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Properties of the Cyanobacterial Coupling Factor ATPase from *Spirulina platensis*

I. Electrophoretic Characterization and Reconstitution of Photophosphorylation

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The coupling factor ATPase (F_1) from photosynthetic membranes of the cyanobacterium *Spirulina platensis* was purified to homogeneity by a combination of ion-exchange chromatography and sucrose density gradient centrifugation. The ATPase activity of purified *Spirulina* F_1 is latent but can be elicited by trypsin treatment, resulting in specific activities (CaATPase) of 27-37 μmol P_i min^{-1} mg protein $^{-1}$. On denaturing sodium dodecyl sulfate-polyacrylamide gradient gels, *Spirulina* F_1 is resolved into five subunits with molecular weights of 53,400, 51,600, 36,000, 21,100, and 14,700, similar to the molecular weights of the subunits of spinach chloroplast coupling factor (CF_1). As determined by native polyacrylamide gradient gel electrophoresis, the molecular weight of the *Spirulina* F_1 holoenzyme was estimated to be 320,000, somewhat smaller than the estimated molecular weight of spinach CF_1 (392,000). *Spirulina* F_1 was shown to be an active coupling factor by its ability to reconstitute phenazine methosulfate-dependent cyclic photophosphorylation in membrane vesicles which had been depleted of coupling factor content by 2 M NaBr treatment. We estimate the *Spirulina* F_1 content of membrane vesicles to be 1 F_1 per 830 chlorophylls or 0.12 mol F_1 mol $P700^{-1}$, based on the specific ATPase activities of the membrane vesicles and the purified *Spirulina* F_1, the molecular weight of F_1, and the P700 content of the vesicles.

Although the bioenergetic properties of photosynthetic membranes of cyanobacteria are not as well characterized as those of higher plant chloroplast thylakoids, in most respects cyanobacterial thylakoids resemble their eukaryotic counterparts. Thus, measurements of the protonmotive force generated during illumination indicate that it is of comparable magnitude to that found in chloroplasts and, like chloroplasts, it consists primarily of a ΔpH, acid interior (1, 2). The phosphorylation efficiency, or $P/2e$, of cyanobacterial thylakoids ranges from 0.9 to 1.5 with artificial electron acceptors of NADP (2, 3) and both uncoupling and photosynthetic control have been observed in a number of instances (2-5).

Further progress in elucidating the bioenergetic properties of cyanobacterial thylakoids and their evolutionary relationships to chloroplasts requires the isolation, purification, and characterization of the components of the energy-transducing apparatus. The F_1-F_0 ATPase[2] complex has

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[2] Abbreviations used: AQS, anthraquinone-2-sulfonate; F_0 and F_1, the hydrophobic and hydrophilic portions, respectively, of the proton-translocating ATPase complex; CF_1, chloroplast F_1; DCCD, *N,N*-dicyclohexylcarbodiimide; PMSF, phenylmethylsulfonyl fluoride; TPCK, L-1-tosylamide-2-phenyleth-

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been extracted from two thermophilic cyanobacteria and each was shown to reconstitute DCCD- and uncoupler-sensitive ATP-$^{32}P_i$ exchange (6, 7). Definitive assignment of polypeptide bands on SDS-polyacrylamide gels was not made. Additional work on the *Synechoccocus* complex has documented the lipid requirement for optimal reconstitution (8). The hydrophilic portion of the complex, F_1, was also purified from *Mastigocladus laminosus* (9). This enzyme, which exhibited latent ATPase activity, contained four subunits, lacking δ, and failed to reconstitute photophosphorylation in depleted membranes; however, reconstitution could be achieved when the purified subunit was added to the four-subunit enzyme (10).

We report here the first purification of an intact five-subunit, reconstitutively active F_1 from a cyanobacterium, *Spirulina platensis*. In the accompanying paper (44), we examine the characteristics of ATP hydrolysis in the membranes and the soluble F_1 from *Spirulina*. A preliminary report on the isolation of a latent CaATPase coupling factor from *Spirulina* has been published (11).

MATERIALS AND METHODS

Culture conditions. Axenic cultures of *Spirulina platensis* (Nordst.) Geitl., clone OPL, were supplied by Dr. Susan Kilham. The growth conditions were as described (12). All subsequent operations were performed at 0-4°C, unless otherwise specified.

Membrane vesicle isolation. Cells of *Spirulina platensis* were pelleted by centrifugation (16,000*g*, 20 min) and the pellet was resuspended to 0.1-0.2 mg chl a/ml in a medium consisting of 0.4 M sucrose, 20 mM Hepes, 15 mM NaCl, 3 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM PMSF, 1 mM *p*-aminobenzamidine, and 0.1 mM TPCK, pH 7.5. The cells were disrupted by sonic oscillation for 5 min in batches of 150-250 ml in a salt-ice bath at three-quarters full output with a Branson W-185 sonicator. Unbroken cells were removed by low-speed centrifugation (1000*g* for 10 min), resuspended in the same medium, sonicated, and centrifuged as above. The supernatants were pooled and centrifuged at 48,000*g* for 1 h to sediment the sonic vesicles. The sonic vesicles were resuspended to between 0.5 and 1.0 mg chl a/ml in 0.4 M sucrose, 20 mM Hepes, 15 mM NaCl, pH 7.5 (SHN), and stored in small aliquots at −70°C or treated immediately as described below.

