Nucleotide Interactions with the Dicyclohexylcarbodiimide-Sensitive Adenosinetriphosphatase from Spinach Chloroplasts†

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ABSTRACT: The intrinsic nucleotide content of the dicyclohexylcarbodiimide-sensitive ATPase (DSA) from spinach chloroplasts and its interactions with ADP have been studied. Both partially purified and sucrose gradient purified DSA contain at least 1 mol of ADP/mol of enzyme and 1 mol of ATP/mol of enzyme, although considerable variation exists between different preparations. Radioactively labeled ADP is incorporated into DSA in the presence of 5 mM $MgCl_2$ and 10 mM octyl glucoside with a half-life of ~30 min. Incorporation of ADP into DSA reconstituted in phospholipid vesicles occurs at about twice this rate, and a slightly slower rate of uptake is observed with [^{3}H]ADP and [^{3}H]ATP in the presence of 2 mM ethylenediaminetetraacetic acid. The [^{3}H]ATP always appears as bound [^{3}H]ADP on the enzyme. Nucleotide analyses indicate that this incorporation represents an exchange with tightly bound ADP. The nucleotide exchange requires binding at another nucleotide site or sites on the enzyme and is essentially a one-turnover process. Even during ATP synthesis <20% of incorporated ^{3}H-labeled nucleotide is removed. Binding studies with forced dialysis indicate the presence of a reversible binding site for ADP distinct from the nucleotide exchange. Similar binding isotherms are obtained for the partially purified enzyme stabilized with 10 mM octyl glucoside, the gradient-purified enzyme stabilized with 0.4% sodium cholate, and the reconstituted, partially purified enzyme. The binding stoichiometry is ~0.5 mol of ADP/mol of DSA and the dissociation constant is ~2 μM, which is similar to the Michaelis constant for ADP estimated from kinetic studies of ATP synthesis.

The ATP-synthesizing complex (DSA)[1] from spinach chloroplasts is composed of a water-soluble portion, CF_1, made up of five different polypeptides probably in the ratio 2:2:1:1:2 (Baird & Hammes, 1976; Binder et al., 1978) and a membrane component, CF_0, which may be comprised of as many as four types of polypeptides with apparent molecular weights of 17 500, 15 500, 13 500, and 8000 (Pick & Racker, 1979). The soluble portion of the complex contains ATPase activity and nucleotide binding sites (Vambutas & Racker, 1965; McCarty & Racker, 1968; Farron & Racker, 1970; Cantley & Hammes, 1975; Magnusson & McCarty, 1976a; Carlier & Hammes, 1979). A low molecular weight (8000) proteolipid in CF_0 has been shown to mediate proton movements in liposomes (Nelson et al., 1977; Criddle et al., 1977) and may form the channel through which protons are pumped during ATP synthesis.

Isolated CF_1 is a latent Ca^{2+}-dependent ATPase which can be activated by a variety of methods (Farron & Racker, 1970). In contrast, DSA, which is activated by dithiothreitol during preparation, shows optimal ATPase activity in the presence of Mg^{2+} (Pick & Racker, 1979). An uncoupler-sensitive [^{32}P]P_i–ATP exchange is catalyzed by DSA after reconstitution into phospholipid vesicles (Pick & Racker, 1979). This enzyme also catalyzes ATP synthesis when reconstituted into liposomes together with bacteriorhodopsin, a light-driven proton pump (Winget et al., 1977; T. G. Dewey and G. G. Hammes, unpublished experiments), or when a pH jump is applied to reconstituted DSA (Pick & Racker, 1979).

The isolation of an ATP-synthesizing complex, which retains biological activity when reconstituted into vesicles, permits physical–chemical investigations of the molecular mechanism of ATP synthesis. Resonance energy transfer already has been used to obtain structural information about reconstituted DSA (Baird et al., 1979). Previous studies have shown that solubilized CF_1 contains one tightly bound ADP and binds two additional ADP molecules (Cantley & Hammes, 1975; Carlier & Hammes, 1979). In the work reported here, nucleotide analyses were performed on partially purified DSA and DSA purified by sucrose gradient centrifugation. The exchange of intrinsic ADP with medium nucleotide and the reversible binding of ADP to both the solubilized and the reconstituted enzyme also were investigated.

Materials and Methods

Chemicals. Cholic acid was purchased from Sigma Chemical Co. and recrystallized prior to use (Kagawa & Racker, 1971). Octyl glucoside was purchased from Calbiochem. Triton X-100 was obtained from Sigma and asolectin (crude soybean phospholipids) was obtained from Associated Concentrates, Woodside, NY. ADP and ATP (vanadium free) were purchased from Sigma; ^{3}H-labeled nucleotides were obtained from New England Nuclear and were purified by paper chromatography on 3 MM Whatman paper with a solvent system of isobutyric acid–1 N ammonia (100:60 v/v). [^{32}P]P_i was obtained from ICN and was heated to 90–100 °C in 2 N HCl for 1–2 h to hydrolyze any pyrophosphate which might be present. All other chemicals used in this study were analytical grade, and all solutions were made with deionized distilled water.

