Isolation, Purification, and Characterization of Coupling Factor 1 from *Chlamydomonas reinhardi*†

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ABSTRACT: Chloroplast thylakoid particles were prepared from wild-type *Chlamydomonas reinhardi* by gentle sonication. These particles catalyzed phenazine methosulfate dependent photophosphorylation with rates ranging from 300 to 700 μmol of adenosine 5'-triphosphate (ATP) formed (mg of chlorophyll)$^{-1}$ h^{-1}. Photophosphorylation was not sensitive to tentoxin but was sensitive to an anticoupling factor 1 (CF_1) antiserum preparation made against spinach CF_1. The *C. reinhardi* chloroplast CF_1 was isolated from thylakoid particles by either chloroform or ethylenediaminetetraacetic acid extraction. The former enzyme appeared to be missing the δ subunit and did not reconstitute with partially resolved thylakoid particles. The latter enzyme reconstituted with partially resolved particles and had a specific activity at 37 °C of 2–5 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}. The enzyme utilized both MnATP and MgATP. CaATP was a poor substrate, and SrATP was not hydrolyzed. The enzyme was not activated by heat or proteolysis but was stimulated ~2-fold by 50 mM dithiothreitol. Alcohols reversibly stimulated the ATPase activity of the enzyme 5–25-fold. Ethanol, 20%, dramatically lowered the temperature optimum from ~75 to ~45 °C and slightly lowered the pH optimum from 8.5 to 8.2. Ethanol had no effect on the activation energy of the ATPase reaction (17 ± 1.7 kcal/mol). The kinetics of the ATPase reaction catalyzed by the *C. reinhardi* enzyme are complex. Both free divalent cations and divalent cation ATP inhibited the activity of the enzyme. The apparent K_M for MgATP (55 μM free Mg^{2+}) was ~0.2 mM.

The chloroplast coupling factor 1 protein (CF_1),[1] the extrinsic membrane protein sector of the energy-transducing reversible ATPase, has been isolated from a large variety of higher plants including spinach, lettuce, Swiss chard, peas, radishes, and various tobacco species and from the protozoan *Euglena gracilis* (Chang & Kahn, 1966; Lien & Racker, 1971; Nelson, 1976; Selman & Durbin, 1978). In all instances where it has been examined, the enzyme appears to contain five nonidentical subunits, although the stoichiometry of the subunit composition is still disputed (Baird & Hammes, 1979). In most cases, with the exception of the *E. gracilis* enzyme (Chang & Kahn, 1966), the ATPase activity of the enzyme is latent and is only expressed after activation by either proteolysis, heat treatment, or incubation of the enzyme in the presence of a high concentration of thiol reducing agents (Farron & Racker, 1970; Petrack et al., 1965; Vambutas & Racker, 1965). The soluble enzyme is usually assayed as a CaATPase (McCarty & Racker, 1968), although Nelson et al. (1972) have described a MgATPase activity for the enzyme isolated from spinach when measured in bicarbonate or maleate buffers.

Reconstitution of ATP synthetase enzymatic activity has been demonstrated by Selman & Durbin (1978), who reconstituted tentoxin-sensitive spinach membranes with tentoxin-resistant CF_1 and titrated the restored activity with tentoxin. It has also been demonstrated by Nelson & Hauska (1979), who, after completely removing CF_1 from spinach membranes, reconstituted photophosphorylation, and by Berzborn & Schröer (1976), who showed that the reconstituted activity of partially resolved membranes was greater than that which could be expected for the residual CF_1 remaining with the membranes after extraction. Reconstitution is usually taken as evidence that the enzyme that binds to extracted membranes does so specifically, although other compounds [e.g., dicyclohexylcarbodiimide (McCarty & Racker, 1967) and triphenyltin chloride (Gould, 1976)] can behave as "coupling factors".

In this paper, we describe the isolation and purification of the chloroplast CF_1 from wild-type *Chlamydomonas reinhardi*. Its subunit composition is similar to that of higher plant ATPases; however, in contrast to other ATPases, it is specific for Mg^{2+} or MnATP, and the ATPase activity is not latent. It cross-reacts with a rabbit anti-CF_1 antiserum made against spinach CF_1, and the antibody inhibits both photophosphorylation with *C. reinhardi* thylakoid particles and the ATPase activity of the isolated enzyme. In addition, we show that the ATPase activity of the *C. reinhardi* enzyme can be stimulated 5–25-fold in 20% ethanol. The effect of ethanol is to slightly change the pH optimum of the enzyme and to dramatically change the temperature optimum. Finally, we demonstrate that the *C. reinhardi* ATPase reconstitutes with partially resolved *C. reinhardi* thylakoid particles.

Materials and Methods

Materials. [γ-^{32}P]ATP (Magnusson et al., 1976), tentoxin (Schadler et al., 1976), spinach CF_1 (Lien & Racker, 1971; Binder et al., 1978; Strotmann et al., 1973), and the anti (spinach) CF_1 antiserum (Frasch et al., 1980) were prepared as previously described. [^{32}P]Phosphate was purchased from New England Nuclear. All other materials were of reagent grade quality or better.

C. reinhardi Cultures. *C. reinhardi* wild-type strains 137c and 4j were the generous gift of Dr. Robert Togasaki, Department of Biology, Indiana University, Bloomington, IN. Small (300-mL) cultures were stirred and grown at room temperature in dim light in a medium containing 15.0 mL of

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[1] Abbreviations used: CF_1, chloroplast coupling factor 1; PMS, phenazine methosulfate; EDTA, ethylenediaminetetraacetic acid; Chl, chlorophyll; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; Tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; PP_i, inorganic pyrophosphate.

