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Mechanism of Phosphorylation Catalyzed by Chloroplast Coupling Factor 1. Stereochemistry†

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ABSTRACT: The reaction mechanism and substrate specificity of soluble chloroplast coupling factor 1 (CF_1) from spinach were determined by using the purified isomers of chromium–nucleotide complexes either as substrates for the enzyme or as inhibitors of the Ca^{2+}-dependent ATPase activity. The isolation of CrADP([^{32}P]P_i) formed upon the addition of the enzyme to [^{32}P]P_i and Λ-bidentate CrADP and the observation that the Λ-bidentate CrADP epimer was 20-fold more effective in inhibiting the Ca^{2+}-dependent ATPase activity than was the Δ epimer suggest that the substrate of phosphorylation catalyzed by CF_1 is the Λ-bidentate metal ADP epimer. Tridentate CrATP was hydrolyzed by soluble CF_1 to CrADP(P_i) at an initial rate of 3.2 μmol (mg of CF_1)$^{-1}$ min^{-1}, indicating that the tridentate metal ATP is the substrate for ATP hydrolysis. From these results a mechanism for the phosphorylation of ADP catalyzed by coupling factor 1 is proposed whereby the bidentate metal ADP isomer associates with the enzyme, phosphate inserts into the coordination sphere of the metal, and the oxygen of the β-phosphate of ADP attacks the inorganic phosphate by an S_N2 type reaction. The resulting product is the tridentate ATP ligand.

Soluble coupling factor 1 (CF_1),[1] the extrinsic membrane protein from spinach chloroplasts, neither has the ability to phosphorylate ADP nor will it catalyze appreciable rates of ATP–P_i or ADP–ATP exchange following isolation (Carmeli & Racker, 1973). Verification that CF_1 is responsible for energy transduction in higher plants has relied, therefore,

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; CrADP(P_i), (phosphato)chromium adenosine 5′-diphosphate; CD, circular dichroism; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Bes, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Mops, 3-(*N*-morpholino)-propanesulfonic acid; Chl, chlorophyll; PEI, poly(ethylenimine); RuBP, ribulose bisphosphate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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primarily on experiments which showed that reconstitution of CF_1 into CF_1-depleted thylakoids restored photophosphorylation activity (McCarty & Racker, 1966, 1967; Selman & Durbin, 1978).

The inability of soluble CF_1 to catalyze phosphorylation has made it difficult to determine the enzymatic mechanism. Elucidation of the mechanism of the energy-transducing system has been complicated further by the observation of multiple binding sites for nucleotides having a range of binding constants (Harris, 1978; Cantley & Hammes, 1975; Carlier & Hammes, 1979), all of which appear to bind too tightly to account for the observed rates of phosphorylation. Recent evidence now suggests that the tight binding of nucleotides to CF_1 regulates the enzymatic activity (Cantley & Hammes, 1979; Dunham & Selman, 1981; Shoshan & Selman, 1979).

Although many mechanisms for the reversible ATPase have been proposed (Kozlov & Skulachev, 1977; Young et al., 1974; Mitchell, 1974), there exists little data to support or reject any of them. Currently, the mechanism of photophosphorylation is believed to be a compulsory ordered bi-uni reaction where the binding of ADP precedes the binding of phosphate (Selman & Selman-Reimer, 1981). Very little information is available concerning the function of the metal in the reaction catalyzed by CF_1 because the magnesium- or calcium-chelated nucleotides exist in solutions as rapidly equilibrating mixtures of isomers which exchange at rates of 10^3–10^5 s^{-1}. However, for a few kinases, it has been possible to determine the isomer of the metal–nucleotide complex which possesses the proper structure to undergo catalysis by the use of purified isomers of chromium–nucleotide complexes (Dunaway-Mariano & Cleland, 1980a,b). Because chromium(III) ligands exchange 10^{10}–10^{13}-fold slower than those of magnesium(II) or calcium(II), such complexes have acted as dead-end inhibitors or, in some cases, as slow substrates for these enzymes (Dunaway-Mariano & Cleland, 1980a,b; DePamphilis & Cleland, 1973; Janson & Cleland, 1974a,b; Danenberg & Cleland, 1975). Recently, the mitochondrial coupling factor, F_1, has been observed to catalyze partial reactions of phosphorylation and ATPase with chromium nucleotides (Bossard et al., 1980).

We have examined the stereospecificity and structural specificity of the substrates for soluble CF_1 using isomers of CrATP and CrADP as probes. The inhibition kinetics of the ATPase activity by such isomers, and their ability to catalyze the formation of the intermediate of the reaction, (phospha-to)chromium adenosine 5′-phosphate [CrADP(P_i)], by soluble CF_1, provide evidence for a mechanism which involves phosphorylation of the Λ epimer of the bidentate metal–ADP ligand to form the tridentate metal–ATP complex as a product.

Experimental Procedures

Preparation of [γ-^{32}P]ATP. The reaction mixture for the synthesis of [γ-^{32}P]ATP contained, in 0.5 mL total volume, spinach thylakoid membranes (Selman & Selman-Reimer, 1981) at 20 μg of Chl/mL, 20 mM Tricine/NaOH (pH 8.0), 0.1 mM phenazine methosulfate, 3 mM ADP, 4 mM $MgCl_2$, 2.0 mM ascorbate, 20 μM [^{32}P]phosphate (3 mCi), and 10 mM KCl. This suspension was illuminated for 5 min at 20 °C. The chloroplasts were removed by centrifugation, and the [γ-^{32}P]ATP was purified by chromatography on PEI–cellulose as described by Magnusson et al. (1976).