Enzyme Preparation. The preparation of chloroplast membranes was similar to the procedure employed by Pick & Racker (1979) with the following modifications: the grinding medium contained 50 mM potassium phosphate (pH 7.4), 10–15 mM ascorbate, and 0.35 M NaCl, and the membranes were washed first in 0.4 M sucrose, 10 mM NaCl, and 50 mM NaTricine (pH 7.8), then in 0.15 M NaCl and 0.01 M NaTricine (pH 8.0), and finally again in the original wash

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[1] Abbreviations used: ATPase, adenosinetriphosphatase; DSA, dicyclohexylcarbodiimide-sensitive ATPase; CF_1, coupling factor 1 of DSA; CF_0, coupling factor 0 of DSA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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mixture. The DSA complex was solubilized as described by Pick & Racker (1979). The fraction which precipitates between 37.5% and 45% saturation of ammonium sulfate is designated as the partially purified enzyme preparation. This preparation can be stored at −70 °C for several months, following quick-freezing in a dry ice–acetone slurry.

Partially purified DSA was further purified by sucrose density centrifugation using a modification of the procedure described by Pick & Racker (1979). The partially purified enzyme, 25–35 mg in 1 mL, was diluted with 4–5 mL of 30 mM Tris–succinate (pH 6.5), 0.2% Triton X-100, 0.1 mM ATP, and 0.5 mM EDTA. This mixture was layered onto 33 mL of a 7–40% sucrose gradient with 0.4% sodium cholate containing 30 mM Tris–succinate (pH 6.5), 0.1 mM ATP, 0.5 mM EDTA, and 0.1% sonicated asolectin. Centrifugation was performed in a Beckman SW-27 rotor for 8.5–9.5 h at 26 500 rpm at 2 °C. Fractions of 2 mL were collected from the top of the gradient tube. The major protein band in these gradients is associated with dark green and brown pigment bands which usually are located 6 and 8 mL from the top of the gradient tube. A smaller protein shoulder is located about three-fourths of the way into the gradient tube (fractions 14 and 15). The position of this shoulder relative to the pigment proteins is nearly identical with the relative positions of these fractions in the sucrose (cholate) gradient profiles reported by Pick & Racker (1979). The peak of $[^{32}P]P_i$–ATP exchange activity corresponds with the peak of the protein shoulder (fractions 14 and 15). The $[^{32}P]P_i$–ATP exchange activity is always resolved from the major protein (pigmented) fractions, but significant activity is frequently observed in the areas between the pigment–protein peak and the smaller protein shoulder, suggesting that some of the DSA does not move into the shoulder. However, only the fractions which comprise the protein shoulder were pooled and used. In some cases ATP was omitted from the gradients to facilitate the study of nucleotide binding to the complex. This results in a slight broadening of the protein shoulder, but the relative location of the shoulder is unchanged and the major $[^{32}P]P_i$–ATP exchange activity remains centered over this area of the gradient. Sodium dodecyl sulfate gel electrophoresis patterns for the gradient-purified enzyme were similar to those obtained by Pick & Racker (1979).

Reconstitution. The DSA was reconstituted into liposomes by diluting partially purified enzyme (final concentration 0.5 mg/mL for activity assays and 1–4 mg/mL for nucleotide uptake and binding experiments) in asolectin (40 mg/mL) which had been sonicated to clarity with a bath-type sonicator (cylindrical ultrasonic tank and generator, Laboratory Supply Co. Inc., Hicksville, NY) in 80 mM NaTricine and 0.5 mM EDTA (Pick & Racker, 1979). Gradient-purified samples were diluted at least 9-fold (v/v) in the phospholipids to minimize detergent interference in the reconstitution. Following the addition of enzyme, the protein–phospholipid mixtures were rapidly frozen in an acetone–dry ice mixture (−70 °C) and then thawed at room temperature.

The partially purified and gradient-purified preparations were assayed for $[^{32}P]P_i$–ATP exchange activity according to Winget et al. (1977). Prior to assay, gradient fractions were incubated in 50 mM dithiothreitol for 15 min (after reconstitution) which increases the observed activity (Pick & Racker, 1979). The specific activity for the partially purified enzyme was typically 40–70 nmol of P_i/(min·mg) (37 °C), although specific activities as low as 20 and as high as 100 were obtained. The specific $[^{32}P]P_i$–ATP exchange activity is more variable in the gradient-purified samples; at best we obtain only slight increases (1.2-fold) in the specific activity relative to the activity of the partially purified preparation, indicating that the enzyme complex loses activity during gradient purification. The protein concentrations for partially purified and gradient-purified samples were determined according to Bensadoun & Weinstein (1976), using bovine serum albumin as a standard.

Analysis for Tightly Bound Nucleotides. Nucleotide analyses were performed on both partially purified and gradient-purified DSA. In most cases, 400–500-μL aliquots of the enzyme preparations were first passed through two disposable 3-mL plastic syringes (Becton-Dickinson) which were partially plugged with glass wool and filled with Sephadex G-50 (medium), equilibrated in 5 mM NaTricine (pH 8.0). Prior to the addition of enzyme, these syringes were placed into test tubes (13-mm i.d. × 100 mm) in an IEC HN-SII table-top centrifuge (Damon/IEC Division) and centrifuged for 2 min at ~1000 rpm. After the addition of enzyme, the columns were again centrifuged for 2 min (1000 rpm). This results in the elution of better than 80% of the DSA. When necessary, the gradient-purified samples were concentrated with a collodion membrane (Schleicher & Schuell) or with Sephadex G-25 (medium). In the latter case, 2 g of Sephadex G-25 was added to 10 mL of gradient-purified enzyme in a plugged syringe, the Sephadex was allowed to swell in the cold (0–4 °C) for 20 min, and then the enzyme–Sephadex suspension was centrifuged for 10 min at 2000 rpm (0 °C). This results in an ~2-fold concentration of the enzyme solution with no change in ionic strength, buffer concentration, or detergent concentration.

