Function of Tightly Bound Nucleotides on Membrane-Bound Chloroplast Coupling Factor†

Deborah Leckband‡ and Gordon G. Hammes*
Department of Chemistry, Cornell University, Ithaca, New York 14853
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ABSTRACT: The kinetic behavior of tightly bound nucleotides on chloroplast coupling factor from spinach was determined under phosphorylating and nonphosphorylating conditions. Chloroplast coupling factor 1 (CF_1) was labeled with tightly bound radioactive ADP and/or ATP at two specific sites and reconstituted with thylakoid membranes depleted of CF_1 by treatment with NaBr. The initial incorporation and dissociation of ADP from one of the sites requires light but occurs at the same rate under phosphorylating and nonphosphorylating conditions. The initial rate is considerably slower than the rate of ATP synthesis, but nucleotide exchange is very rapid during steady-state ATP synthesis. A direct correspondence between this nucleotide binding site and a site on soluble CF_1 that hydrolyzes ATP was demonstrated. A second site binds MgATP very tightly; the MgATP does not dissociate during ATP synthesis nor does its presence alter the rate of ATP synthesis. This is analogous to the behavior found for soluble CF_1 during ATP hydrolysis. These results demonstrate that the tight-binding nucleotide sites on soluble CF_1 and membrane-bound coupling factor are essentially identical in terms of binding properties and kinetic behavior during ATP hydrolysis and synthesis.

The chloroplast coupling factor of thylakoid membranes consists of a membrane-embedded proton pore [chloroplast coupling factor o (CF_o)][1] and a soluble peripheral portion (CF_1) which contains the catalytic sites. The solubilized enzyme retains an ATPase activity, but the CF_o portion and a pH gradient are required for ATP synthesis.

Studies of the kinetics and ligand binding properties of the soluble enzyme have characterized three high-affinity nucleotide binding sites and their functions (Bruist & Hammes, 1981; Leckband & Hammes, 1987). One of the sites, site 1, binds ADP tightly but is a catalytic site during steady-state ATP hydrolysis. A second site, site 2, binds MgATP tightly, with a half-time for MgATP dissociation of many hours; it is not a catalytic site. In addition, a third site binds and dissociates ADP and ATP rapidly, with an equilibrium dissociation constant in the micromolar range, and also is a catalytic site. Studies with intact thylakoids and purified CF_1–CF_o have suggested two tightly bound nucleotides, one ADP and one ATP, and a third site that binds nucleotides with an equilibrium dissociation constant in the micromolar range (Cerione & Hammes, 1981; Harris & Slater, 1975).

Though similarities in nucleotide binding behavior have suggested a correlation between the nucleotide binding sites of CF_1 and CF_1–CF_o, the functional equivalence of these sites has not yet been demonstrated. In this work, soluble CF_1 with radioactive nucleotides at site 1 and/or site 2 has been reconstituted onto membranes depleted of CF_1. The behavior of these bound nucleotides under both phosphorylating and nonphosphorylating conditions has been examined. The results obtained demonstrate the equivalence of nucleotide binding sites 1 and 2 on soluble CF_1 with sites on CF_1–CF_o, in terms of both nucleotide binding properties and function during ATP hydrolysis and synthesis.

Materials and Methods

Chemicals. ADP, ATP (vanadium free), ascorbate, *N*-methylphenazonium methosulfate, and gramicidin were from Sigma Chemical Co. Nucleotide purity was assessed by thin-layer chromatography using Brinkmann poly(ethylenimine)–cellulose plates and 1.4 M aqueous LiCl (pH 6.5). [^{3}H]ADP and [^{3}H]ATP were from Amersham Corp., [γ-^{32}P]ATP and [α-^{32}P]ATP were from ICN, and *N*-[^{3}H]-ethylmaleimide was from New England Nuclear. All other reagents were high-quality commercial grades. Aqueous solutions were prepared with deionized–distilled water.

Enzyme. CF_1 was prepared from fresh market spinach (Binder et al., 1978; Lien & Racker, 1971). Enzyme with a 340:305-nm fluorescence emission ratio (280-nm excitation) of >1.5 was stored as an ammonium sulfate precipitate for not more than 1 month. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Chua, 1980) was used to check enzyme preparations for purity as well as for proteolysis of the minor subunits. Prior to use, the enzyme was pelleted and resuspended in 40 mM Tricine (pH 8.0) and 50 μM EDTA. CF_1 was desalted on two Sephadex G-50 fine centrifuge columns (Penefsky, 1977) equilibrated with the Tricine–EDTA buffer. Enzyme concentrations were determined spectrophotometrically using an extinction coefficient of 0.484 mL/(mg·cm) at 277 nm (Bruist & Hammes, 1981) and a molecular weight of 400 000 (Moroney et al., 1983). Enzyme absorbances were corrected for light scattering.