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Beijerinck's solution, 0.3 mL of Hutner's trace elements (Hutner, 1950), 1.0 mM potassium phosphate, and 20 mM Tris–acetate (pH 7.3) until they reached an absorbance of about 0.8–1.2 at 650 nm. These cultures were then used either directly for photophosphorylation experiments or for inoculation of 1.0-L flasks for the isolation of small quantities of the ATPase. The 1.0-L cultures were maintained at room temperature and in dim light and grown to an absorbance of ~1 at 650 nm. For isolation of larger quantities of the ATPase, four 1-L cultures (absorbance ~1 at 650 nm) were used to inoculate an 80-L fermentor. The growth medium in the fermentor was identical with the growth medium for the small 300-mL cultures. The temperature was maintained at 25 °C, the culture was illuminated with dim incandescent light, and the medium was well aerated. The cells were grown to an absorbance of 0.8–to 1.0 at 650 nm, harvested by continuous centrifugation, and further treated as described below. Identical results were obtained with either wild-type strain.

Preparation of Thylakoid Particles. The cells from one 300-mL culture flask were collected by centrifugation (200*g* for 5 min) and washed in 50 mM Tricine–NaOH (pH 8.0) buffer. The cells were resuspended in 50 mM Tricine–NaOH (pH 8.0) buffer to an equivalent concentration of 0.5 mg of chlorophyll/mL and sonicated in 3.0-mL batches at room temperature in a bath-type sonicator (Laboratory Supplies Co., Inc., power output 80 kHz, 80 W) for 20 s. When large batches of cells were processed, a flow-through cell was devised such that the average time that any cell was exposed to sonication was 20 s. The resulting suspension was centrifuged at 200*g* for 5 min to remove unbroken cells. These cells were resuspended in Tricine buffer and recycled. Thylakoid particles were pelleted by centrifugation at 30000*g* for 10 min.

When used for phosphorylation experiments, the particles were resuspended in buffer containing 20 mM Tricine–NaOH (pH 8.0), 0.5 mM $MgCl_2$, 0.3 M sucrose, and 10 mM NaCl and maintained at 0–4 °C until used. When used to prepare ATPase, the particles were resuspended and washed 3 times in 10 mM $NaPP_i$ (pH 7.8) (~0.5 mg of Chl/mL) and centrifuged at 30000*g* for 10 min.

Photophosphorylation. Reaction mixtures for photophosphorylation contained, in a total volume of 1.0 mL, thylakoid particles equivalent to 10 μg of chlorophyll/mL, 20 mM Tricine–NaOH (pH 8.0), 100 μM phenazine methosulfate (PMS), 3 mM ADP, 5 mM [^{32}P]phosphate (~2 × 10^6 cpm/mL), 0.5 mM $MgCl_2$, 10 mM NaCl, and 1 mM sodium ascorbate. Reaction mixtures were maintained at 19 °C and illuminated for 0.5 min with white light isolated from a 150-W tungsten–halogen lamp (~40 000 lx). The light beam was focused on the reaction mixture after passing through 5 cm of 5% $CuSO_4$. Esterified phosphate was extracted as previously described (Selman, 1976) and counted in a Packard 460 C liquid scintillation counter.

Isolation of C. reinhardi ATPase. (*A*) *Chloroform Extraction.* After having been washed 3 times with 10 mM $NaPP_i$ (pH 7.8), the *C. reinhardi* thylakoid particles were resuspended to a concentration equivalent to 1–3 mg of chlorophyll/mL in 20 mM Tricine–NaOH (pH 8.0) buffer containing 1 mM EDTA, 1 mM ATP, and 2 mM dithiothreitol. An equivalent half-volume of redistilled chloroform was added and the suspension stirred at room temperature for 20 s. The phases were broken by slow-speed centrifugation (200*g* for 1.0 min), and the chloroform phase was removed. The supernatant was centrifuged for 1.5 h at 30000*g* at 15 °C. The supernatant was concentrated to ~5.0 mL by pressure dialysis (Amicon PM 10 filter) and dialyzed for 18 h against 1 L of buffer containing 20 mM Tris–SO_4 (pH 8.0), 1 mM EDTA, 0.1 mM ATP, and 1.0 mM dithiothreitol with two changes of buffer. The dialyzate was centrifuged at 30000*g* for 1 h at 15 °C to remove precipitated protein and applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm) equilibrated with 20 mM Tris–SO_4 (pH 8.0), 10 mM ammonium sulfate, 1.0 mM ATP, and 1.0 mM EDTA. The column was washed with a 600-mL linear ammonium sulfate gradient (10–300 mM) in 20 mM Tris–SO_4 (pH 8.0), 1.0 mM ATP, and 1.0 mM EDTA. The ATPase eluted from the column in 160–200 mM ammonium sulfate (total volume ~60 mL). Fractions were analyzed for ATPase activity and by nondissociating polyacrylamide gel electrophoresis. Fractions of highest purity were combined and concentrated to ~2.0 mL by pressure dialysis, and the protein was precipitated by the addition of an equal volume of saturated ammonium sulfate in 20 mM Tris–SO_4 (pH 8.0) buffer containing 1.0 mM EDTA. The protein was stored as an ammonium sulfate suspension at 0–4 °C without any noticeable loss of ATPase activity.