The extraction of nucleotides from the enzyme samples was carried out according to the procedure of Rosing & Slater (1972). Perchloric acid (final concentration 4% w/v) was added to 8–20 mg of partially purified DSA or 2–3 mg of gradient-purified DSA in ~1 mL at 0 °C, and the mixture was centrifuged for 10 min at 10 000 rpm (0 °C). The supernatant was neutralized with 20% KOH, and the insoluble $KClO_4$ salt was eliminated by centrifugation at room temperature. The nucleotides were analyzed by chromatography on poly(ethylenimine)–cellulose (0.5 × 2.5 cm, Sigma; Magnusson et al., 1976). The nucleotides were eluted with 15–20 mL of 0.1 M LiCl, 5 mL of 0.3 M LiCl, 6 mL of 1.0 M LiCl, and 6–10 mL of 1.5 M LiCl. The amount of eluted nucleotide was determined spectrophotometrically (Cary 118 spectrophotometer) by assuming a nucleotide extinction coefficient of 15 400 M^{-1} cm^{-1} at 259 nm, pH 7.0 (Beaven et al., 1955). Corrections for the loss of solution in the protein and $KClO_4$ precipitates were made both by measurement of the initial volume and the collected volume and by measurement of a small amount of added radioactive nucleotide, before and after the centrifugations. The corrections obtained with these two methods were in good agreement.

Nucleotide Uptake Measurements. In experiments monitoring the time course of nucleotide uptake, partially purified DSA was incubated with radioactive nucleotide for the desired time period, and then the reaction was stopped by application of the material to two consecutive 3-cm disposable syringes containing Sephadex G-50 (medium) as previously described. Control experiments indicate that the elution of DSA through two 3-cm columns results in essentially complete separation of free nucleotide from the protein, for nucleotide concentrations as high as 100 μM. When DSA was not reconstituted into liposomes, 10 mM octyl glucoside was included in the incubation solution and column buffer to minimize inactivation due to aggregation. Following elution from the Sephadex G-50

columns, protein concentrations for enzyme which was not reconstituted were determined by the method of Bensadoun & Weinstein (1976). For reconstituted enzyme, the protein concentrations were determined by the method of Peterson (1977). The amount of radioactive nucleotide bound to DSA was determined by scintillation counting of an aliquot in 10 mL of ACS aqueous scintillation fluid (Amersham).

Experiments also were performed to monitor uptake and displacement of [^{3}H]ADP after a pH gradient was introduced across DSA vesicles by bacteriorhodopsin. The bacteriorhodopsin was purified from the purple membranes of *Halobacterium halobium* (a gift from Professor Russell MacDonald) by sucrose gradient centrifugation for 16 h at 25 000 rpm (2 °C), with a step gradient of 20% (6 mL), 36% (15 mL), 40% (6 mL), and 46% (3 mL) sucrose. Reconstitution of bacteriorhodopsin was achieved by adding 2 mL of the purified protein (~2 mg/mL) to 2 mL of sonicated asolectin (40 mg/mL) in 50 mM NaTricine (pH 8.0), 0.15 M KCl, and 0.5 mM EDTA and to 0.38 mL of 0.5 M octyl glucoside. This mixture was incubated on ice for 15 min, and the bacteriorhodopsin vesicles were concentrated by spinning down the vesicles at 40 000 rpm for 40 min (2 °C). Partially purified DSA was added to the bacteriorhodopsin vesicles (final DSA concentration 2–3 mg/mL) and reconstituted by the freeze–thaw procedure described above. When the displacement of [^{3}H]ADP was monitored during ATP synthesis, the reconstituted DSA was first incubated with 20 μM [^{3}H]ADP for 3 h and then passed through two 3-mL Sephadex G-50 (medium) columns (in disposable syringes) equilibrated with 50 mM NaTricine (pH 8.0), 5 mM $MgCl_2$, and 0.15 M KCl. The eluted DSA (0.8 mL containing tightly bound [^{3}H]ADP) was then added to 0.1 mL of [^{32}P]P_i (0.1 M) and valinomycin (10 μM). This solution (0.9 mL) was placed in front of a light source (300-W bulb) for 15 min to allow the proton pumping by the bacteriorhodopsin to reach a steady state. ATP synthesis and the displacement of labeled ADP were initiated by the addition of 0.1 mL of ADP (~200 μM). Aliquots were removed at various times and either passed through two 3-mL Sephadex G-50 (medium) columns for analysis of the amount of [^{3}H]ADP remaining on the enzyme or added to an equal volume of 10% trichloroacetic acid for the determination of the amount of [^{32}P]P_i incorporated into ATP (using the same procedure as that described to determine [^{32}P]P_i–ATP exchange).

Binding Measurements. The binding of ADP to DSA was studied at 4 °C by the forced dialysis technique (Cantley & Hammes, 1973) using XM 50 membranes. In studies with the reconstituted enzyme, the protein concentration of the partially purified sample, after reconstitution, was ~4 mg/mL, and the concentration of asolectin was 30–35 mg/mL. Similar protein concentrations were used in binding studies where the partially purified enzyme was not reconstituted; however in these cases, all solutions contained 10 mM octyl glucoside. Prior to a binding experiment, gradient-purified enzyme was put through two 3-mL Sephadex G-50 (medium) columns (in disposable syringes), which were equilibrated in 50 mM NaTricine (pH 8.0), 0.5 mM EDTA, 0.12 M NaCl, 0.4% sodium cholate, and 0.1% sonicated asolectin to remove any dissociable nucleotides which may have been bound during the gradient purification. In all binding experiments, 250 μL of enzyme (or enzyme vesicle) solution was added to 50 μL of solution containing different concentrations of [^{3}H]ADP. The enzyme–nucleotide mixture was incubated for 45 min before the pressure was applied (10–15 psi) and free ligand (5–10 μL) was collected. The radioactivity of free nucleotide (effluent)