CF_1 was labeled with radioactive nucleotides by incubation for 1 h in 40 mM Tricine, 50 μM EDTA, and 200 μM nucleotide at pH 8.0. Exogenous nucleotides were removed by passage of the enzyme through two consecutive centrifuge columns. When site 2 was labeled, 1 mM $MgCl_2$ was included in the incubation mixture. Labeling at site 1 was with either [^{3}H]ATP or [α-^{32}P]ATP. Site 2 was labeled wth [γ-^{32}P]ATP. Stoichiometries were measured by determination of the radioactivity of a known amount of protein. Scintillation counting was with a Beckman LS 1801 scintillation counter.

Ca^{2+}-ATPase activity of CF_1 was assayed at 37 °C for 5 min in the presence of 40 mM Tricine (pH 8.0), 4 mM ATP, and 5 mM $CaCl_2$ after heat activation of the enzyme (Lien & Racker, 1971). Phosphate formed in the assay was de-

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; CF_o, chloroplast coupling factor o; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; EDTA, ethylenediaminetetraacetic acid.

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termined colorimetrically (Tausskey et al., 1953). Typical hydrolysis activities of enzyme used for reconstitution were 20–30 μmol/(mg·min). Mg^{2+}-dependent ATPase activities of stripped and reconstituted sodium bromide particles were determined by assaying in 50 mM octyl glucoside, 4 mM $MgCl_2$, 4 mM ATP, 40 mM Tricine, and 50 μM EDTA (pH 8.0) at 37 °C for 5 min (Pick & Basillan, 1981; Patrie, 1984). In these assays, reactions were quenched with 50% trichloroacetic acid containing 1% sodium dodecyl sulfate (Patrie, 1984).

Membranes. Preparation of CF_1-depleted thylakoid membranes (sodium bromide particles) was by the method of Nelson and Eytan (1979). Sodium bromide particles were stored at −70 °C for up to 2 months. During this period, a 10–15% loss in *N*-methylphenazonium methosulfate dependent photophosphorylation by reconstituted membranes was detected. The efficiency of light-dependent proton pumping and formation of the pH gradient was determined by monitoring the light-induced quinacrine fluorescence quenching in the presence or absence of 50 μM dicyclohexylcarbodiimide (Patrie, 1984). Residual CF_1 bound to the membranes was determined by assaying the octyl glucoside activated Mg^{2+}-ATPase activity of stripped membranes relative to reconstituted membranes.

Reconstitution. The CF_1 was reconstituted onto stripped thylakoid membranes by incubation of 0.5 mg of CF_1 with membranes containing 0.1 mg of chlorophyll in ~0.5 mL of 40 mM Tricine, 50 μM EDTA, and 6 mM $MgCl_2$ (pH 8.0) on ice for 15 min. After incubation, the membranes were pelleted in a microcentrifuge (Eppendorf) at 14000*g* for 2 min at room temperature. Pellets were washed 2 or 3 times with 0.4 M sucrose, 40 mM Tricine, 50 mM NaCl, and 5 mM dithiothreitol at pH 8.0 (STN–DTT). Activities of unpelleted preparations ranged from 1 to 2 μmol of ATP synthesized (mg of chlorophyll)$^{-1}$ min^{-1}. Pelleted and washed membranes had activities of 0.5–1.2 μmol of ATP synthesized (mg of chlorophyll)$^{-1}$ min^{-1}. The amount of radioactive CF_1 bound to the membranes was determined by scintillation counting and correction for color quenching with an external standard.