Isolation of CF_1 from Spinach Thylakoid Membranes. CF_1 was prepared from spinach leaves by using a modification of the procedures described by Hesse et al. (1976) and Lien & Racker (1971). Deveined spinach leaves were homogenized for 20 s in 0.3 M sucrose and 10 mM sodium pyrophosphate buffer, pH 7.8, and strained through four layers of nylon mesh. The thylakoids were pelleted by centrifugation at 2000*g* for 15 min, resuspended to approximately 500 μg of Chl/mL in 10 mM sodium pyrophosphate, pH 7.8, and washed 3 times in this buffer at 4 °C to remove RuBP carboxylase. The membranes were pelleted by centrifugation at 8000*g* for 15 min.

After the third wash, the membranes were resuspended in buffer A, which contained 2.0 mM Tris–Tricine, pH 7.8, 0.3 M sucrose, and 0.1 mM ATP, to a concentration equivalent to 200 μg of Chl/mL and stirred slowly at room temperature for 20 min. The suspension was centrifuged at 30000*g* for 30 min, and the membranes were again resuspended in buffer A as before and removed by centrifugation. The above supernatants were combined and mixed with 90 mL of settled DEAE-Sephadex A-50, which had been equilibrated in 50 mM $(NH_4)_2SO_4$ and buffer B (40 mM Tris–SO_4, pH 7.1, 2 mM EDTA, and 1 mM ATP). All subsequent steps in the purification of CF_1 contained buffer B.

The DEAE-Sephadex A-50 slurry was stirred slowly for 1 h at room temperature. The gel was allowed to settle, and the clear supernatant was discarded. The gel was gently layered into a 2.5 × 25 cm column on top of 7 cm of DEAE-Sephadex equilibrated in the same buffer. The column was washed with 600 mL of buffer containing 50 mM $(NH_4)_2SO_4$ prior to the elution of CF_1 with 600 mL of buffer containing 300 mM $(NH_4)_2SO_4$. The protein was desalted by dialysis, adsorbed to a second DEAE-Sephadex A-50 column (2.5 × 25 cm), and eluted in a linear gradient of 100–300 mM $(NH_4)_2SO_4$ (total volume 800 mL). The protein ran as a single band on analytical polyacrylamide electrophoresis gels (Davis, 1964) and contained five subunits as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969). The protein was stored as a suspension in 55% ammonium sulfate and 2 mM ATP at 4 °C. Prior to use, aliquots of the suspension were centrifuged, and the protein was redissolved in 20 mM Tricine–NaOH, pH 8.0. The protein was desalted by the rapid centrifugation–chromatography procedure described by Penefsky (1977) and activated by incubation for 4 min at 60 °C in 35 mM ATP and 5 mM dithiothreitol as described by Lien & Racker (1971). Protein concentrations were determined by the Coomassie dye binding method (Bradford, 1976) using bovine serum albumin as the standard. In experiments to determine the substrate activity of chromium nucleotides by CF_1, the heat-activated protein was eluted with 40 mM Tricine, pH 8.0, through a 0.7 × 50 cm column containing Sephadex G-50 fine to remove ATP.

Assay for ATPase Activity. Reaction mixtures for ATPase activity contained, in 1.0 mL total volume, 200 mM 3-(*N*-morpholino)propanesulfonic acid–NaOH (pH 7.0), 10 mM $CaCl_2$ and 0.4–10 mM [γ-^{32}P]ATP (containing approximately 2 × 10^6 cpm/mL). In experiments where chromium–nucleotide complexes were used as inhibitors of the ATPase activity, the complex was added less than 2 s before the reaction was started by the addition of CF_1. Although most of the nucleotide complexes were stored at pH values below 5.0, the assay mixture was sufficiently buffered so that no change in pH was observed upon addition of the inhibitor. Reaction mixtures were incubated at 37 °C for 10 min followed by the addition of 5 mL of a mixture of 1.0% ammonium molybdate in 0.8 M $HClO_4$ and 0.2 mM KH_2PO_4. The complexed phosphate was extracted at 4 °C into 5 mL of a 1:1 (v/v) mixture of water-saturated isobutyl alcohol and benzene, and 0.5 mL of the organic phase was counted by liquid scintillation in a Packard 460 C liquid scintillation counter. Controls were treated identically but received no $CaCl_2$. Maximum rates

of ATPase activity were determined to be approximately 25–30 μmol of ATP hydrolyzed (mg of CF_1)$^{-1}$ min^{-1} in 20 mM Tricine, pH 8.0, 10 mM $CaCl_2$, and 10 mM [γ-^{32}P]ATP.

Preparation of Chromium–Nucleotide Complexes. The preparation of (phosphato)chromium adenosine 5′-diphosphate [CrADP(P_i)] was accomplished by the procedure outlined by Danenberg & Cleland (1975). First, bidentate CrADP was synthesized by heating distilled H_2O to 80 °C in a water bath after which solutions of $CrCl_3$ and ADP were added to a final concentration of 20 mM each. The temperature of this solution was maintained at 80 °C for 10 min and then rapidly cooled to 4 °C in a bath containing ethanol and ice. The bidentate CrADP was purified from unreacted reagents on a 2.5 × 25 cm Dowex 50-H^+ column at 4 °C. The Cr(III) and bidentate CrADP formed a bluish green band at the top of the column. This column was washed with water to remove unreacted ADP prior to the elution of the bidentate CrADP with 400 mM $HClO_4$.

Second, CrADP(P_i) was synthesized from bidentate CrADP. The pH of the purified bidentate CrADP solution was increased to 3.0 by the addition of solid K_2HPO_4. The solution was filtered, and then the temperature was raised to 80 °C where it was maintained for 5 min. The solution was rapidly cooled to 4 °C in a bath of ethanol and ice and filtered. Just prior to use, the CrADP(P_i) solution was passed through a 2 × 25 cm column of Sephadex G-10 by elution with distilled H_2O in order to lower its ionic strength.