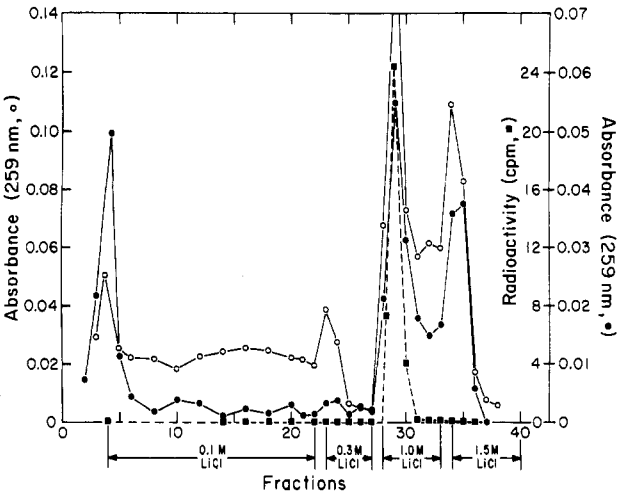

FIGURE 1: Elution of neutralized supernatant from perchloric acid precipitated partially purified DSA, 0.89 mol of [^{3}H]ADP/mol of DSA (○), and gradient-purified DSA (●), on a poly(ethylenimine)–cellulose column, monitoring absorbance at 259 nm (○, ●) and radioactivity (■). Fractions of 1 mL were collected as indicated on the abscissa.

Table I: Intrinsic Nucleotide Stoichiometry of DSA

DSA	mol of ADP/ mol of DSA	mol of ATP/ mol of DSA	no. of determinations
partially purified	0.90–2.48	0.27–3.14	5
av	1.79	1.74	
purified	0.88–1.86	0.11–2.0	4
av	1.40	1.18	

and total nucleotide (the original incubation solution) was determined in 10 mL of ACS (Amersham) scintillation fluid.

Results

Intrinsic Nucleotides. Chromatography of the neutralized supernatants from perchloric acid precipitated partially purified DSA on poly(ethylenimine)–cellulose resulted in the resolution of four fractions containing material absorbing at 259 nm (Figure 1). The material eluted at 0.1 and 0.3 M LiCl is colored (yellow green) and has a maximum absorbance at 263 nm. The third and fourth fractions eluted at 1.0 and 1.5 M LiCl, respectively, have maximum absorbances at 259 nm. Control experiments monitoring the separation of AMP, ADP, and ATP in the absence of protein and inclusion of small amounts of [^{3}H]ADP in the supernatants obtained from the perchloric acid precipitation of DSA samples indicate that the third and fourth fractions were ADP and ATP, respectively. Chromatography of the supernatant from perchloric acid treated gradient-purified enzyme yields a similar profile (Figure 1), although nearly all of the pigment is eluted at 0.1 M LiCl.

The range and the average amounts of ADP and ATP per DSA (mol/mol) are listed in Table I. The number of moles of DSA present in the partially purified samples was determined by assuming a molecular weight of 405 000[2] for the complex and 0.27 mg of DSA/mg of total protein in the partially purified preparation. The value 0.27 represents the ratio of the amount of protein found in the peak for [^{32}P]P_i–ATP exchange activity to the total amount of protein

[2] A molecular weight of 405 000 has been determined for sucrose gradient purified DSA with small angle X-ray scattering methods (H. Paradies, U. Pick, and B. Baird, unpublished data).

Table II: Incorporation of Nucleotide into DSA[a]

nucleotide (μM)	DSA	r_0	r[b]
[^{3}H]ADP (80–90)	partially purified	0.85	0.96
[^{3}H]ADP (90)	partially purified	0.92	1.06
[^{3}H]ADP (85–115)	partially purified	0.94	1.10
[^{3}H]ADP (20)	partially purified	1.15	1.26
[^{3}H]ADP (110–130)	partially purified	0.71	0.72
[^{3}H]ADP (93)	gradient purified	0.54	0.57
[^{3}H]ATP (90–95)	partially purified	0.66	
[^{3}H]ADP (20)	reconstituted, partially purified	0.50[c]	0.52

[a] DSA was incubated with ^{3}H-labeled nucleotide at the concentrations indicated within the parentheses for 2.5–3 h in 50 mM NaTricine (pH 8.0), 5 mM $MgCl_2$ and 10 mM octyl glucoside at 4 °C. In all cases, except rows 1 and 2, labeled enzyme was separated from free nucleotide by elution through two 3-cm Sephadex G-50 columns. For row 1, labeled enzyme was separated from free nucleotide by elution on a 50-cm Sephadex G-25 (medium) column. For row 2, enzyme was separated from free nucleotide by elution through a 3-cm Sephadex G-50 column followed by dialysis overnight. In rows 5 and 7, 2 mM EDTA was substituted for $MgCl_2$, and octyl glucoside was not present with the reconstituted enzyme. The values listed in columns 3 and 4 represent the average of at least two experiments (except for row 6 which is the result of one experiment). [b] The values of r (mol of ADP/mol of DSA) were calculated from the observed values, r_0, by 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 nucleotide from the enzyme. ([^{3}H]ADP) represents the initial concentration of labeled ADP, and (E_0) is the molar concentration of DSA. [c] The amount of [^{3}H]ADP incorporated into reconstituted DSA has been corrected for nonspecific uptake of radioactivity by using vesicles without protein as a control.

on the gradient. It is the average from five gradient purifications, with the range being 0.23–0.30 mg of DSA/mg of total protein.