The amount of soluble CF_1 that binds to the NaBr-treated thylakoid membranes was determined by *N*-[^{3}H]ethylmaleimide modification of CF_1 followed by reconstitution. The *N*-[^{3}H]ethylmaleimide-labeled CF_1 was prepared by incubation of the latent enzyme with 2 mM *N*-[^{3}H]ethylmaleimide in 40 mM Tricine and 50 μM EDTA (pH 8.0) for 30 min. The unreacted label was removed by passage of the enzyme through two centrifuge columns equilibrated with 40 mM Tricine and 50 μM EDTA (pH 8.0). The disulfide on the γ-polypeptide was labeled by first reducing the disulfide by a 3-h incubation of the enzyme in 50 mM dithiothreitol, 40 mM Tricine, and 50 μM EDTA (pH 8.0). Unreacted dithiothreitol was removed by passage of the enzyme solution through two centrifuge columns. The exposed sulfhydryls were then labeled by incubation of the enzyme in 2 mM *N*-[^{3}H]-ethylmaleimide for 30 min as above. The label stoichiometry ranged from 2.5 to 3.2 mol of *N*-[^{3}H]ethylmaleimide/mol of CF_1, in agreement with previous work (Musier & Hammes, 1987). The *N*-ethylmaleimide modification of CF_1 did not affect the efficiency of reconstitution or the final activity of the preparation. The amount of *N*-[^{3}H]ethylmaleimide–CF_1 bound to the membranes was determined by scintillation counting of the washed membranes and correcting for color quenching.

Photophosphorylation and Nucleotide Exchange. *N*-Methylphenazonium methosulfate dependent photophosphorylation was assayed in 40 mM Tricine, 50 μM EDTA, 50 mM NaCl, 4 mM $MgCl_2$, 4 mM ADP, 2 mM [^{32}P]P_i at 10^4 cpm/mmol, 50 μM *N*-methylphenazonium methosulfate, and 2 mM ascorbate (pH 8.0). Samples containing 20–50 μg of chlorophyll were added to 1.0 mL of assay solution. The light source was two Kodak 600 H slide projectors equipped with ELH lamps (300 W) focused on the sample. With a single projector, the light intensity was approximately 10^6 erg/(cm^2·s) (Krupinski & Hammes, 1986). Sample temperatures were controlled by use of a Haake circulating water bath and a transparent sample cell that allowed water to circulate between the light source and the sample tube. The temperature for all assays was maintained at 24 °C. Specific activities were highest when the concentration of chlorophyll in the assay was ~20–50 μg/mL. After 2 min of illumination, the reaction was quenched with 100 μL of 50% trichloroacetic acid. The precipitate was removed by centrifugation for 1 min at 1200 rpm in an IEC HN-SII bench-top centrifuge (Damon/IEC Division). The [γ-^{32}P]ATP synthesized was determined by molybdate extraction (Winget et al., 1977).

Light-dependent incorporation of nucleotides was by methods described previously (Strotmann et al., 1976; Strotmann, 1984). Reaction mixtures were the same as in photophosphorylation assays except 50 μM [^{3}H]ADP at 10^{11} cpm/mmol was used rather than 4 mM nonradioactive ADP. For determination of nucleotide incorporation during illumination under nonphosphorylating conditions, phosphate was omitted from the medium. Aliquots containing 20–50 μg of chlorophyll were added to 0.50 mL of assay solution. Reactions were initiated by illumination and quenched with 200 μL of 0.175 mM gramicidin, 0.7 mM ADP, and 20 mM EDTA in 50% ethanol. Samples were pelleted in the microcentrifuge as described, and pellets were washed 3 times with ice-cold STN–DTT buffer (Strotmann et al., 1976). After the pellets were washed and resuspended in STN–DTT buffer, the radioactivity was determined by scintillation counting and the amount of chlorophyll by the method of Arnon (1949).

Light-dependent release of nucleotides was monitored under the same conditions as in the incorporation assays. The reaction was quenched with 200 μL of the gramicidin–ADP–EDTA solution or by direct application on a Whatman GF/F (2.4 cm) glass fiber filter rinsed with 5 mL of 5 mM ATP (pH 6.5–7.0). The gramicidin-quenched samples were also applied to ATP-washed filters. Samples were rinsed with 15 mL of 100 mM NaCl, and 40 mM Tricine at pH 8.0. Filters were immersed in 10 mL of ACS scintillation cocktail (Amersham) for determination of radioactivity. Identical results were obtained with both quenching methods.

Steady-state rates of nucleotide incorporation were measured by first illuminating samples under phosphorylating conditions for a sufficient time to exchange the rapidly exchanged nucleotide (~30 s). The time necessary for complete exchange was determined by a control experiment with 50 μM [^{3}H]ADP at ~10^{11} cpm/mmol. Samples were quenched with 200 μL of gramicidin–ADP–EDTA quenching buffer, pelleted, and washed 3 times with STN–DTT buffer as described above.

