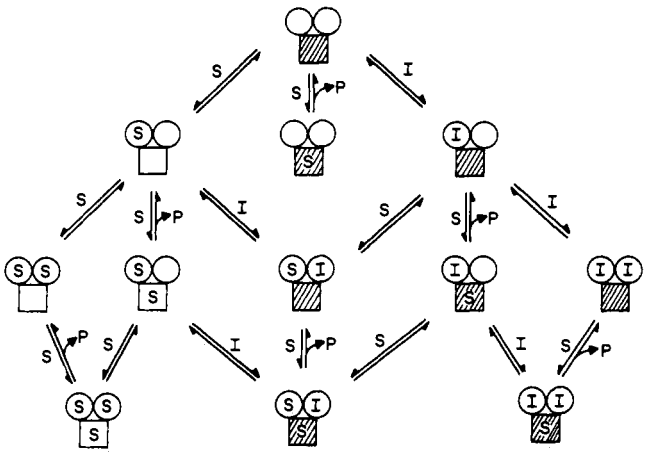
Because of the complexity in structure and function of both the mitochondrial and chloroplast proteins, F_1 and CF_1, and the important role the membrane obviously plays in both phosphorylation and photophosphorylation, it is very tenuous to infer in vivo functions from studies with the solubilized enzymes. However, since AMP-PNP inhibits ATP hydrolysis in both F_1 and submitochondrial particles (Penefsky, 1974), it is likely that the ATPase active site of the soluble and intact enzymes is the same. The fact that AMP-PNP does not inhibit ATP synthesis by mitochondria led to the suggestion of different sites for ATP synthesis and ATP hydrolysis (Penefsky, 1974). This finding is not consistent with our results with soluble CF_1 in which AMP-PNP is a potent inhibitor for all ADP sites. However, synthesis and hydrolysis experiments are carried out under quite different experimental conditions. Furthermore, the energized states of F_1 or CF_1 on the membrane might ex-

hibit drastically altered nucleotide binding and turnover rates.

Appendix: Mechanism for the Effect of ADP on the ATPase Activity of CF_1

The proposed mechanism is shown below, where the circles represent control sites and the squares the active site.

Chart I



The hatched squares represent the state of low catalytic activity and the open squares represent the state of high catalytic activity. If all the control sites are assumed to be in equilibrium with ATP and ADP, while the species with an active site occupied is assumed to be in a steady state, the initial velocity, v, divided by the total enzyme concentration, (E_0), is given by eq A1. In this equation K_m and k_s are

$$\frac{v}{(E_0)} = \frac{k_s}{1 + [K_m/(S)][C_1 + C_2(S)/K_m']/C_3} + \frac{k_s'}{1 + [K_m'/(S)][C_1 + C_3(S)/K_m]/C_2} \quad \text{(A1)}$$

$$C_1 = (1 + (S)/K_1 + (I)/K_I)^2$$

$$C_2 = 1 + 2(I)/K_I + 2(I)(S)/K_1K_I + (I)^2/K_I^2$$

$$C_3 = 2(S)/K_1 + (S)^2/K_1^2$$

the Michaelis constant and turnover number for the high activity state, K_m' and k_s' are the corresponding parameters for the low activity state, K_1 is the intrinsic dissociation constant for ATP binding to the control site, K_I is the corresponding constant for ADP binding, S is ATP, and I is ADP.

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