Isolation of ATPase-depleted membrane vesicles. Prior to treatment, sonic vesicles were washed once with 10 mM sodium pyrophosphate (pH 7.5) to partially remove RuBP carboxylase. The washed vesicles were treated with 2 M NaBr to remove F_1 exactly as described by Nelson (13), except that the chl concentration for NaBr treatment and for storage (−70°C) was 0.5 to 1.0 mg chl a/ml.

Extraction of Spirulina ATPase. In preliminary stages of this work, the ATPase was extracted from sonic vesicles prepared from fresh cultures. Subsequently, it was found that *Spirulina* filaments could be stored as a pellet at −70°C for weeks to months, with no changes in the isolated and purified protein in terms of ATPase activity, molecular weight of the enzyme, or reconstitutive activity. Frozen pellets were warmed gradually at room temperature or in lukewarm water baths and resuspended in the sonication medium described above. All other procedures regarding membrane isolation were as described. All solutions contained the following protease inhibitors: 0.1 mM PMSF, 1 mM *p*-aminobenzamidine, 50 μM TPCK. Prior to F_1 extraction, the membranes were washed three times with sodium pyrophosphate to remove most of the membrane-bound phycocyanin and RuBP carboxylase. This was accomplished by resuspension of the membrane vesicles in 10 mM sodium pyrophosphate, pH 7.5, 0.1-0.2 mg chl/ml followed by centrifugation at 48,000*g* for 1 h.

A. Chloroform extraction. Washed sonic vesicles were resuspended in a medium slightly modified from (14), consisting of 10% glycerol, 5 mM Hepes, 5 mM ATP, 5 mM DTT, and 1 mM EDTA, pH 7.5, to a concentration of about 1 mg chl a/ml. The solution was divided into portions of 12-15 ml in 25-ml corex tubes. One-half volume of chloroform (reagent grade, not further purified) was added to the tube, and the suspension was mixed vigorously for 10-15 s with a Vortex stirrer. The phases were separated by brief (1000*g*, 1 min) centrifugation, and the upper aqueous phase was carefully removed. This solution was subjected to 10,000*g* centrifugation for 15-min, followed by ultracentrifugation at 73,000*g* for 30 min to remove membranes. The resulting supernatant fluid was light blue or pale yellow-brown, depending on the amount of phycocyanin solubilized during the pyrophosphate washes.

B. Low-ionic-strength extraction of Spirulina ATPase. The pyrophosphate-washed membranes were resuspended to between 0.05 and 0.1 mg chl a/ml either in

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ylchloromethyl ketone; SHN, 0.4 M sucrose, 20 mM Hepes, 15 mM NaCl; PMS, phenazine methosulfate; chl, chlorophyll; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; chl, chlorophyll; RuBP, ribulose bisphosphate; Tricine, *N*-tris(hydroxymethyl)methylglycine; BSA, bovine serum albumin; TCA, trichloroacetic acid.

2 mM Tricine/1 mM EDTA or in 50 mM sucrose/2 mM Tricine titrated to pH 7.5 with solid Tris (15, 16). The solution was stirred for 30 min, centrifuged (48,000*g* for 1 h), and extracted a second time as described above. The light green supernatants were pooled and further purified as described below.

Purification of the ATPase. All solutions contained the protease inhibitors described under Extraction of *Spirulina* ATPase. The crude extract was applied to a DEAE Sephadex A-25 column, which was then washed with several bed volumes of 0.1 M NaCl to elute phycocyanin; a linear 0.1–0.5 M NaCl gradient was developed to elute the ATPase. In addition to NaCl, column solutions contained 10% glycerol, 20 mM Tris-Cl, 1 mM ATP, 2 mM EDTA, 3 mM sodium azide, and the protease inhibitors. The pH of these solutions was adjusted to 7.15 at room temperature and cooled to 4°C. The column dimensions were 2 × 20 to 25 cm, the gradient volume was 800 ml, and fractions of 5.5 ml were collected. Before chromatography, chloroform extracts were dialyzed against the above solution, minus NaCl. Tris-Tricine and EDTA extracts were batch-adsorbed onto 100 ml of settled, equilibrated exchanger prior to loading onto a column containing an equivalent volume of fresh equilibrated exchanger. These extracts were eluted by a step gradient in place of a linear gradient. The column was washed with 200 mM NaCl and the enzyme was eluted with 400 mM NaCl. Active fractions were pooled and precipitated with saturated ammonium sulfate added to a final concentration of 60% (w/v).