The other CrADP and CrATP complexes were synthesized according to the procedures outlined by Dunaway-Mariano & Cleland (1980a,b). Purification of these complexes also followed the procedures of Dunaway-Mariano & Cleland (1980a,b) with the exception of β,γ-bidentate CrATP. This isomer was purified from the unreacted ATP and Cr(III) by chromatography on a 2.5 × 25 cm column of Dowex 50X-H^+ at 4 °C. The Cr(III) and β,γ-bidentate CrATP formed a bluish green band at the top of the column. The column was washed with distilled H_2O to remove unreacted ATP. This wash caused three bands to become apparent: a blue band at the top of the column containing the Cr(III), a clear band containing Na^+, and a blue-green band containing γ-monodentate, and β,γ-bidentate CrATP. Elution of the β,γ-bidentate CrATP was obtained with 100 mM acetate, pH 4.0. The green β,γ-bidentate CrATP elutes from the column first, followed by the elution of γ-monodentate CrATP. This method of purification minimized the conversion of β,γ-bidentate CrATP to the tridentate form.

Identification of each complex was made: (i) by spectrophotometry (Cleland & Mildvan, 1979), (ii) by calculating stoichiometric amounts of nucleotide from extinction coefficients and chromium and phosphate by the methods of Postmus & King (1955) and Chen et al. (1956), respectively, (iii) by circular dichroism spectroscopy (where applicable), (iv) by high-voltage electrophoresis (Bossard et al., 1980), and (v), for β-monodentate CrADP, by the ability to inhibit the enzymatic activity of hexokinase (Danenberg & Cleland, 1975).

The stereoisomers of bidentate CrADP were purified by chromatography on a 0.7 × 200 cm cycloheptaamylose column made by cross-linking cycloheptaamylose with epichlorohydrin (Cornelius & Cleland, 1978). Passing freshly made bidentate CrADP through the column 3 times (concentrating the epimers after each elution by lyophilization) yielded stereoisomers with molar ellipticities approximately equivalent to those reported by Dunaway-Mariano & Cleland (1980a,b). All experiments involving the stereoisomers were carried out within 24 h of their final purification to prevent the possibility of racemization or polymer formation.

Spectroscopic Measurements. Circular dichroism spectra were measured in quartz cuvettes having a 10-cm path length with a Jasco J-41C spectropolarimeter (Dunaway-Mariano & Cleland, 1980a,b). Dual-beam spectrophotometry was measured with an Aminco DW-2a spectrophotometer equipped with a Midan microprocessor data analyzer. The temperature of the samples was maintained by a block mounted in the cuvette chamber, thermostatically controlled by a Lauda K2/R water bath.

Data Analysis. For competitive inhibition, the kinetic data were fit to the equation

$$v_i = \frac{VS_i}{S_i + K_M(1 + I/K_{is})}(1 + e_i)$$

where v_i is the experimentally determined rate at a substrate concentration of S_i in the presence of the inhibitor I. The variance in the rate is e_i. The data were analyzed by computer to minimize $\sum e_i^2$, and points with deviations greater than 2.6σ were omitted. The data were also fit to the equation

$$v_i = \frac{VS_i}{S_i(1 + I/K_{ii}) + K_M(1 + I/K_{is})}(1 + e_i)$$

for noncompetitive inhibition (as above) for comparison.

Results

Stereochemistry of the ADP + P_i → ATP Reaction Catalyzed by CF_1. Chromium–nucleotide complexes have been found to serve as slow substrates with a few kinases (Dunaway-Mariano & Cleland, 1980a,b). However, due to the slow rate of exchange of chromium ligands, stable intermediates of the reaction are usually formed. Thus, the total number of turnovers of the enzyme with chromium nucleotides is small, and the product of catalysis is predominantly an intermediate of the reaction. This intermediate formed by the reactions catalyzed by CF_1 would be a (phosphato)chromium adenosine 5′-diphosphate complex [CrADP(P_i)]. Evidence that heat-activated, soluble CF_1 can catalyze the formation of CrADP(P_i) from bidentate CrADP and phosphate is presented in Figure 1. In this experiment, 2 mg of CF_1 was added to a solution containing 1 mM concentrations of bidentate CrADP and [^{32}P]phosphate. After the incubation of this solution, the protein was precipitated by the addition of concentrated acetic acid. The CrADP(P_i) formed during the reaction was separated from the unreacted substrate by chromatography on Dowex 50-H^+. The elution profile from the Dowex column of synthetic CrADP(P_i) is represented in Figure 1 as solid circles. The addition of CF_1 to the reaction mixture (open circles) resulted in a 4-fold increase in the amount of CrADP([^{32}P]P_i) that was formed from bidentate CrADP. This represents approximately 13 turnovers of the enzyme. These results are a direct indication that soluble CF_1 can catalyze this partial forward reaction, which strongly suggests that CF_1 is a reversible ATPase. This reaction was examined under a variety of conditions. The isomers of CrADP were found to be much more stable in Pipes buffer than in either Bes or Mops, and thus, using Pipes buffer in the assay yielded better results. Although long incubation times at 37 °C were initially employed to observe the synthesis of CrADP(P_i) mediated by CF_1, in later experiments incubation times as short as 90 min at 25 °C were found to be sufficient.

In Figure 1, (phosphato)chromium adenosine 5′-diphosphate was observed to elute from the Dowex column as two partially separated peaks. It has been shown previously (Dunaway-