Variations in the ADP and ATP stoichiometries are observed for different preparations of the partially purified and gradient-purified enzyme. In every preparation tested, at least ~1 mol of ADP/mol of DSA is found. In some preparations, ≤0.3 mol of ATP/mol of DSA occurs. However, about equal amounts of ADP and ATP usually were observed. The amounts of intrinsic ADP and ATP are not correlated with specific activity, as determined by [^{32}P]P_i–ATP exchange. For example, one of the more active preparations [sp act. = 70 nmol of P_i/(min·mg of total protein)] contained 1.1 mol of ADP/mol of DSA and <0.3 mol of ATP/mol of DSA. The nucleotide stoichiometries determined for the partially purified samples are generally higher than those determined for gradient-purified enzyme preparations. This may result from some residual pigment which elutes at 1.0 and 1.5 M LiCl and contributes to the total 259-nm absorbance of these fractions. An accurate determination of the amount of intrinsic AMP (if any) is not possible since the AMP standards are eluted with the pigment at 0.1 M LiCl. In the profile for gradient-purified enzyme shown in Figure 1, if all the material eluted at 0.1 M LiCl were AMP, the stoichiometry for this nucleotide would be ~0.9 mol of AMP/mol of DSA. However, this fraction is mostly pigment (it is colored and has a maximum absorbance at 263 nm); thus, any AMP must be present in an amount much lower than this stoichiometry.

Incorporation of ADP. When partially purified DSA (7–16 μM) was incubated with [^{3}H]ADP (80–90 μM) in 50 mM NaTricine (pH 8.0), 5 mM $MgCl_2$, and 10 mM octyl glucoside for 3 h (4 °C), ~0.9 mol of [^{3}H]ADP/mol of DSA was found to be tightly bound after elution of the protein from a 50-cm Sephadex G-25 (medium) column (Table II, row 1). When the incubation was continued for 11 h, no change in the amount of radioactivity associated with DSA was found. Similar amounts of tightly bound ^{3}H-labeled nucleotide were observed if the enzyme was dialyzed 19–24 h (Table I, row 2) or if the enzyme was eluted from two 3-cm Sephadex G-50 nmedium) centrifuge columns (Table II, rows 3 and 4). An uptake of [^{3}H]ADP also occurs with the gradient-purified enzyme (Table II, row 6). The lower stoichiometry in this case may be due to the fact that the gradient-purified enzyme was concentrated several times prior to the experiment; such treatment results in substantial activity losses.

Experiments were performed to determine if the uptake of [^{3}H]ADP represents the binding of an additional nucleotide molecule or if it reflects an exchange with the intrinsic nucleotides. In one study, half of a partially purified DSA preparation was incubated for 2.5 h (4 °C) with 100 μM [^{3}H]ADP in 50 mM NaTricine (pH 8.0), 5 mM $MgCl_2$, and 10 mM octyl glucoside, while the other half of the preparation was incubated in the absence of nucleotide as a control. A nucleotide determination (following elution of the protein samples through two 3-cm Sephadex G-50 columns) showed that all of the radioactivity which was incorporated (0.89 mol of [^{3}H]ADP/mol of DSA) was eluted as ADP and that no increase in the total amount of ADP tightly bound to the DSA preparation (~2.2 mol of ADP/mol of DSA) occurred. A similar experiment was performed with the gradient-purified enzyme. All of the radioactivity which was incorporated (0.54 mol of [^{3}H]ADP/mol of DSA) again was eluted as ADP, and no increase in tht total amount of bound ADP was found (~1.8 mol of ADP/mol of DSA). Thus, the experimental results indicate that the incorporation of ^{3}H-labeled nucleotide represents an exchange with the intrinsic nucleotide of the protein. Since the dissociation of intrinsic ADP is not observed in the absence of medium ADP, this exchange requires binding at an additional nucleotide site or sites.

Incubations of partially purified DSA with [^{3}H]ADP in the presence of 2 mM EDTA for 3 h (4 °C) consistently results in a lower uptake of radioactivity (Table II, row 5). This indicates that the presence of Mg^{2+} increases the rate of nucleotide incorporation. A comparable incorporation of radioactivity occurs when the partially purified enzyme is incubated with [^{3}H]ATP (90–95 μM) for 3 h in 50 mM NaTricine, 10 mM octyl glucoside, and 2 mM EDTA (Table II, row 7). When DSA, which was preincubated with [^{3}H]ATP, was precipitated with perchloric acid and the supernatant was analyzed for nucleotides by poly(ethylenimine)–cellulose chromatography, all of the radioactivity was found to be ADP. Freeze–thaw-reconstituted DSA consistently shows a reduced incorporation of radioactivity when incubated with 20 μM [^{3}H]ADP (Table II, row 8).