Mg[γ-^{32}P]ATP was incorporated into reconstituted CF_1 lacking tightly bound MgATP by incubation in the same medium used to label site 1, except that 50 μM [γ-^{32}P]ATP was used. After a 1-min incubation, the reaction was quenched with a gramicidin solution as described, except 0.7 mM ATP was used rather than 0.7 mM ADP.

Results

When *N*-[^{3}H]ethylmaleimide-labeled CF_1 is reconstituted onto sodium bromide treated membranes, about 1 nmol of

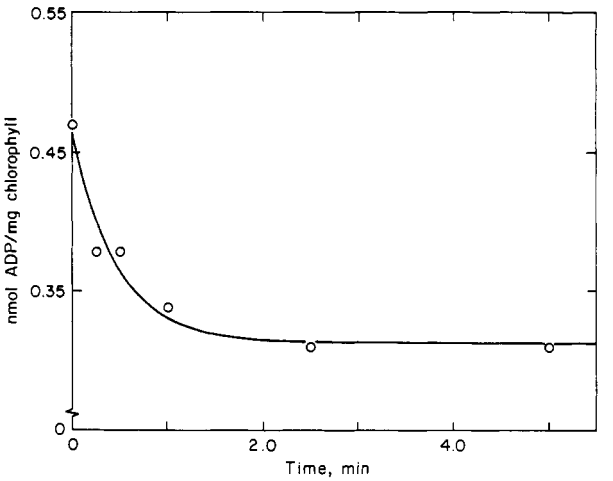

FIGURE 1: Typical plot of the stoichiometry of radioactive ADP bound to reconstituted membranes vs time during photophosphorylation. The final concentrations were 40 mM Tricine, 50 mM NaCl, 50 μM EDTA, 4 mM $MgCl_2$, 2 mM phosphate, 50 μM *N*-methylphenazonium methosulfate, 2 mM ascorbate, 50 μM ADP, and 50 μg/mL chlorophyll at pH 8.0 and 24 °C. The curve was calculated with eq 1 and the best-fit parameters $k = 2.1 \pm 0.4$ min^{-1}, $A_r = 0.16 \pm 0.03$ nmol of ADP/mg of chlorophyll, and $A(\infty) = 0.31 \pm 0.03$ nmol of ADP/mg of chlorophyll.

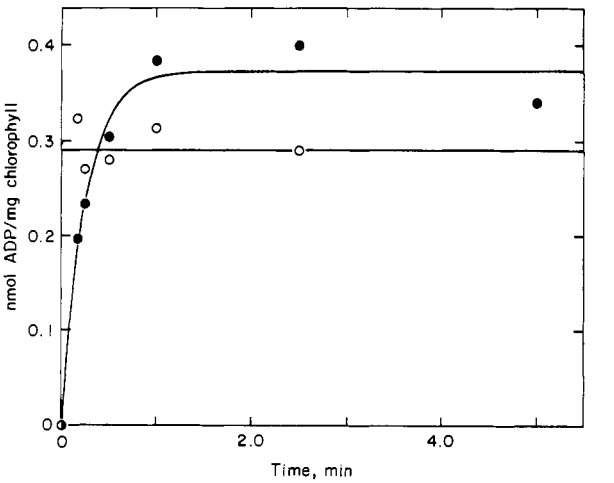

FIGURE 2: Representative plots of the time courses of initial ADP incorporation into reconstituted membranes (●) and ADP incorporation during steady-state ATP synthesis (○). Experiments were carried out at 24 °C in 50 μM [^{3}H]ADP, 4 mM $MgCl_2$, 2 mM phosphate, 40 mM Tricine, 50 mM NaCl, 50 μM EDTA, 2 mM ascorbate, and 50 μM *N*-methylphenazonium methosulfate. The curve for the initial ADP incorporation (●) was calculated with eq 2 and the best-fit parameters $A(\infty) = 0.49 \pm 0.05$ nmol of ADP/mg of chlorophyll and $k = 3.1 \pm 0.7$ min^{-1}.

CF_1/mg of chlorophyll is found to be associated with the reconstituted membranes. The *N*-ethylmaleimide has no effect on the synthetic activity, in agreement with published work (Patrie, 1984). Octyl glucoside activated Mg^{2+}-ATPase assays of reconstituted and CF_1-depleted membranes indicated 5–15% of the total CF_1 on reconstituted membranes is residual enzyme that was not removed by the sodium bromide treatment. A further indication of residual CF_1 on the stripped membranes is that the β-polypeptide was faintly visible after electrophoresis of NaBr-treated membranes on 13% sodium dodecyl sulfate–polyacrylamide gels.