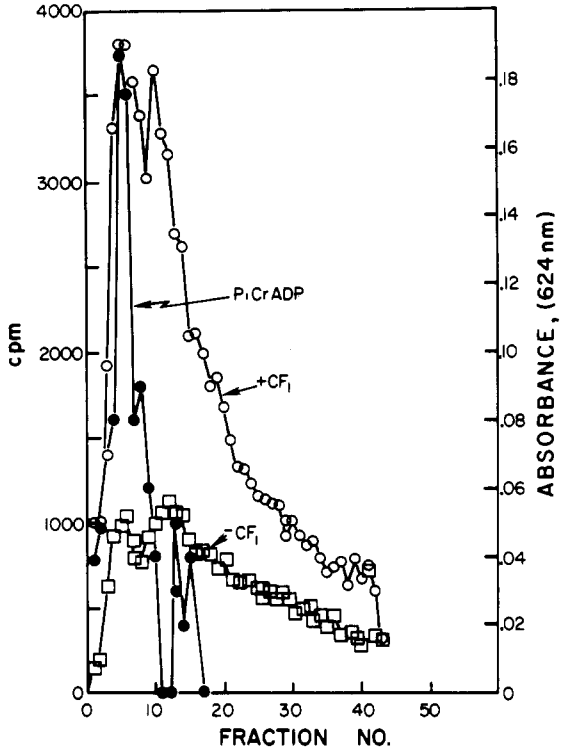

FIGURE 1: Chromatography of CrADP(P_i) formed from bidentate CrADP following incubation with CF_1. The reaction mixture contained 200 mM Pipes, pH 6.3, 1 mM [^{32}P]phosphate (5.85×10^6 cpm/mL), and 1 mM bidentate CrADP (5.3 mL total volume) and was incubated for 5 h at 30 °C in the presence (○) or absence (□) of 2 mg of heat-activated CF_1. The reaction was terminated by precipitating the protein with 2 drops of glacial acetic acid, and the protein was removed by centrifugation. An aliquot from each sample containing approximately 5×10^6 cpm was chromatographed on a 0.7×30 cm Dowex 50X-H^+ column. Unreacted [^{32}P]phosphate was removed with 120 mL of 10 mM HCl. The CrADP([^{32}P]P_i) was then eluted with 100 mL of 100 mM HCl, and 2-mL fractions were counted by liquid scintillation. The elution of synthesized CrADP(P_i) from an identical Dowex column was measured by its absorbance at 624 nm (●).

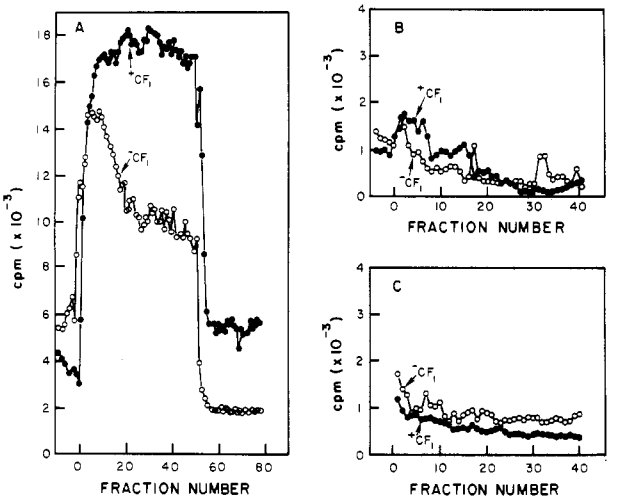

FIGURE 2: Comparison of the ability of CF_1 to catalyze the formation of CrADP(P_i) from CrADP isomers. The reaction mixture contained 150 mM Pipes, pH 6.3, 1 mM [^{32}P]phosphate (2×10^7 cpm/mL), and 1 mM freshly purified Λ-bidentate CrADP (A), Δ-bidentate CrADP (B), or β-monodentate CrADP (C) in 0.5 mL. The solutions were incubated at 25 °C in the presence (●) or absence (○) of 2 mg of heat-activated CF_1 for 90 min before termination of the reactions and chromatography of each sample as described in Figure 1.

Mariano & Cleland, 1980a,b) that the cis and trans epimers of CrADP(glucose-6-P) (the Cr forming a ligand to glucose-6-P via the phosphate) can be separated with Dowex 50-H^+ by using a similar elution protocol to that shown in Figure 1. Since the separation of the screw-sense isomers (Δ, Λ) has, to date, never been achieved by chromatography on Dowex 50-H^+, the two peaks of CrADP(P_i) apparent in the elution profile of Figure 1 and subsequent figures probably represent the separation of cis,trans isomers. It is likely that the enzymatically made CrADP(P_i) is enriched in the cis forms since, for ATP to be synthesized, the phosphate must coordinate adjacent to the β-phosphate of ADP. However, when CrADP(P_i) is synthesized, steric hindrance favors the formation of the trans epimers. This may explain why the enzymatically made CrADP(P_i) is enriched in the peak that elutes from Figure 1 second while the synthesized CrADP(P_i) contains more of the peak which is first to elute.

Purification of the two stereoisomers of bidentate CrADP (the Δ and Λ epimers) has been achieved by chromatography on cycloheptaamylose (Dunaway-Mariano & Cleland, 1980a,b). The ability of soluble CF_1 to catalyze the synthesis of CrADP(P_i) using the purified epimer of bidentate CrADP as substrates is shown in Figure 2. Neither monodentate CrADP nor the Δ epimer of bidentate CrADP demonstrated substrate activity. However, when the Λ-bidentate CrADP was incubated with [^{32}P]phosphate, the addition of heat-activated CF_1 increased the amount of CrADP(P_i) formed 2-fold above the amount which had formed in the absence of the enzyme. Such results suggest that the Λ epimer of ADP, which is bidentate to a divalent cation, has the correct conformation of the substrate for phosphorylation of CF_1.

In the experiment of Figure 2A, phosphate was able to coordinate the chromium(III) of Λ-bidentate CrADP to a significant extent in the absence of enzyme as observed for the racemic mixture of bidentate CrADP epimers in Figure 1. Although differences in stability between the monodentate CrADP and the bidentate CrADP under the conditions of the assay may explain the proportional lack of formation of CrADP(P_i) in solution from the monodentate isomer, we currently do not understand why so little CrADP(P_i) was formed in solution by the Δ-bidentate CrADP epimer. Despite the fact that such results have been replicated, this discrepancy appeared to indicate variability in the background from experiment to experiment.