(*B*) *EDTA Extraction.* Three times $NaPP_i$ washed thylakoid particles were resuspended in distilled water to a concentration equivalent to 0.1 mg of chlorophyll/mL. At room temperature, EDTA was added to a final concentration of 0.75 mM. The pH was maintained between 7.2 and 7.6 by the addition of 0.02 N NaOH. The suspension was stirred for 10 min, and the membranes were pelleted by centrifugation at 30000*g* for 1.5 h. Initially, the ATPase was isolated as described above for the chloroform-extracted enzyme (see Table II); however, in later preparations it was found that the batch absorption procedure described by Binder et al. (1978) is much quicker. To the clarified supernatant, 100 mL of settled DEAE-Sephadex A-50, equilibrated with 20 mM Tris/SO_4 (pH 8.0), 10 mM ammonium sulfate, and 1 mM EDTA were added. The suspension was stirred slowly at room temperature for 30 min and then gently layered over a bed (2.5 × 15 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. The protein was eluted as described above.

ATPase Assay. For routine ATPase assays the following reaction mixture in a total volume of 0.1 mL was used: 20 mM Tricine–NaOH (pH 8.0), 1 mM EDTA, 5 mM $MgCl_2$, 10 mM [γ-^{32}P]ATP (~2 × 10^5 cpm), 50 mM dithiothreitol, and 0.5 μg of protein. Reaction mixtures were incubated for 15 min at 37 °C. Although not shown, the assay is linear for up to 60 min. The reaction was terminated by the addition of 2.0 mL of a solution of 0.8 N $HClO_4$ and 1% (w/v) ammonium molybdate. In order to push the complex formation between P_i and ammonium molybdate, we added unlabeled phosphate (2 mM) and extracted the inorganic phosphate into 2.0 mL of a 1:1 v/v mixture of water-saturated 2-butanol and benzene. After being mixed for 20 s, the sample was centrifuged at 1800*g* for 2 min, and 1.0 mL of the organic phase was removed and counted in a Parkard 460 C liquid scintillation counter.

Partial Depletion of CF_1 from C. reinhardi Thylakoid Particles. The *C. reinhardi* thylakoid particles described above were suspended in distilled water to a concentration equivalent to 0.2 mg of chlorophyll per mL, and EDTA (pH 7.5) was added to a final concentration of 0.75 mM. The suspension was stirred for 5 min at 0–4 °C. The membranes were collected by centrifugation (30000*g* for 10 min) and resuspended in 20 mM Tricine–NaOH (pH 8.0) buffer containing 0.25 M sucrose, and 10 mM NaCl.

Reconstitution. Incubation mixtures for reconstitution contained in a total volume of 0.15 mL *C. reinhardi* thylakoid

particles that had been extracted with EDTA as described above, equivalent to 100 μg of chlorophyll/mL, 20 mM Tricine–NaOH (pH 8.0), 10 mM $MgCl_2$, and variable amounts of ATPase. This suspension was incubated at 19 °C for 1.0 min and then diluted to 1.0 mL. Photophosphorylation was measured as described above.

Miscellaneous Methods. Nondenaturing (Davis, 1964) and sodium dodecyl sulfate (Weber & Osborn, 1969) polyacrylamide slab gels were run as described and stained with either Coomassie G-250 or Coomassie R-250, respectively. Nondenaturing polyacrylamide gels were stained for activity by visualizing inorganic phosphate with the method described by Chen et al. (1956). Protein was determined by the Coomassie dye binding method (Bradford, 1976) using bovine serum albumin as the standard. Chlorophyll was determined by extraction into 80% acetone (Arnon, 1949). Ouchterlony double-diffusion plates were run in 1.5% agarose as previously described (Ouchterlony, 1962).

Results

PMS-Dependent Photophosphorylation with C. reinhardi Chloroplast Particles. *C. reinhardi* chloroplast thylakoid particles can be prepared by disrupting the cells. Two common methods are a mild sonication and passage of a cell suspension through a pressure cell (Brand et al., 1975; Bar-Nun et al., 1977; Hudock et al., 1979). Both methods yield variable rates of PMS-dependent phosphorylation, probably because of variability in the intactness of the chloroplast thylakoid particles. With a cell suspension equivalent to 0.5 mg of Chl/mL, maximal rates of PMS-dependent phosphorylation are obtained after 20 s of sonication at 23 °C. Phosphorylation with these fragments is only linear for ~2 min; thereafter, the rate of phosphorylation decreases rapidly (data not shown). Over the course of this study, phosphorylation rates (measured in the linear range) varied from 225 to 800 μmol of ATP synthesized (mg of Chl)$^{-1}$ h^{-1}.

PMS-dependent photophosphorylation with *C. reinhardi* thylakoid particles can be inhibited up to 85% by an antiserum prepared against spinach CF_1. However, tentoxin, a species-specific inhibitor of higher plant coupling factor 1 proteins (Arntzen, 1972; Steele et al., 1976), is virtually ineffective (data not shown).