Reconstitution of CF_1 containing nucleotide bound at site 1 (0.8–0.9 mol of [^{3}H]ADP/mol of CF_1) on NaBr-treated thylakoid membranes results in 0.3–0.5 nmol of [^{3}H]ADP bound/mg of chlorophyll. Several washings of the pellet or filtration on Whatman GF/F filters in the dark failed to remove the label. A negligible amount of the bound [^{3}H]ADP exchanges in the dark. Radioactive nucleotide bound at site 1 does, however, dissociate from the enzyme in a light-dependent manner. The initial light-induced release of [^{3}H]ADP from site 1 was measured in 50 μM *N*-methylphenazonium methosulfate, 2 mM ascorbate, 40 mM Tricine, 50 μM EDTA, 4 mM $MgCl_2$, 2 mM phosphate, and 50 μM ADP. Samples were illuminated and quenched as described under Materials and Methods. A typical time course is shown in Figure 1. Approximately 25–35% of the radioactive nucleotide is lost in the first 30 s. The time course can be described by a single-exponential decay:

$$A(t) = A_r \exp(-kt) + A(\infty) \quad (1)$$

where $A(t)$ is the nanomoles of [^{3}H]ADP per milligram of chlorophyll, A_r is the amount of rapidly exchanging A, $A(\infty)$ is the value of A at $t = \infty$, and k is the rate constant. The curve in Figure 1 was obtained by a nonlinear least-squares fit of the data to eq 1 with $k = 2.1 \pm 0.4$ min^{-1}, $A_r = 0.16 \pm 0.03$ nmol/mg, and $A(\infty) = 0.31 \pm 0.03$ nmol/mg. Under nonphosphorylating conditions (in the absence of 2 mM phosphate), the time course is similar: $k = 2.1 \pm 0.8$ min^{-1}, $A_r = 0.12 \pm 0.07$ nmol/mg, and $A(\infty) = 0.34 \pm 0.05$ nmol/mg.

Light-dependent incorporation of [^{3}H]ADP into CF_1 was compared to the initial release. The enzyme was reconstituted onto sodium bromide particles, and the membranes were washed to remove unbound CF_1. Nucleotide incorporation was under the same conditions as light-induced release during photophosphorylation, except [^{3}H]ADP was used in place of nonradioactive ADP. Unbound nucleotide was removed by three washes of the pellet. Time courses are well described by an exponential increase:

$$A(t) = A(\infty)[1 - \exp(-kt)] \quad (2)$$

where $A(t)$ is the amount of membrane-bound radioactivity at time t and k is the rate constant. The curve in Figure 2 was obtained by a nonlinear least-squares fit of the data to eq 2, with $A(\infty) = 0.49 \pm 0.05$ nmol of [^{3}H]ADP/mg of chlorophyll and $k = 3.1 \pm 0.7$ min^{-1}. Time courses obtained under nonphosphorylating conditions were similar. The levels of nucleotide incorporated ranged from 0.35 to 0.83 nmol of [^{3}H]ADP/mg of chlorophyll.

When radioactive nucleotide was incorporated into CF_1 by illuminating reconstituted membranes in the presence of 50 μM [^{3}H]ADP, the time course of light-induced release during photophosphorylation was similar to those in which [^{3}H]-ADP-labeled CF_1 was reconstituted with NaBr-treated membranes. The rate constant in this case was 3 ± 1 min^{-1} with 50 μM ADP. This indicates that the residual CF_1 does not alter the kinetics. Only about 40% of the radioactive nucleotide was released rapidly. The fact that only a fraction of the radioactive nucleotide in site 1 can be dissociated even when the radioactive nucleotide is incorporated directly into the reconstituted system suggests this behavior is an artifact of the experimental procedure rather than an intrinsic property of the binding site. The incomplete dissociation can be shown to be due to the pelleting and washing of the reconstituted system required to eliminate radioactive nucleotides and/or unbound CF_1. Pelleting and resuspension reduce both the ATP synthetic activity and the amount of exchangeable nucleotide in site 1, probably due to incomplete suspension and/or damage to the membranes. That this is not an intrinsic property of site 1 was unequivocally demonstrated by the following experiment. Reconstituted CF_1 was labeled at site 1 with 50 μM [^{3}H]ADP as previously described. Rather than measurement of dissociation of the [^{3}H]ADP after pelleting and

washing of the reconstituted membranes, nonradioactive ADP was added to 5 mM, and the rate of light-induced dissociation of [^{3}H]ADP from site 1 was measured as above. More than 85% of the [^{3}H]ADP was released from the reconstituted enzyme within 2 min, as compared to a theoretical release of 99%. Thus, site 1 behaves as a single population when the reconstituted enzyme is exactly the same during incorporation and dissociation of [^{3}H]ADP.