Some typical time courses for the incorporation of [^{3}H]ADP (20 μM) into partially purified DSA (2.3 μM) in the presence of 10 mM octyl glucoside and into reconstituted partially purified DSA at 4 °C are shown in Figure 2. The rate of uptake of the nucleotide can be approximated as a first-order process (solid lines in Figure 2), although the initial reaction is slightly faster than predicted by the first-order fit. The total uptake of nucleotide is less in the reconstituted enzyme, and the rate of incorporation is ~2-fold faster (Table III, rows 1 and 2). Experiments were performed to determine if the introduction of a pH gradient across DSA-reconstituted vesicles had any effect on the total amount of nucleotide incorporated or on the rate of incorporation. DSA (2.3 μM) reconstituted into phospholipid vesicles with bacteriorhodopsin shows about the same uptake of [^{3}H]ADP at room temperature as that observed in reconstituted DSA at 4 °C. However, the rate of nucleotide incorporation at room temperature is

Table III: Kinetics of Uptake and Displacement of ADP[a]

DSA	conditions	ADP uptake (mol of ADP/ mol of DSA)	$t_{1/2}$ (min)	ADP displaced (mol of ADP/ mol of DSA)	$t_{1/2}$ (min)
partially purified	4 °C	0.94	36		
partially purified (reconstituted)	4 °C	0.40	18		
partially purified (reconstituted with bacteriorhodopsin)	room temp, dark	0.42	~1		
partially purified (reconstituted with bacteriorhodopsin)	room temp, light	0.34	~1		
partially purified (reconstituted)	4 °C	0.59		0.09	~2
partially purified (reconstituted with bacteriorhodopsin)	4 °C, ATP synthesis	0.52		0.1	<2

[a] All experiments were performed in 50 mM NaTricine (pH 8.0) and 5 mM $MgCl_2$. The values for ADP uptake were calculated by using the relationship $r = r_0\{([^3H]ADP)/[([^3H]ADP) - r_0(E_0)]\}$, as described in the legend for Table II. The values listed for the amount of ADP displaced represent the amount of radioactive nucleotide remaining on DSA (corrected for isotopic dilution as described above) subtracted from the original amount of radioactive nucleotide (corrected for isotopic dilution) incorporated into DSA.

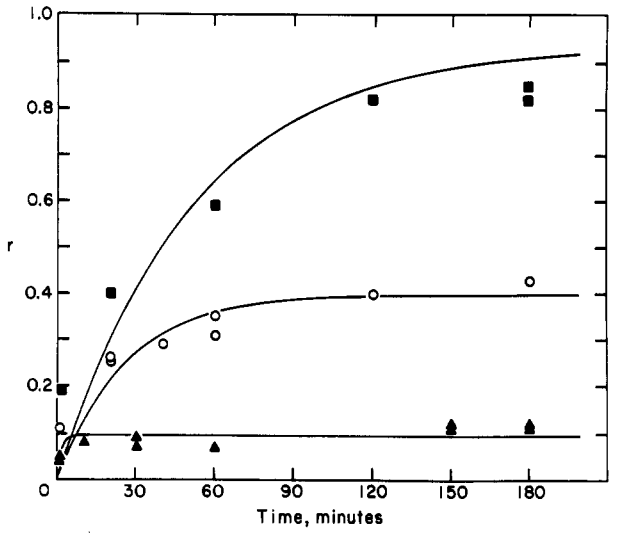

FIGURE 2: Plot of r, moles of tightly bound [^{3}H]ADP incorporated per mole of partially purified DSA (■) and reconstituted DSA (○) and moles of [^{3}H]ADP displaced per mole of reconstituted DSA (▲), vs. time. In the incorporation study, DSA was incubated with [^{3}H]ADP in 50 mM NaTricine and 5 mM $MgCl_2$ at 4 °C and analyzed for tightly bound [^{3}H]ADP at the times indicated. In the displacement study, enzyme was first incubated with [^{3}H]ADP for 3 h, then eluted through two consecutive Sephadex G-50 columns, and reincubated with cold ADP, with all solutions containing 50 mM NaTricine (pH 8.0) and 5 mM $MgCl_2$. Correction for isotopic dilution was made as described in the legend for Table II.

significantly faster (Table III, row 3). When DSA is reconstituted with bacteriorhodopsin and then incubated with [^{3}H]ADP in front of a high-intensity light, a pH gradient is generated and ATP synthesis occurs (see below). However, no significant effect is observed on the parameters of the ADP exchange process (Table III, row 4).

Experiments also were performed to determine the reversibility of the ADP incorporation. Reconstituted, partially purified DSA (16 μM) was incubated for 3 h with 20.6 μM [^{3}H]ADP in 50 mM NaTricine (pH 8.0) and 5 mM $MgCl_2$ at 4 °C. Aliquots were passed through two 3-cm Sephadex G-50 (medium) columns, and the eluted protein was incubated with 200 μM ADP. The amount of [^{3}H]ADP displaced from the enzyme (cf. Figure 2) and the average half-time for the removal of the label are included in Table III (row 5). The total amount of label displaced is only ~15% of the original amount incorporated, and the half-time is about one-tenth that of [^{3}H]ADP uptake. The amount of nucleotide removed and the time course for the displacement appear to correspond to the initial, rapid phase observed in the uptake plots, but this may be fortuitous (Figure 2). In experiments where DSA is not reconstituted into liposomes, an initial displacement of 10% or less of the total label in 1 h is observed. A very slow displacement of radioactivity has been observed in enzyme which is not reconstituted: ~50% of the total label is removed after 21 h. If the enzyme (which is not reconstituted) is incubated with [^{3}H]ADP and a 10–15-fold excess of unlabeled ADP is added after only 1 h, no increase in the amount of label removed is found. Also, essentially no displacement of radioactive nucleotide was observed when DSA labeled prior to its isolation from the chloroplasts (0.36 mol of [^{3}H]ADP/mol of enzyme) was incubated with 200 μM unlabeled ADP for 3 h. Experiments where labeled enzyme was preincubated with 50 mM dithiothreitol, an activator of the enzyme, also showed no change in the rate or displacement of ADP.