Isolation and Purification of C. reinhardi ATPase. Several different approaches were tried to solubilize the CF_1 fraction from photosynthetically competent *C. reinhardi* particles. The low-salt, Tris–Tricine extraction described by Strotmann et al. (1973) removes negligible amounts of ATPase activity from the thylakoid membrane particles. In contrast, almost all of the ATPase activity is solubilized when the membranes are treated with chloroform (Younis et al., 1977). This protein, however, is not homogeneous when eluted from a DEAE-Sephadex A-50 column and runs as two very closely spaced equally staining bands on nondenaturing polyacrylamide gels. Both bands stain for ATPase activity. The specific activity of this protein ranged from 4.5 to 9.5 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1} (assayed at 37 °C with 5 mM MgATP). This protein contained only four subunits (see Figure 1), was incapable of reconstituting photophosphorylation with partially resolved thylakoid particles, and was probably lacking the δ subunit. These findings are similar to those that have been reported for the isolation of higher plant coupling factors in the presence of chloroform (Younis et al., 1977). The specific activity of this protein was, however, always substantially higher (2–3-fold) than the activity of the protein isolated by extracting the chloroplast particles with EDTA. In all other respects, no substantial differences were noted between this protein and the one isolated by EDTA extraction.

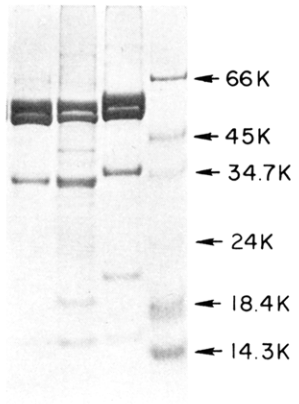

FIGURE 1: Sodium dodecyl sulfate gel of various ATPases. Chloroform-released ATPase from *C. reinhardi* (wild type 137c) (lane 1), EDTA-released ATPase from *C. reinhardi* (wild type 4j) (lane 2), and Tris–Tricine-released CF_1 from spinach chloroplasts (lane 3) were prepared as described under Materials and Methods. A 10% sodium dodecyl sulfate–polyacrylamide slab gel was run as described by Weber & Osborn (1969) and stained with Coomassie R-250. Lane 4 contained standards with molecular weights as indicated in kilodaltons.

Table I: Flow Chart for Purification of *C. reinhardi* ATPase[a]

fraction	total protein[b]	total act.	sp act.[c]	x-fold purification
thylakoid membranes	155	34.1	0.22	
thylakoid membranes after first EDTA wash	76	14.4	0.19	
thylakoid membranes after second EDTA wash	50	1.5	0.03	
EDTA extract	10	10	1.0	4.6
DEAE-Sephadex A-50	2	5.4	2.7	12.3

[a] Conditions were as described under Materials and Methods. The yield was 16%. [b] mg. [c] μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}.

The most reproducible method for the isolation of the *C. reinhardi* ATPase is a modification (Binder et al., 1978) of the EDTA extraction described by Lien & Racker (1971). A representative flow chart is shown in Table I. The yield in total activity (assuming that the activity of the enzyme bound to the crude membrane particles is the same as that of the soluble enzyme) is ~20% with a total purification of about 10–15-fold. After the first extraction with EDTA, ~50% of the protein (activity) remains with the thylakoid particles. A second EDTA wash removes more than 90% of the activity; however, this extract contains several proteins and was not usually used for further purification. The protein elutes off of the DEAE-Sephadex A-50 column (2.5 × 30 cm) at ~160 mM ammonium sulfate and appears to be >95% pure as judged from nondenaturing polyacrylamide gels (not shown).

Figure 1 shows the polypeptide profile of several ATPases run on sodium dodecyl sulfate–polyacrylamide gels. Clearly, the protein isolated by chloroform extraction (lane 1) is missing δ. The protein isolated by EDTA extraction (lane 2) has five major bands that appear to migrate a little faster than the corresponding bands from the spinach enzyme (lane 3). The δ subunit seems to have the largest difference in apparent molecular weights (18 000 for the *C. reinhardi* enzyme vs. 21 000 for the spinach enzyme). Upon prolonged storage as an ammonium sulfate suspension at 0–4 °C, the δ subunit from

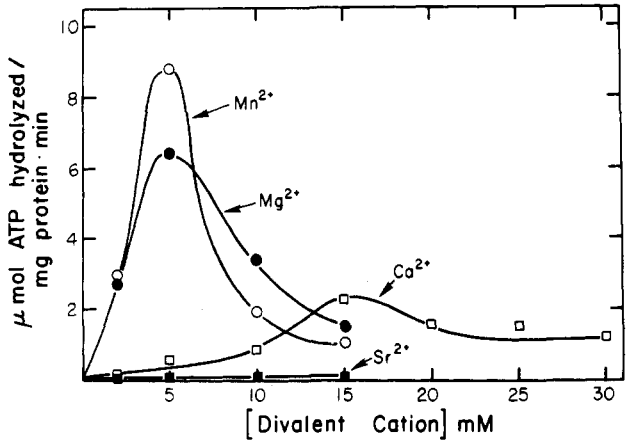

FIGURE 2: Cation concentration curves for the *C. reinhardi* ATPase. Chloroform-released ATPase from *C. reinhardi* (wild type 137c) was prepared and assayed at 37 °C as described under Materials and Methods. The ATP concentration was 10 mM.