The possibility exists that initial rates of nucleotide exchange differ from those during steady-state ATP synthesis. The rate of [^{3}H]ADP incorporation during steady-state ATP synthesis was determined by illuminating reconstituted membranes (50–75 μg of chlorophyll/mL) for 30 s under phosphorylating conditions. In a separate experiment, it was determined that 30 s was sufficient for exchange of bound nucleotide from the rapidly exchanging population. After the initial incubation period, the [^{3}H]ADP was added to a final concentration of 50 μM (10^{11} cpm/mmol). Although complete exchange from the rapidly exchanging enzyme population requires 30 s, nucleotide incorporation during steady-state ATP synthesis is complete within the first 10 s of the assay (Figure 2). Examination of the reaction at earlier times was not possible with the method used. The photophosphorylation time course is linear within 5 s of initiation of the reaction. Prior to the addition of radioactive ADP, approximately 15% of the substrate was consumed. At 50 μM ADP, with the same enzyme preparation, the steady-state phosphorylation rate is 550 nmol (mg of chlorophyll)$^{-1}$ min^{-1}.

When CF_1 containing a stoichiometric amount of Mg[γ-^{32}P]ATP at site 2 is added to CF_1-depleted membranes, 0.35–0.55 nmol of [γ-^{32}P]ATP/mg of chlorophyll is detected. This label is not removed by repeated washings or by filtration and washing on Whatman GF/F filters. Retention of bound label on filters was 90–97%. Illumination of samples under conditions described for [^{3}H]ADP release assays resulted in no loss of label. Prolonged illumination (15 min) under steady-state photophosphorylating conditions also resulted in no loss of label. Approximately 0.2–0.3 nmol of Mg[γ-^{32}P]ATP/mg of chlorophyll is incorporated into reconstituted CF_1 lacking MgATP at site 2 above the background when MgATP is at site 2. This incorporation is light independent. Approximately 0.1 nmol of Mg[γ-^{32}P]ATP/mg of chlorophyll is incorporated in a light-dependent manner, presumably at site 1, with or without MgATP at site 2. The reduced incorporation into site 1 can be attributed to ATP hydrolysis at site 1 (Magnussen & McCarty, 1976). The rate of ATP synthesis is the same, with or without MgATP at site 2.

Discussion

The most important conclusion of this work is that the number and function of tight-binding nucleotide sites on soluble CF_1 are essentially identical with those of the membrane-bound coupling factor. The correspondence between the ATPase and synthetic activities of the enzyme also is demonstrated.

When either site 1 or site 2 is stoichiometrically labeled with radioactive nucleotides, ~0.4–0.6 nmol of CF_1 with radioactive nucleotide is bound per milligram of chlorophyll. If [^{3}H]ADP is incorporated into site 1 in the reconstituted system, a slightly higher stoichiometry is obtained (~0.4–0.8 nmol/mg) due to the additional residual CF_1 that is not stripped from the membranes by sodium bromide. The higher stoichiometry obtained when *N*-[^{3}H]ethylmaleimide-labeled CF_1 is reconstituted (~1 nmol/mg) is probably due to the binding of denatured CF_1 to the membrane: denatured enzyme would release nucleotides but not *N*-ethylmaleimide. Thylakoid membranes have been reported to have ~1 nmol of CF_1/mg of chlorophyll (Frasch et al., 1980). Although the method of reconstitution used produces vesicles of only modest activity, it permits almost quantitative separation of CF_1 from the membranes, thus permitting nucleotides to be placed on CF_1 in a well-defined manner.