The removal of tightly bound ADP has been monitored under conditions where the enzyme is synthesizing ATP. When partially purified DSA (~1 μM, 0.49 mol of [^{3}H]-ADP/mol of DSA), which has been reconstituted with bacteriorhodopsin, is incubated at 4 °C with 200 μM ADP under conditions where ATP synthesis can occur [0.71 nmol of P_i incorporated into ATP/(min·mg of DSA)], the amount of ^{3}H-labeled nucleotide displaced is similar to that observed in earlier studies (Table III, row 6). However, the displacement appears to be essentially complete within 3 min, indicating that the rate of removal of the labeled nucleotide is increased during the synthesis of ATP.

Binding Experiments. The binding of ADP to partially purified and gradient-purified DSA was studied by using the forced dialysis technique in 50 mM NaTricine (pH 8.0) and 5 mM $MgCl_2$ at 4 °C. In all cases the binding isotherms were fit by least-squares analysis to

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

where r represents the moles of ligand bound per mole of enzyme, L is the concentration of free ligand, n is the total number of ligand binding sites per mole of enzyme, and K is the dissociation constant. The results obtained are summarized in Table IV, and a typical binding isotherm is shown in Figure 3. ADP binds to the partially purified enzyme with an average stoichiometry of ~0.5 mol of ADP/mol of DSA and an average dissocation constant of ~2 μM. When partially purified enzyme was preincubated for 3 h with ~90 μM [^{3}H]ADP and then a forced dialysis experiment was performed by using [^{3}H]ADP with the same specific activity as that of the labeled nucleotide in the initial incubation, similar binding parameters were obtained (Table IV, row 3). This indicates that the nucleotide binding observed in these forced dialysis experiments represents a distinct interaction from the tight uptake of ^{3}H-

Table IV: Parameters for Binding of ADP to DSA

DSA	r	K (μM)
partially purified[a] (36)[b]	0.54	2.96
partially purified[a] (73)[b]	0.48	1.58
partially purified[a] (0.89 mol of [^{3}H]ADP/mol of DSA)	0.57	2.85
gradient-purified[c]	0.57	1.69
reconstituted,[d] partially purified	0.31–0.54 (0.42)[e]	1.05–2.73 (1.86)[e]
reconstituted,[f] partially purified	0.56	1.17

[a] 50 mM NaTricine (pH 8.0), 5 mM $MgCl_2$, and 10 mM octyl glucoside, 4 °C. [b] Specific [^{32}P]P_i–ATP exchange activities [nmol of P_i/(min·mg of protein)]. [c] 50 mM NaTricine (pH 8.0), 5 mM $MgCl_2$, 0.4% sodium cholate, 0.1 M NaCl, and 0.1% sonicated asolectin, 4 °C. [d] 50 mM NaTricine (pH 8.0) and 5 mM $MgCl_2$, 4 °C. [e] Average for three experiments. [f] 50 mM NaTricine (pH 8.0), 5 mM $MgCl_2$, and 0.1 M NaCl, 4 °C.

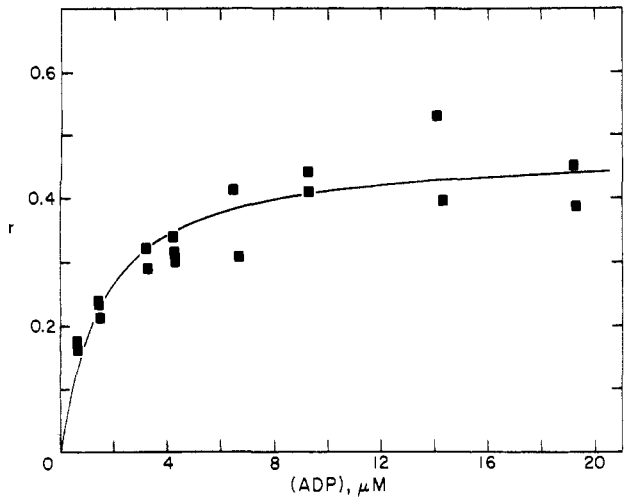

FIGURE 3: Plot of moles of [^{3}H]ADP per mole of partially purified DSA, r, vs. the free ADP concentration as determined by forced dialysis technique in 50 mM NaTricine (pH 8.0), 5 mM $MgCl_2$, and 10 mM octyl glucoside at 4 °C.

labeled nucleotide described earlier. This is because the rate of incorporation of tightly bound nucleotide is not appreciable at the ADP concentrations used in the forced dialysis experiments. No correlation between the amount of [^{3}H]ADP which binds to a partially purified enzyme preparation and the specific activity of the preparation, as determined by [^{32}P]P_i–ATP exchange assays, was found. However, the activity is probably a measure of the efficiency of reconstitution rather than of the catalytic potential of the partially purified enzyme.

Similar binding parameters are obtained with the gradient-purified enzyme (Table IV, row 4) which demonstrates that the nucleotide binding is specific for DSA. The presence of different detergents does not alter the binding parameters since the studies with gradient-purified DSA were performed in the presence of 0.4% sodium cholate, while the binding studies with partially purified enzyme were done in the presence of 10 mM octyl glucoside. The binding isotherms for reconstituted DSA are similar to those for enzyme which is not reconstituted, although the average stoichiometry is slightly lower for the reconstituted system. No significant changes are observed in the binding parameters with changes in ionic strength.