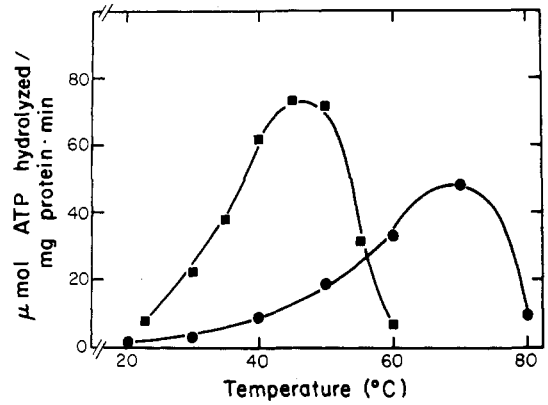

FIGURE 3: Temperature profiles for the *C. reinhardi* MgATPase. Conditions were as described in Figure 2 except that the concentration of $MgCl_2$ was 5 mM. The temperature was varied as indicated, and the assay was terminated after 10 min. The assay was performed in the absence (●) and presence (■) of ethanol (20%). The Arrhenius replots are linear to 70 and 45 °C without and with ethanol, respectively, and the activation energies are 18 and 21 kcal/mol, respectively (not shown).

the *C. reinhardi* ATPase tends to dissociate from the complex (data not shown).

Characterization of ATPase. Most higher plant CF_1 ATPases usually have a much higher catalytic activity with CaATP than with MgATP (Nelson, 1976; McCarty & Racker, 1968), although soluble spinach CF_1 can, under some conditions, use MgATP (Nelson et al., 1972). Figure 2 shows concentration curves for the *C. reinhardi* ATPase activity as a function of various divalent cations. In contrast to other CF_1 ATPases, the *C. reinhardi* enzyme has a substantially higher activity with Mn^{2+} and Mg^{2+} than with Ca^{2+}. The order of activity is MnATP > MgATP > CaATP ≫ SrATP. The optimal Mn^{2+} concentration is sharper than the optimal Mg^{2+} concentration. In addition, calcium (10 mM) severely inhibits (75%) the MgATPase activity of the enzyme (not shown).

Although dithiothreitol stimulates the MgATPase activity of this enzyme ~2-fold, it is not required for activity (data not shown). The dithiothreitol stimulation saturates at ~50 mM. In contrast to higher plant ATPases (Vambutas & Racker, 1965), the *C. reinhardi* enzyme is not activated by protease digestion or heat (data not shown). The activity of the enzyme at 37 °C, after being incubated at 65 °C for 0–5 min, is unaltered (data not shown).

As shown in Figure 3, the *C. reinhardi* ATPase is fairly heat stable and has a temperature optimum at ~70 °C. The

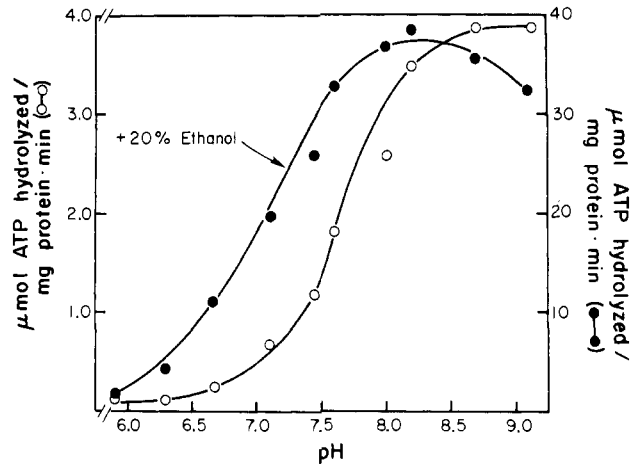

FIGURE 4: pH profiles for the *C. reinhardi* ATPase. EDTA-released ATPase from *C. reinhardi* (wild type 4j) was prepared and assayed as described under Materials and Methods. Reaction mixtures contained 10 mM ATP, 5 mM $MgCl_2$, and 40 mM of the following buffers: pH 6.2–6.5, 2-(*N*-morpholino)ethanesulfonic acid; pH 6.8–7.6, 3-(*N*-morpholino)propanesulfonic acid; pH 7.9–8.1, *N*-tris(hydroxymethyl)methylglycine; pH 8.4–8.9, *N,N*-bis(2-hydroxyethyl)glycine; pH 9.3, glycylglycine. Assays were performed in the absence (○) and presence (●) of ethanol (20%).

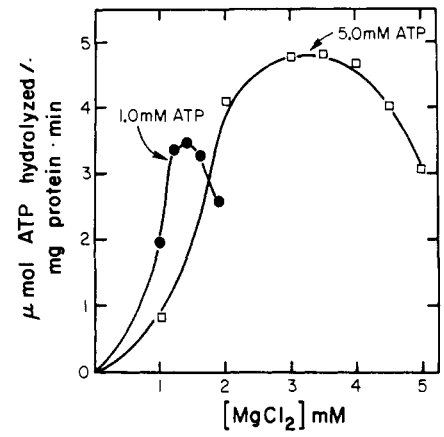

FIGURE 5: $MgCl_2$ concentration curves for the *C. reinhardi* ATPase activity. Conditions were as described in Figure 2 except that the ATP concentration was either 1.0 (●) or 5.0 mM (□). The $MgCl_2$ concentration was varied as indicated. Note that the concentration of EDTA was maintained at 1.0 mM.

activation energy for the ATPase reaction is 17 ± 1.7 kcal/mol, consistent with other ATPases (Farron & Racker, 1970).

The pH optimum for the ATPase reaction is fairly broad (Figure 4). Maximal activity is obtained at pH 8.8 and half-maximal activity at pH 7.7.