Stereochemistry of the ATP → ADP + P_i Reaction Catalyzed by CF_1. Evidence that tridentate CrATP is the substrate for the ATPase activity catalyzed by CF_1 is presented in Figure 3A. The heat-activated enzyme was incubated with tridentate [γ-^{32}P]CrATP for 60 min. The protein was precipitated, and the solution was placed on a Dowex column equilibrated with 10 mM HCl. After the column was washed with 10 mM HCl to remove nucleotide which had dissociated from the chromium, CrADP([^{32}P]P_i) was eluted from the column with 25 mM NaCl. As observed with the elution profile in Figures 1 and 2, the CrADP(P_i) eluted as a double peak which probably consisted of cis and trans isomers (Dunaway-Mariano & Cleland, 1980a,b). The formation of CrADP([^{32}P]P_i) from tridentate CrATP was increased 3.5-fold by the presence of the enzyme. In contrast, when β,γ-bidentate [γ-^{32}P]CrATP was used as a substrate for the ATPase reaction of CF_1, the formation of CrADP([^{32}P]P_i) was not observed (Figure 3B). It was not possible to use this assay to determine the ability of γ-monodentate CrATP as a substrate because of its short lifetime at pH 6.3 (M. J. Bossard, G. S. Samuelson, and S. M. Schuster, unpublished results).

The visible absorbance spectra of equimolar concentrations of CrADP(P_i) and tridentate CrATP are shown in Figure 4.

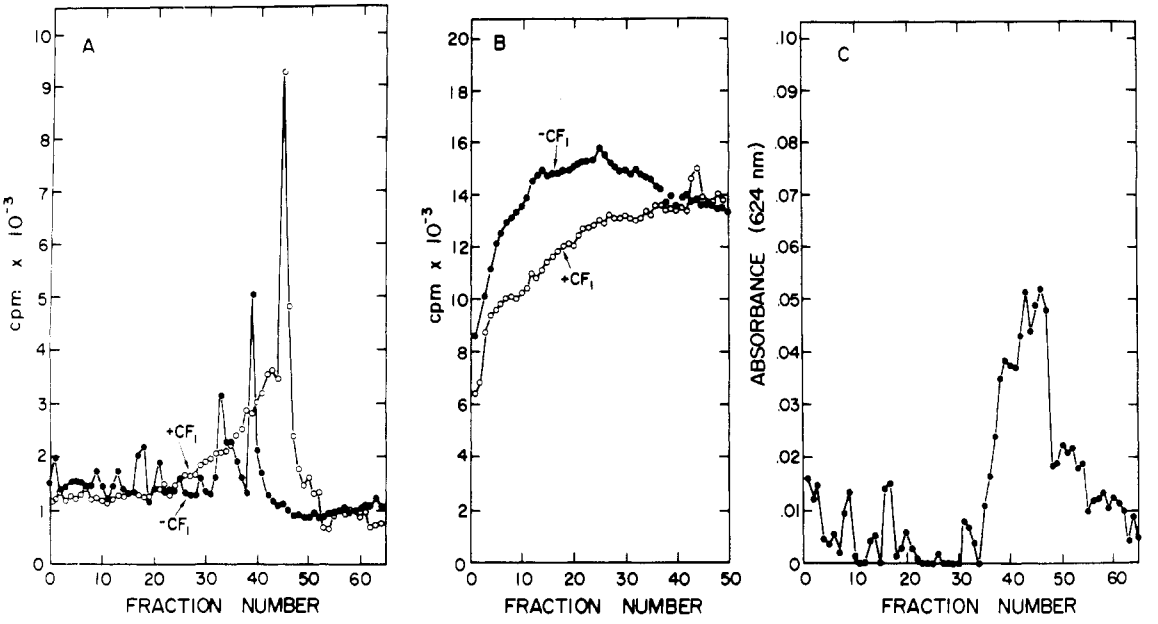

FIGURE 3: Comparison of the ability of CF_1 to catalyze the hydrolysis of tridentate CrATP vs. β,γ-bidentate CrATP. The reaction mixture contained 200 mM Pipes, pH 6.3, and either 1 mM tridentate [γ-^{32}P]CrATP (A) or 1 mM β,γ-bidentate [γ-^{32}P]CrATP (B) in 1.0 mL total volume. The solutions were incubated for 60 min at 23 °C in the presence (○) or absence (●) of 2 mg of heat-activated CF_1. The reaction was terminated by precipitating the protein with acid. An aliquot of each sample containing 6.7×10^5 cpm for the tridentate CrATP samples and 3.7×10^6 cpm for the β,γ-bidentate CrATP samples was chromatographed on a 0.7 × 10 cm Dowex 50X-H^+ column equilibrated with 10 mM HCl. ATP, which had dissociated from the chromium, was removed with 75 mL of 10 mM HCl. CrADP([^{32}P]P_i) was then eluted with 25 mM NaCl and collected in 2-mL fractions. The elution of synthesized CrADP(P_i) from an identical Dowex column was measured by its absorbance at 624 nm with cuvettes having a 5-cm path length (C).

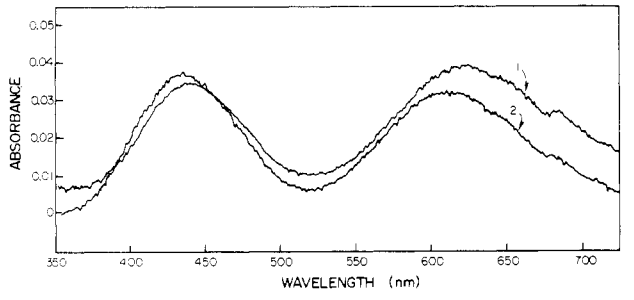

FIGURE 4: Visible spectra of 1.5 mM concentrations of CrADP(P_i) (curve 1) and tridentate CrATP (curve 2) in Pipes buffer, pH 6.3. The spectra were corrected for base-line deviations by a Midan computer. Concentrations were determined by analysis of chromium, phosphate, and adenine as described under Experimental Procedures.