Discussion

The results summarized in Table I show that DSA, upon isolation from the thylakoids, contains at least one tightly bound ADP. In most preparations, at least one tightly bound ATP molecule per molecule of enzyme also is found. An average stoichiometry of 2.5 mol of nucleotide/mol of DSA has been estimated from studies on sucrose gradient purified enzyme preparations. Three tight nucleotide sites per chloroplast coupling factor have been found in studies on intact chlorplasts (Harris & Slater, 1975). The ATP/ADP ratio in the DSA preparations is about unity, while a ratio of ~2 was reported in the chloroplast studies. Apparently, a significant reduction occurs in the binding affinity at some of the nucleotide sites during the isolation of the soluble component of the ATP-synthesizing complex, CF_1, which contains only one tightly bound ADP and no intrinsic ATP (Carlier & Hammes, 1979).

The incubation of DSA with [^{3}H]ADP results in the exchange of 0.5–1 mol of intrinsic ADP with the labeled nucleotide. A similar incorporation of radioactive nucleotide has been observed in energized chloroplasts (Magnusson & McCarty, 1976a,b; Strotmann et al., 1976, 1979; Strotmann & Bickel-Sandkötter, 1977) and in CF_1 (Carlier & Hammes, 1979). Although the rate of nucleotide exchange in DSA (half-time ~1 min at room temperature) is significantly slower than the light-driven exchange observed in chloroplasts (half-time ~0.1 s; Magnusson & McCarty, 1976b), it is faster than the exchange observed in either latent (half-time ~4 h) or heat-activated CF_1 (half-time ~1 h; Carlier & Hammes, 1979). The nucleotide incorporation observed in DSA and CF_1 (Carlier & Hammes, 1979) appears to be similar; binding at some additional nucleotide site(s) appears necessary for exchange, the rates of ADP incorporation are slightly faster in the presence of Mg^{2+}, and incorporation of ATP is accompanied by hydrolysis of the nucleotide to ADP. The rate of incorporation of radioactive nucleotide is faster in reconstituted DSA than in DSA in a detergent solution. This may be because the protein conformation of the DSA complex in a membranelike environment facilitates the exchange of nucleotide. A reduction in the total amount of label incorporated occurs with the reconstituted DSA, suggesting that some of the DSA has become less accessible for nucleotide exchange.

Whether DSA is incubated directly with nucleotide in the presence of 10 mM octyl glucoside or is reconstituted prior to the incubation, the nucleotide-exchange process is only partially reversible. This lack of reversibility is not due to inactivation of the enzyme. No increase in the percentage of radioactively labeled nucleotide removed from DSA is observed when a 10–15-fold excess of unlabeled ADP is added 1 h after incubation with [^{3}H]ADP or when excess ADP is added to enzyme labeled with [^{3}H]ADP prior to isolation from the chloroplasts. A possible explanation for this lack of reversibility in the nucleotide-exchange reaction is that the DSA molecules exist in different protein conformations, one where the intrinsic nucleotide is tightly held but primed for release upon nucleotide binding at another site and a second where the intrinsic nucleotide is nonexchangeable. Activation by dithiothreitol during the isolation of DSA from thylakoid membranes could convert most of the enzyme molecules to the former conformation. Incubation of the isolated (activated) DSA with nucleotide might then promote a release of intrinsic nucleotide from the exchange site which is accompanied by a return of most of the DSA molecules (≥85%) to a nonexchangeable protein conformation. A similar interconversion between a nonexchangeable protein conformation and an exchangeable conformation, with the latter conformation predominating in the light or under conditions of a pH gradient, has been proposed for the ATP-synthesizing complex in intact chloroplasts (Strotmann et al., 1976, 1979; Bickel-Sandkötter

& Strotmann, 1976; Strotmann & Sandkötter, 1977).

The rate of displacement of the reversibly incorporated ^{3}H-labeled nucleotide appears to increase when the enzyme is synthesizing ATP. Under these conditions, turnover at the exchange site (which only occurs in ~15% of the total DSA molecules) is comparable to the turnover rate at the catalytic sites. Nevertheless, the high percentage of DSA molecules showing irreversible incorporation of [^{3}H]ADP, even when ATP synthesis is observed, suggests that the nucleotide exchange is not occurring at a catalytic site. This suggestion is in accord with the evidence obtained from studies on intact chloroplasts which show that the nucleotide specificity is different for exchange and photophosphorylation (Magnusson & McCarty, 1976b). However, since the fraction of DSA molecules which are actively synthesizing ATP cannot be determined, a catalytic function for the exchange site cannot be unequivocally ruled out.

Forced dialysis experiments performed with DSA prelabeled with [^{3}H]ADP indicate that a reversible binding of ADP to the enzyme occurs in addition to the exchange of medium nucleotide with intrinsic ADP. Unlike the case of nucleotide exchange, no significant changes in the binding stoichiometry occur when DSA is reconstituted into phospholipid vesicles. The binding affinity for ADP also is similar for the reconstituted enzyme and for enzyme in the presence of different detergents. The stoichiometry of binding is consistently <1 mol of ADP/mol of DSA. This may be because this binding is a more sensitive indicator of the enzyme being catalytically active than the intrinsic nucleotide stoichiometry. The estimated dissociation constant for ADP binding to DSA (~2 μM) is similar to the Michaelis constant for ADP determined from kinetic investigations of ATP synthesis (T. G. Dewey and G. G. Hammes, unpublished experiments).

In summary, the results obtained suggest the purified DSA contains at least three nucleotide binding sites: one contains tightly bound ADP, another contains tightly bound ATP, and the third binds ADP reversibly. Radioactive ADP or ATP can be exchanged into the site containing tightly bound ADP, but this exchange is almost irreversible. Nucleotide turnover at the sites containing tightly bound nucleotides during catalysis appears to be insufficient for their participation in catalysis, but an unambiguous interpretation is not possible because the percentage of active enzyme cannot be determined.

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