The kinetics of the ATPase reaction are quite complex (cf. Figure 6). Excess divalent cations inhibit the ATPase activity as shown in Figure 2. Figure 5 shows Mg^{2+} concentration curves for two concentrations of ATP, 1 and 5 mM. Both curves show optimal rates at a ratio of ATP to Mg^{2+} of ~2.0. At a 1:1 ratio of ATP to Mg^{2+} at 1 mM ATP, the ATPase activity is substantially lower (25%) than with 5 mM ATP, which again suggests that free Mg^{2+} inhibits the ATPase activity. In addition, Figure 6 demonstrates that, at a constant concentration of free Mg^{2+}, MgATP is also a substrate inhibitor at high MgATP concentrations. The apparent K_M for MgATP in the presence of 55 μM free Mg^{2+} is 0.2 mM.

ATPase Inhibitors. On Ouchterlony plates, the anti (spinach) CF_1 antiserum shows a precipitation band indicating partial identity of spinach CF_1 to the *C. reinhardi* ATPase. The antiserum clearly inhibits the ATPase activity of the *C. reinhardi* enzyme. Figure 7 shows an antiserum titration curve

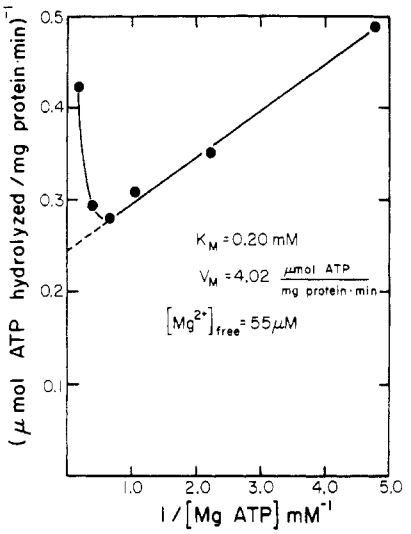

FIGURE 6: Lineweaver–Burk plot for the *C. reinhardi* MgATPase. Conditions were as described in Figure 2 except that the ATP and $MgCl_2$ concentrations were varied to keep the free Mg^{2+} concentration at 55 μM (assuming a log K_d of 4.2 for MgATP) and EDTA was omitted. The linear portion of the plot (at MgATP concentrations <5.0 mM) was fit to a nonlinear regression analysis as previously described (Frasch & Selman, 1980).

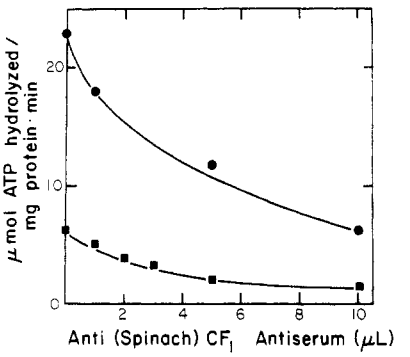

FIGURE 7: Antibody titration curve for the *C. reinhardi* MgATPase activity. Conditions were as described in Figure 2. Reaction mixtures contained 10 mM ATP, 5 mM $MgCl_2$, and various amounts of an anti (spinach) CF_1 antiserum (2 mg of protein/mL) as indicated. Assays were performed in the absence (■) and presence (●) of 20% ethanol.

for the inhibition of the Mg^{2+} ATPase activity of the *C. reinhardi* ATPase. At least some of the determinants important for the recognition of the anti (spinach) CF_1 antiserum are present on the *C. reinhardi* enzyme, and the interaction of the antibody with the enzyme leads to a partial (80%) inhibition of its activity.

Table II summarizes the effect of various ATPase inhibitors on the MgATPase activity of the *C. reinhardi* enzyme. Quercetin, tentoxin, oligomycin, and vanadate have no effect on the activity of the enzyme. Dicyclohexylcarbodiimide at 200 μM inhibits the activity slightly (30%), and phlorizin at 5 mM inhibits the activity severely (70%).

Alcohol Stimulation of ATPase Activity. In the course of the examination of the effects of various inhibitors on the MgATPase activity of the *C. reinhardi* enzyme, it was observed that the solvents for the inhibitors markedly affected the control rates of ATP hydrolysis. In particular, ethanol seemed to have the most dramatic effect on the ATPase activity, stimulating the control rate 5–25-fold. Organic solvent stimulation of various enzymes has been observed previously (Tan & Lovrien, 1972), and Schuster (1979) and Tiefert (1980) have observed alcohol stimulation of the F_1 from mitochondria and CF_1 from spinach chloroplasts, respectively.

Table II: Effect of Energy-Transfer Inhibitors on MgATPase Activity of *C. reinhardi* ATPase[a]

inhibitor[b]	concn (μM)	% control
quercetin	100	97
tentoxin	25	100
oligomycin	10	108
vanadate	100	106
dicyclohexylcarbodiimide	200	71
phlorizin	5[c]	29

[a] Conditions were as described in Figure 2. Reaction mixtures contained 10 mM ATP and 5 mM $MgCl_2$. [b] The control rates for quercetin, tentoxin, oligomycin, vanadate, dicyclohexylcarbodiimide, and phlorizin were 15.0, 35.5, 14.9, 6.8, 12.5, and 9.6 μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}, respectively. [c] mM.