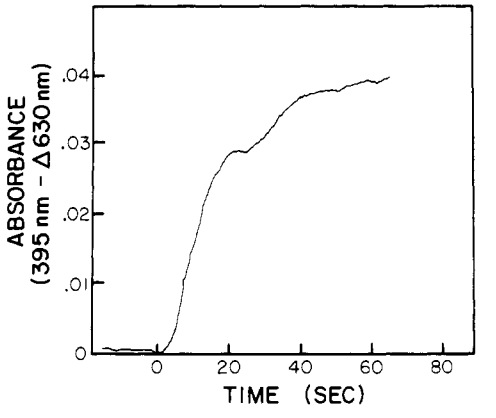

FIGURE 5: Initial rate of hydrolysis of tridentate CrATP by CF_1. A cuvette with a 10-cm path length contained 200 mM Pipes, pH 6.3, and 1 mM tridentate CrATP. The reaction was started by addition of 2 mg of CF_1 and followed at 30 °C by dual wavelength spectrophotometry.

The difference in absorbance between the CrADP(P_i) complex and tridentate CrATP in the 600–650-nm region of the spectrum was large enough to measure the conversion of nanomolar quantities of tridentate CrATP to CrADP(P_i) in a cuvette having a 10-cm path length.

Dual wavelength spectrophotometry was used to follow the conversion of tridentate CrATP to CrADP(P_i) as a function of time (Figure 5). The reaction was started by the addition of 2 mg of heat-activated CF_1 (1.2 μM final concentration) to a 10-cm cuvette containing the CrATP isomer at a concentration of 1 mM. The initial rate of formation of CrADP(P_i) at 30 °C was calculated to be 3.2 μmol (mg of CF_1)$^{-1}$ min^{-1} as measured by the absorbance change at 630 nm. This rate continued for approximately 30 s.

Complexes of γ-monodentate, β,γ-bidentate, and tridentate chromium ATP, purified by chromatography on Dowex-50X-2H^+ (Dunaway-Mariano & Cleland, 1980a,b), were examined to determine if they were dead-end inhibitors of the calcium-dependent ATPase activity of soluble CF_1. When the CrATP complexes were added to heat-activated CF_1 in the presence of variable amounts of substrate just prior (<2 s) to the assay of calcium-dependent ATPase activity, the resulting pattern of initial velocities for each of the three complexes demonstrated competitive kinetics. A summary of the K_{is} values of the CrATP complexes for ATPase activity with respect to Ca-ATP, calculated as the average of four repli-

Table I: Inhibition Constants for Cr-Nucleotide Complexes[a]

	K_{is} (μM)
γ-monodentate CrATP	155 ± 18.5
β,γ-bidentate CrATP	724 ± 56.0
tridentate CrATP	186 ± 3.5
β-monodentate CrADP	17 ± 3.1
bidentate CrADP	111 ± 36.8

[a] K_{is} values were determined by computer analysis as described under Experimental Procedures. The K_{is} values presented below are the average values of four replications.

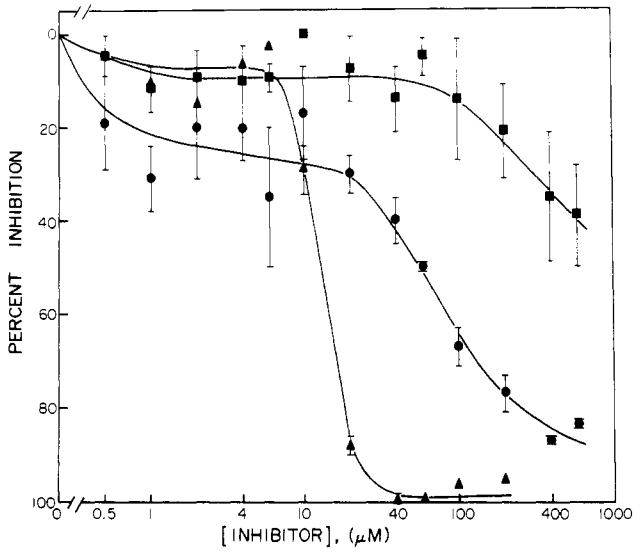

FIGURE 6: Dependence of the inhibition of calcium-dependent ATPase activity, catalyzed by CF_1, on the concentration of the stereoisomers of CrADP. Purification of the stereoisomers of bidentate CrADP and assay of ATPase activity are described under Experimental Procedures. The isomers used were the Λ-bidentate CrADP (●), Δ-bidentate CrADP (■), and β-monodentate CrADP (▲). The data from each curve are expressed as the average of three experiments. The substrate concentration was 0.5 mM CaATP (pH 6.3), and the average rate of ATPase activity was 3.0 μmol (mg of CF_1)$^{-1}$ min^{-1} without inhibitor. The average rate at pH 8.0 with 10 mM CaATP was 28 μmol (mg of CF_1)$^{-1}$ min^{-1}.

cations, is presented in Table I. The K_{is} for β,γ-bidentate CrATP (724 μM) is approximately 4-fold higher than the K_{is} for γ-monodentate CrATP (155 μM) or tridentate CrATP (186 μM). These results indicate that all three types of CrATP complexes can interact with the active site of CF_1 and confirm the earlier observation of the greater preference of CF_1 for tridentate CrATP than for β,γ-bidentate CrATP (Figure 3). The K_{is} of the γ-monodentate CrATP was comparable to that for tridentate CrATP, and thus, the possibility that the monodentate isomer can act as a substrate or product cannot be eliminated.

The evidence presented in Figure 2 suggests that the substrate of CF_1-catalyzed phosphorylation is the Λ-bidentate metal–ADP ligand. However, it cannot be concluded a priori that this isomer is also the product of the ATPase of CF_1. The isomers of CrADP were examined as inhibitors of the ATPase activity of soluble CF_1 in order to test this hypothesis. Both β-monodentate CrADP and bidentate CrADP competitively inhibited the ATPase activity of heat-activated CF_1. The average K_{is} values of four replications for each of these complexes (Table I) were 111 μM and 17 μM for bidentate CrADP and β-monodentate CrADP, respectively. These results suggest that β-monodentate CrADP is a much more potent inhibitor of the ATPase activity than is the bidentate form.