The light-dependent incorporation and release of ADP and ATP to and from CF_1 on thylakoids have been well documented [cf. Strotmann and Bickel-Sandkötter (1977), Magnussen and McCarty (1976), and Schumann (1984, 1987)]. In this work, the light-dependent exchange rate and stoichiometry of nucleotide binding have been shown to be the same in the reconstituted system, regardless of whether CF_1 was labeled at site 1 before or after reconstitution. This directly demonstrates the equivalence of site 1 on soluble CF_1 with the rapidly exchanging nucleotide site of intact thylakoids. However, a pH gradient apparently is required for rapid exchange with the membrane-bound enzyme whereas only exogenous nucleotide is required with CF_1.[2]

The initial release and incorporation of nucleotides are somewhat slower than for thylakoids [cf. Strotmann (1984) and Schumann (1984)]. The reason for this difference is not apparent; the slower rate may be due to formation of a smaller pH gradient. However, the incorporation of [^{3}H]ADP during steady-state photophosphorylation is significantly faster than the initial exchange and comparable to catalytic turnover. The initial release of ADP from thylakoids during photophosphorylation also has been reported to be slower than the initial rate of photophosphorylation (Strotmann & Bickel-Sandkötter, 1977; Strotmann et al., 1976; Strotmann, 1984). With soluble CF_1, the initial rate of nucleotide dissociation from site 1 is considerably slower than the nucleotide exchange rate during steady-state turnover. The exchange rate during steady-state ATP hydrolysis is comparable to catalytic turnover (Leckband & Hammes, 1987). This again demonstrates the similarity in functional behavior of site 1 on and off the membrane and during ATP hydrolysis and synthesis.

When CF_1 containing radioactive MgATP at site 2 is reconstituted, the radioactive nucleotide is not lost either during illumination or during photophosphorylation. Similarly, MgATP remains tightly bound to site 2 on soluble CF_1 during ATP hydrolysis (Bruist & Hammes, 1982; Leckband & Hammes, 1987). This tightly bound MgATP clearly does not occupy a catalytic site. This site may serve a regulatory function. However, earlier studies with CF_1 demonstrated that occupation of this site by MgATP had no effect on the kinetics of ATP hydrolysis (Cantley & Hammes, 1975; Bruist & Hammes, 1982; Leckband & Hammes, 1987). Occupation of noncatalytic nucleotide binding sites of F_1 from beef heart mitochondria and *Escherichia coli* also has no effect on catalysis (Myers & Boyer, 1983; Perlin et al., 1984; Wise & Senior, 1985). The finding of two tightly bound nucleotides (sites 1 and 2) is consistent with the finding of approximately one ADP and approximately one ATP per purified coupling factor molecule (Cerione & Hammes, 1981). Harris and Slater (1975) report one ADP and two ATP's per enzyme molecule tightly bound to chloroplast membranes.

[2] Results obtained with CF_1–CF_o reconstituted into bacteriorhodopsin–phospholipid vesicles have demonstrated that both a pH gradient and exogenous nucleotide are required for rapid release of tightly bound ADP. The rate of nucleotide release is a linear function of the light intensity, with a slow residual exchange occurring in the absence of light (half-time of 15 min with 50 μM exogenous ADP). ADP dissociation in the absence of exogenous nucleotide is not observed (Leckband, 1988).

The number of nucleotide binding sites on chloroplast coupling factor is controversial. Studies with the membrane-bound enzyme and soluble CF_1 reveal *at least* three binding sites per molecule (Harris & Slater, 1975; Bruist & Hammes, 1981; Rosen et al., 1979). In addition to sites 1 and 2 studied in this work, a nucleotide binding site with a dissociation constant in the micromolar range has been found with CF_1 (Bruist & Hammes, 1981) and intact coupling factor (Cerione & Hammes, 1981). Equilibrium binding experiments with CF_1 indicate that additional binding sites have dissociation constants $>\sim 1$ mM (Bruist & Hammes, 1981). Evidence for additional binding sites has been recently reported (Xue et al., 1987). However, this work is flawed by the fact that only a single centrifuge column was used to remove loosely bound nucleotides from mixtures of the enzyme and relatively high concentrations of radioactive nucleotides. Although a single centrifuge column adequately removes free ligand, in our experience, a *minium* of two columns is necessary to remove all loosely bound ligands. Loosely bound ligands are not necessarily associated with specific nucleotide binding sites: specificity of binding must be established to interpret these experiments quantitatively.