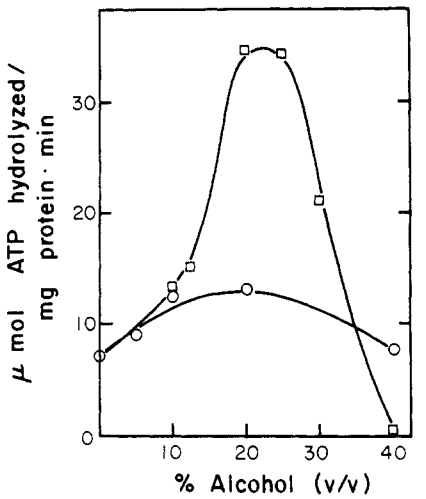

FIGURE 8: Alcohol concentration curves for the stimulation of the *C. reinhardi* MgATPase activity. Conditions were as described in Figure 2 except that the reaction mixtures contained 10 mM ATP, 5 mM $MgCl_2$, and varying amounts of ethanol (□) and methanol (○) (v/v) as indicated.

Figure 8 shows concentration curves for the stimulation of the ATPase activity by methanol and ethanol. Methanol is maximally effective at a slightly lower concentration (20%) than is ethanol (23%), but the maximal stimulation with methanol is only ~2-fold compared to the 6-fold stimulation seen in Figure 8 with ethanol. Other longer chain alcohols are not as effective as ethanol (data not shown). The ATPase isolated by chloroform extraction is only stimulated by a factor of ~6, whereas the enzyme isolated by EDTA extraction is stimulated 10–25-fold (see Figure 4). However, since the specific activity of the chloroform-released enzyme is already higher than that of the EDTA-released enzyme, the maximal specific activities of both enzymes are about the same. Figure 3 shows that ethanol has a dramatic effect on the temperature optimum of the ATPase, decreasing it from about 70 to 45 °C. Ethanol has, however, no marked effect on the activation energy of the ATPase reaction, which is ~21 kcal/mol in 20% ethanol. Ethanol also slightly alters the pH optimum of the ATPase, decreasing it from 8.8 to ~8.2 (Figure 4).

Ethanol does not change the substrate specificity of the enzyme, which remains most active with Mn and MgATP (data not shown). In addition, ethanol is not activating a contaminating latent ATPase because the activity of the ethanol-stimulated enzyme is quite sensitive to the anti (spinach) CF_1 antiserum (Figure 7).

In order to test whether or not the ethanol stimulation of the *C. reinhardi* ATPase activity was reversible, we performed two types of experiments. In one experiment, the ATPase was incubated in 20% ethanol at 23 and 37 °C for 5 min and then removed from the ethanol solution by centrifugation chro-

Table III: Reversible Stimulation by Ethanol of *C. reinhardi* ATPase Activity[a]

preincubation conditions prior to gel filtration		act. after gel filtration [μmol of ATP hydrolyzed (mg of protein)$^{-1}$ min^{-1}]	
temp (°C)	ethanol	−20% ethanol	+20% ethanol
23	−	6.7	34.3
23	+	6.7	40.1
37	−	6.3	38.9
37	+	5.2	37.4

[a] Chloroform-released *C. reinhardi* (wild-type 137c) ATPase was isolated as described under Materials and Methods. Protein samples (0.1 mg/mL) were incubated for 5.0 min at the indicated temperatures with or without 20% ethanol, then removed from the incubation solution by centrifugation chromatography (Penefsky, 1976), and assayed for ATPase activity with or without 20% ethanol.

Table IV: Reconstitution of PMS-Dependent Photophosphorylation with Partially Resolved *C. reinhardi* Thylakoid Particles by the Addition of *C. reinhardi* ATPase[a]

	amount of ATPase added (mg)	μmol of ATP synthesized (mg of Chl)$^{-1}$ h^{-1}
control		225
EDTA washed		30
EDTA washed	0.09	46
EDTA washed	0.18	56
EDTA washed	0.36	65
EDTA washed	1.00	67
EDTA washed	1.50	75

[a] Partially resolved *C. reinhardi* thylakoid particles and EDTA-released ATPase from *C. reinhardi* (wild-type 4j) were prepared and reconstitution was performed as described under Materials and Methods. The ATPase was desalted (Penefsky, 1976) prior to use.

matography on Sephadex G-50 (Penefsky, 1976). The activity of the enzyme was then determined in the absence or presence of 20% ethanol. These results are shown in Table III. Clearly, when ethanol is removed from the enzyme, the activity of the enzyme reverts back to the minus ethanol control rate. Furthermore, the readdition of ethanol stimulates the enzyme. In the second type of experiment, the ATPase was incubated with ethanol and then diluted into reaction mixtures with or without ethanol. When the ethanol concentration is reduced by dilution, the activity of the enzyme returns to the control level (data not shown). Thus, whatever the effect of ethanol on the enzyme activity is, it is completely reversible when ethanol is removed.

Reconstitution of Photophosphorylation with Partially Resolved C. reinhardi Thylakoid Particles and the C. reinhardi MgATPase. The suggestion that the EDTA-extracted enzyme is truly the *C. reinhardi* chloroplast ATPase and not some other ATPase comes from the similarity of its subunit composition to that of spinach CF_1 (Figure 1) and the cross-reactivity of the enzyme with the anti (spinach) CF_1 antiserum (Figure 7). However, neither of these observations prove that the ATPase is equivalent to the chloroplast CF_1.