Figure 6 shows the average results of three experiments in which various concentrations of the purified epimers of bidentate CrADP were added to heat-activated CF_1 in the presence of 0.5 mM CaATP, a rate-limiting substrate concentration. The necessity of using low substrate concentrations in order to observe inhibition as well as the requirement of assaying at pH 6.3 (for stability of the CrADP complexes) limited the control rates of ATPase activity to 3 μmol (mg of CF)$^{-1}$ min^{-1} and, thus, contributed to the large amount of error observed in this experiment. Whereas millimolar concentrations of the epimer of bidentate CrADP having a negative CD absorbance were required to observe 50% inhibition of CF_1-catalyzed ATPase activity, the epimer of bidentate CrADP with a positive CD absorbance was approximately 20-fold more effective, inhibiting to 50% at 60 μM. Since CF_1 can catalyze the formation of CrADP(P_i) from the Λ-bidentate CrADP isomer, the increased ability of the epimer of bidentate CrADP having a positive CD spectrum (the Λ epimer) to inhibit ATPase activity supports the hypothesis that it, and not the Δ epimer, is the substrate of phosphorylation catalyzed by CF_1.

The concentration dependency of β-monodentate CrADP on the inhibition of ATPase activity is also shown in Figure 6. The concentration required to observe 50% inhibition with the monodentate isomer (approximately 15 μM) was lower than that with either of the bidentate stereoisomers. The effectiveness of the monodentate Cr–nucleotide complexes to inhibit Ca^{2+}-dependent ATPase activity may be explained by the ability of the adenine to rotate about the chromium ligand. This flexibility allows these complexes to fit into the active site, but because they lack the proper ligands, catalysis cannot occur.

Discussion

Our data suggest that the substrate of phosphorylation catalyzed by CF_1 is the Λ (positive) epimer of the bidentate metal–ADP complex (a) by the observation of an increase of the CrADP([^{32}P]P_i) intermediate that is formed in solution from phosphate and Λ-bidentate CrADP upon addition of the enzyme and (b) by a 20-fold difference in the concentration of the Λ vs. the Δ epimer of bidentate CrADP required to inhibit Ca-dependent ATPase activity. The substrate of ATPase activity (and, therefore, the most plausible product of the ATP synthesis reaction) was shown to be the tridentate metal ATP ligand by means of (a) isolating the CrADP([^{32}P]P_i) intermediate that is formed as a product of hydrolysis of tridentate Cr[γ-^{32}P]ATP in the presence of CF_1, (b) measuring an increase in absorbance of tridentate CrATP at 630 nm using dual wavelength spectrophotometry, and (c) determining that the K_{is} of tridentate CrATP for Ca^{2+}-dependent ATPase activity is 4-fold lower than the K_{is} observed for β,γ-bidentate CrATP. From these results, we propose the stereochemical mechanism shown in Figure 7 for chloroplast coupling factor 1. The features of this mechanism will be described in the ADP to ATP direction.

In the first step, the binding of Λ epimer of the bidentate metal ADP complex to the enzyme (Figures 1 and 6) precedes the association of the enzyme with phosphate (Selman & Selman-Reimer, 1981). Delocalization of the negative charges of the α- and β-phosphates of ADP by the metal allows one of the oxygens of the inorganic phosphate to be inserted into the coordination sphere of the metal (Figure 1), thus bringing the nucleotide and phosphate into close proximity.

In the second step, we propose that catalysis may occur by the attack of the hydroxyl associated with the β-phosphate of the ADP into the inorganic phosphate by an S_N2 mechanism similar to the mechanism previously proposed by Mitchell (1974). A number of other enzymes which transfer phosphate via a metal ATP ligand are also thought to use this mechanism (Dunaway-Mariano & Cleland, 1980a,b; Jaffe & Cohn, 1979; Yee & Eckstein, 1980). It is tempting to postulate that the enzyme induces the susceptibility of phosphate to nucleophilic attack by selectively protonating its free hydroxyl, making this hydroxyl a good leaving group. Such a hypothesis would imply a direct catalytic role for protons supplied from the protonmotive force. The resulting product of phosphorylation is the tridentate metal ATP ligand (Figure 3).

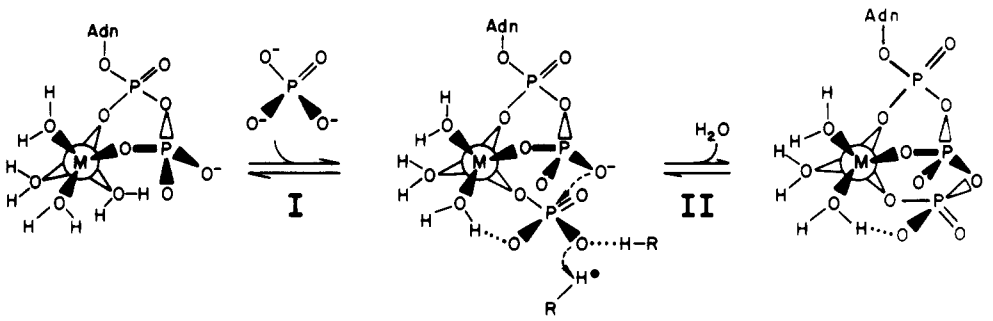

FIGURE 7: Proposed mechanism of phosphorylation catalyzed by CF_1. Step I: Inorganic phosphate forms a ligand to the metal (M) of the Λ-bidentate metal–ADP complex. Step II: The oxygen of the β-phosphate attacks the phosphorus of the inorganic phosphate by an S_N2-type mechanism. The resulting product is the tridentate metal–ATP complex.