Analogy with the mitochondrial and *E. coli* enzymes suggests that chloroplast coupling factor contains six nucleotide binding sites, three catalytic and three noncatalytic [cf. Kironde and Cross (1986) and Senior and Wise (1983)]. However, convincing evidence exists only for three nucleotide binding sites, two catalytic and one noncatalytic [cf. Bruist and Hammes (1981) and Leckband and Hammes (1987)]. This work, in conjunction with other results discussed above, demonstrates that the same three sites are associated with ATP hydrolysis by CF_1 and ATP synthesis by intact coupling factor. The incomplete loss of radioactive nucleotide from site 1 that is observed in this work has been shown to be the result of damage to the system caused by the pelleting–washing treatment required to obtain a sufficiently low background radioactivity. Some heterogeneity exists in the kinetic behavior of site 1 during the hydrolysis of ATP by CF_1 (Leckband & Hammes, 1987). This might be due to site 1 representing two different sites, but this is difficult to reconcile with the observed stoichiometry. A likely alternative is the existence of multiple conformational states of CF_1. Since additional nucleotide binding sites apparently will be characterized by relatively weak binding, the demonstration of specificity will be difficult. Perhaps the use of very tight-binding nucleotide analogues will provide a practical approach to this problem.

References

Arnon, D. I. (1949) *Plant Physiol. 24*, 1–15.

Binder, A., Jagendorf, A., & Ngo, E. (1978) *J. Biol. Chem. 253*, 3094–3100.

Bruist, M. F., & Hammes, G. G. (1981) *Biochemistry 20*, 6298–6305.

Bruist, M. F., & Hammes, G. G. (1982) *Biochemistry 21*, 3370–3377.

Cantley, L. C., Jr., & Hammes, G. G. (1975) *Biochemistry 14*, 2968–2975.

Cerione, R. A., & Hammes, G. G. (1981) *Biochemistry 20*, 3359–3365.

Chua, N. H. (1980) *Methods Enzymol. 69*, 434–446.

Frasch, W. D., Deluca, C. R., Kulzick, M. J., & Selman, B. (1980) *FEBS Lett. 122*, 125–128.

Harris, D. A., & Slater, E. C. (1975) *Biochim. Biophys. Acta 387*, 335–348.

Kironde, F. A. S., & Cross, R. L. (1986) *J. Biol. Chem. 261*, 12544–12549.

Krupinski, J., & Hammes, G. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4233–4237.

Leckband, D. (1988) Ph.D. Thesis, Cornell University.

Leckband, D., & Hammes, G. G. (1987) *Biochemistry 26*, 2306–2312.

Lien, S., & Racker, E. (1971) *Methods Enzymol. 23*, 547–555.

Magnussen, R. P., & McCarty, R. E. (1976) *J. Biol. Chem. 251*, 7417–7422.

Moroney, J. V., Lopresti, L., McEwen, B. F., McCarty, R. E., & Hammes, G. G. (1983) *FEBS Lett. 158*, 58–62.

Musier, K., & Hammes, G. G. (1987) *Biochemistry 26*, 5982–5988.

Myers, J. A., & Boyer, P. D. (1983) *FEBS Lett. 162*, 277–281.

Nelson, N., & Eytan, E. (1979) in *Cation Fluxes Across Biomembranes* (Mukahata, Y., & Packer, L., Eds.) pp 409–416, Academic, New York.

Patrie, W. (1984) Ph.D. Thesis, Cornell University.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Perlin, D. S., Latchney, L. R., Wise, J. G., & Senior, A. E. (1984) *Biochemistry 23*, 4998–5003.

Pick, U., & Basillan, S. (1981) *FEBS Lett. 133*, 289–297.

Rosen, G., Gresser, M., Vinkler, C., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 10654–10661.

Schumann, J. (1984) *Biochim. Biophys. Acta 766*, 334–342.

Schumann, J. (1987) *Biochim. Biophys. Acta 890*, 326–334.

Senior, A. E., & Wise, J. G. (1983) *J. Membr. Biol. 73*, 105–124.

Strotmann, H. (1984) in *Advances in Photosynthesis Research* (Sybesma, C., Ed.) Vol. 2, pp 477–484, Martinus Nijhoff, Boston.

Strotmann, H., & Bickel-Sandkötter, S. (1977) *Biochim. Biophys. Acta 460*, 126–135.

Strotmann, H., Bickel-Sandkötter, S., & Huchzermeyer, B. (1976) *FEBS Lett. 61*, 194–198.

Tausskey, H. H., & Shorr, E. (1953) *J. Biol. Chem. 202*, 675–685.

Winget, G. D., Kanner, N., & Racker, E. (1977) *Biochim. Biophys. Acta 460*, 490–499.

Wise, J. G., & Senior, A. E. (1985) *Biochemistry 24*, 6949–6954.

Xue, Z., Zhou, J., Melese, T., Cross, R. L., & Boyer, P. D. (1987) *Biochemistry 26*, 3749–3753.