In order to more firmly identify the nature of the *C. reinhardi* ATPase, we extracted *C. reinhardi* thylakoid particles with EDTA to remove some of the chloroplast CF_1 and uncouple the membranes. These membranes were then mixed with the *C. reinhardi* ATPase, and PMS-dependent photophosphorylation was measured. The results are shown in Table IV. Under these conditions, EDTA extraction of the thylakoid particles inhibits phosphorylation by ~90%. The addition of the *C. reinhardi* ATPase to the extracted particles results in an increase in phosphorylation which is dependent upon the amount of enzyme added to the reaction mixtures. In Table IV, a maximum of 150% increase in phosphorylation is observed.

Discussion

Three lines of evidence indicate that the enzyme that we have isolated from photosynthetically competent *C. reinhardi* thylakoid particles is the chloroplast CF_1: (i) both photophosphorylation by *C. reinhardi* thylakoid particles and the ATPase activity of the isolated, purified enzyme are inhibited by the rabbit anti (spinach) CF_1 antiserum (Figure 7), (ii) the subunit composition of the *C. reinhardi* enzyme on sodium dodecylsulfate–polyacrylamide gels is very similar to the subunit composition of spinach CF_1 (Figure 1), and (iii) photophosphorylation with partially resolved *C. reinhardi* particles can be reconstituted by the addition of the purified *C. reinhardi* ATPase (Table IV).

In contrast to higher plant CF_1 proteins, the *C. reinhardi* enzyme is activated by neither heat nor proteolysis. Thiol reducing agents do increase the specific activity of the enzymes ~2-fold, but not to the extent observed with spinach CF_1 (McCarty & Racker, 1968). In this respect, the *C. reinhardi* enzyme more closely resembles the *E. gracilis* ATPase (Chang & Kahn, 1966). Alcohols, which have been reported to activate CF_1 (Tiefert, 1980), have a very dramatic effect on the *C. reinhardi* ATPase, stimulating the activity of the enzyme up to 25-fold (Figures 4 and 8). The effect of ethanol is, however, completely reversible (Table III), indicating that ethanol does not cause the release of any inhibitory subunits (Nelson et al., 1973) or nucleotides (Reimer & Selman, 1979) from the enzyme. Ethanol causes a slight shift in the pH optimum of the protein from 8.8 to 8.2 (Figure 4). Ethanol also causes a marked 25 °C drop in the temperature optimum of the protein (Figure 3) which may be due to a change in the dielectric constant of the medium. Ethanol does not, however, alter the activation energy of the ATPase reaction which is ~17 ± 1.7 kcal/mol.

The kinetics of the *C. reinhardi* ATPase is complex and requires more study. Excess divalent cation inhibits the activity of the ATPase (Figures 2 and 5) which is the highest with MnATP as the substrate. Calcium inhibits the MgATPase activity of the enzyme. The apparent K_M for MgATP in the presence of 55 μM free Mg^{2+} is ~0.2 mM (Figure 6). This is ~10-fold lower than the K_M for CaATP for spinach CF_1 (Farron & Racker, 1970) but is comparable to the K_M for MgATP determined by Nelson et al. (1972) for spinach CF_1 in 2-(*N*-morpholino)ethane buffer at pH 6.0. MgATP is also a substrate inhibitor of the *C. reinhardi* ATPase (Figure 6).

The advantage of studying the CF_1 ATPase from *C. reinhardi* as opposed to higher plants is that this system lends itself to genetic approaches that are impractical with higher plants. Indeed, ATPase mutants of *C. reinhardi* are already available (Hudock et al., 1979), and it should be possible to answer questions regarding the function of subunits in both the F_1 and F_0 sectors of the energy-transducing complex that have not been explored with higher plant coupling factors.

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Binding of Phytochrome to Liposomes and Protoplasts†

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ABSTRACT: The physiologically active form of oat phytochrome, Pfr, increases its binding to egg lecithin unilamellar liposomes with increasing ionic strength of the medium while the binding of Pr is almost constant. The preferential binding of Pfr is as much as twice that of Pr at KCl concentrations above 0.2 M, in 0.1 M phosphate buffer (pH 7.2) at 27 °C. The binding of phytochrome to liposomes is also enhanced by ~80% at 27 °C compared to that at 3 °C. Thus, it appears that the binding between Pfr phytochrome and liposomes is hydrophobic in nature whereas the binding of Pr is not predominantly through hydrophobic interactions. The binding of both Pfr and Pr to multilamellar liposomes increases with increasing cholesterol content in the liposomes. The extent of phytochrome's binding is higher in the neutral pH region than above pH 7.5. It takes several hours to reach an equilibrium of binding. The photoreversion of liposome-bound Pfr is inhibited by 40% compared to that of free Pfr, while the phototransformation of liposome-bound Pr to Pfr is promoted by 30%. The rate of dithionite-accelerated dark reversion of liposome-bound Pfr is lower by 50% than that of the free form. These results are consistent with the proposal that the hydrophobic binding site involved results from a vacancy produced by the reorientation or displacement of the Pfr chromophore from the protein. Upon binding to phytochrome, unilamellar liposomes undergo fusion to form larger diameter liposomes. No preferential binding of the Pfr form was found with intact oat protoplasts in vitro.

The mode of action of phytochrome in the red light triggered morphogenic responses of plants remains elusive, in spite of many attempts to elucidate its nature at different levels of study. An attractive hypothesis for the mechanism of phytochrome-mediated photomorphogenesis is based on the proposal that phytochrome, at least in its physiologically active Pfr form, modulates membrane properties by binding to an as yet unidentified receptor [Marmé, 1974; Schäfer, 1975; Schäfer et al., 1976; Pratt, 1978; Rüdiger, 1980 (review)]. A

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