We have, thus far, tacitly assumed that CF_1 uses the identical form of the substrate of the ATPase reaction (tridentate MgATP) as the product in the ATP synthesis reaction. Bossard & Schuster (1981) have recently proposed that beef heart mitochondrial coupling factor, F_1, hydrolyzes ATP at one active site and synthesizes ATP at a second site which has a different stereochemistry from the ATPase site. The low K_{is} values of CF_1-catalyzed ATPase activity observed for γ-monodentate CrATP and β-monodentate CrADP in Table I might suggest that CF_1 has separate active sites for the synthesis and hydrolysis of ATPase proposed for F_1. However, much more substantial proof is required to confirm it.

Support for the model presented in Figure 7 is found in experiments using thiophosphate analogues of ADP as substrates for these reactions (Strotman et al., 1977, 1979). Divalent magnesium prefers to ligate to oxygen rather than sulfur (Cohn, 1979); thus, the bidentate complex between the A form of the ADPαS analogue and Mg^{2+} has been determined to be the Λ epimer (Burgers & Eckstein, 1978, 1980). Strotmann et al. (1977, 1979) have observed that chloroplasts will phosphorylate the A form of ADPαS at a rate almost equivalent to that of ADP, while the ADPαS,B analogue is phosphorylated much more slowly in the presence of Mg(II). We have recently confirmed these results, observing that the maximal velocity of photophosphorylation of ADPαS,A with Mg(II) was 5-fold greater than that observed with ADPαS,B while the K_M of ADPαS,A was almost 3-fold smaller than the K_M of the B isomer (W. D. Frasch and B. R. Selman, unpublished results). Strotmann et al. (1977, 1979) have also observed that ATPαS,A is hydrolyzed by soluble CF_1 faster than ATPαS,B in the presence of Ca(II) (a metal which also prefers coordination to oxygen than to sulfur). These results lend further support to the hypothesis that the Λ epimer of the bidentate metal ADP ligand is the substrate of phosphorylation and that the tridentate ATP ligand is the substrate of the ATPase. However, Jaffe & Cohn (1979) have found that although creatine kinase prefers the B isomer of ATPαS with Mg, the specificity was not reversed with Cd (cadmium prefers to coordinate sulfur to oxygen). Thus, they concluded that the substrate of creatine kinase was not the tridentate ATP ligand but a β,γ-bidentate isomer of ATP. Similar experiments using Cd with the nucleotide phosphorothioate analogues as substrates of CF_1 must be examined before the results of Strotmann et al. (1977, 1979) can be considered conclusive.

We believe that the mechanism presented in Figure 7 is not in conflict with data that have given rise to earlier models (Kozlov & Skulachev, 1977; Young et al., 1974; Mitchell, 1974) but that the results presented here specify the role of the metal and also perhaps of the protons from the protonmotive force used in the synthesis of ATP mediated by CF_1.

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Compartmental Analysis of Light-Induced Proton Movement in Reconstituted Bacteriorhodopsin Vesicles†

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ABSTRACT: Purified bacteriorhodopsin from purple membrane sheets isolated from *Halobacter halobium* was solubilized with a bile salt detergent, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS). The detergent-solubilized protein was then incorporated into lecithin vesicles at either high (450:1) or low (65:1) lipid to protein ratios. Circular dichroism studies showed that the bacteriorhodopsin incorporated was in a monomeric form in the 450:1 vesicles. The 65:1 vesicles exhibited an exciton splitting characteristic of the aggregated state of bacteriorhodopsin. We then examined the light-induced movement of protons for these two preparations. Compartmental analysis was used to derive a kinetic model for the observed proton movement. The pumping was qualitatively the same for monomeric and aggregated protein. A three-compartment model provided an excellent description of proton movement in both sets of vesicles and at four different light intensities. This model demands two independent processes to account for the proton movement. The rate coefficients for both are linearly related to light intensity. However, the total flux of protons via one of these processes diminishes as a function of the hydrogen ion accumulation within the vesicles.

For the past decade bacteriorhodopsin (BR) has been an object of intensive study both as a membrane protein whose detailed structure has been extremely amenable to study (Oesterhelt & Hess, 1971; Henderson, 1977) and as a light-driven proton pump (Oesterhelt & Stoeckenius, 1973; Racker & Stoeckenius, 1974; Bogomolni & Stoeckenius, 1974). Information on the photocycle has provided the basis for ideas about the mechanism linking capture of photons to translocation of protons across the membrane. Nevertheless, the exact molecular details of pumping are not known (Slifkin & Caplan, 1975; Marcus & Lewis, 1977). Several groups have reconstituted BR into phospholipid vesicles in order to examine, in controlled lipid environments, the process of light-induced proton movement (Racker, 1973; Hellingwert et al., 1978). By using a detergent dialysis technique, we have reconstituted BR into uncharged phosphatidylcholine vesicles. By variation of the lipid to protein ratio, the resulting vesicles contain either monomeric or multimeric BR as judged by the absence or presence, respectively, of an exciton splitting as determined by circular dichroism (Heyn et al., 1975). We then measured light-induced pumping in order to analyze the kinetics of proton movement over time periods that were long with respect to the photocycle in order to observe the characteristics of the pump under the "physiologic" condition of prolonged irradiation. The purpose of this study is 2-fold: (1) to investigate whether the numerical data on buildup and decay of light-induced pH gradients point to the existence of multiple proton compartments and (2) to determine whether any differences are apparent between the proton pumping of monomeric vs. multimeric BR. Compartmental analysis is used in this study to characterize proton movement. One of the great advantages of compartmental analysis is that it allows an association of discrete processes with each compartment. Previous work by Eisenbach et al. (1978), using a standard fit to sums of exponentials, suggested that two processes are involved in light-induced proton movement. The results of our compartmental analysis support those conclusions and provide an explicit model to explain both the light-induced pH changes and the reversal of those changes when illumination ceases. By examining pH changes as a function of illuminating intensities, we can relate the rate constants of the various processes to the energy input. Recently, proton pumping has been observed in lipid vesicles containing monomeric BR (Dencher & Heyn, 1979). The studies reported here show that the

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