The final purification step(s) consisted of sucrose density gradient ultracentrifugation. Sucrose gradients, prepared according to Jagendorf (16), consisted of a linear 12–28% (w/w) sucrose gradient with a volume of 38 ml. In addition to sucrose, the solutions contained 20 mM Tricine, pH 8.0, 1 mM ATP, 3 mM azide, and protease inhibitors. The gradients were loaded with 1 ml of partially purified ATPase that had been dialyzed against the same solution, minus the sucrose, and centrifuged in a VTi 50 rotor at 48,000 rpm for 4.5 h at 4°C. Fractions were collected from the bottom of the tube (16). Active fractions at the highest sucrose densities, which would overlap RuBP carboxylase, were discarded and the other active fractions were pooled and precipitated with 60% saturated ammonium sulfate.

Purification of CF_1. Spinach CF_1 was purified from Tris-Tricine extracts by ion-exchange chromatography and sucrose density gradient centrifugation as detailed above, except that all procedures were carried out at room temperature.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed on 13.5 × 12.5-cm slab gels of 1.2-mm thickness in a discontinuous buffer system (17, 18). Electrophoresis was carried out overnight at a constant voltage of 50–70 V. Nondenaturing gel electrophoresis was carried out on 3–22% linear polyacrylamide gels with a 3% stacking gel as described in (17), except that 10% glycerol was also present. Molecular weight standards (Sigma), detailed in the legend to Fig. 3, were electrophoresed alongside the samples. The standard molecular weight curves were determined by linear regression; the correlation coefficient was 0.99 or greater in every case. Visualization of the ATPase on the polyacrylamide gels was accomplished either by enzymatic staining using the method of Horak and Hill (19) or by Coomassie brilliant blue R staining. SDS-polyacrylamide gel electrophoresis was performed according to (17) and as described above. The molecular weight standards (Sigma Dalton Mark VII-L) are detailed in the legend to Fig. 4.

ATPase assay. Trypsin activation of CaATPase activity was performed either by including trypsin (5 mg/ml in 1 mM H_2SO_4) in the reaction mixture (trypsin:ATPase, 10–20:1 w/w) or by pretreatment with trypsin for 15 min at room temperature at a trypsin:ATPase ratio of 5:1 (w/w) in 50 mM Tricine, pH 8.0. The digestion was stopped with a threefold excess of soybean trypsin inhibitor. The reaction mixture (1 ml) for CaATPase contained 50 mM Tricine, pH 8.0, 10 mM $CaCl_2$, and 5 mM ATP. The reaction was allowed to proceed for 5 min at 37°C, and the liberated phosphate was measured by the method of LeBel *et al.* (20) as described by Jagendorf (16), except that after the blue color had developed for 5 min, 0.1 ml of 34% sodium citrate was added to prevent further color development (21).

Reconstitution of ATP synthesis. Reconstitution of PMS-dependent cyclic photophosphorylation activity was assayed by incubating the purified ATPase with ATPase-depleted membranes (about 33 μg chl a) in a 1-ml volume containing 50 mM Tricine, pH 8.0, 25 mM $MgCl_2$, and 0.2 mg/ml BSA, for 15 min on ice. Aliquots (0.4 ml) were transferred to reaction mixtures (1.5 ml) containing 50 mM Tricine, pH 8.0, 50 mM NaCl, 6.7 mM $MgCl_2$, 1 mM ADP, 5 mM sodium phosphate (containing about 8×10^5 cpm/μmol), 0.4 mM PMS, 1 mg/ml BSA, and sonic vesicles equivalent to 13 μg chl a. The assays were performed at room temperature at an intensity of approximately 3×10^6 ergs cm^{-2} s^{-1} (white light). The reaction was quenched after 1 min with 0.2 ml of 30% TCA, and unreacted phosphate was extracted as described by Avron (22). Rates were not altered by the presence of hexokinase and glucose. [^{32}P]ATP was detected by Cerenkov counting (23). Appropriate dark control rates were subtracted from the experimental rates.

P700 measurement. P700 was detected optically by oxidized-minus-reduced spectra in a Cary 17 spectrophotometer fitted with a scattered transmission accessory. The sample (50 μg chl a/ml) was oxidized by addition of solid ferricyanide and the reference cuvette was reduced with solid ascorbate. A molar extinction coefficient of 70,000 cm^{-1} (24) was used to estimate the concentration of P700.

Other assays. Protein was estimated by the method

of Lowry *et al.* (25), using bovine serum albumin as a standard. Chlorophyll was determined according to Arnon (26).

RESULTS

The latent ATPase activity of sonic vesicles of *S. platensis* (see accompanying paper) could be extracted by either the chloroform procedure (14) or low ionic strength treatment (15). Both methods extract about one-half to three-fourths of the activity, but the specific activity of chloroform extracts is four to six times greater than that of low ionic strength extracts (4.3–8.0 μmol P_i min^{-1} mg $protein^{-1}$ compared to 0.6–1.5 μmol P_i min^{-1} mg $protein^{-1}$). Although not shown, no differences were detected in F_1 preparations from either method in terms of subunit composition or ability to reconstitute photophosphorylation, in contrast to chloroform-extracted ATPases from spinach (27), *Chlamydomonas* (28), and *M. laminosus* (10), all of which lacked the δ subunit and failed to reconstitute photophosphorylation. Prior to F_1 extraction, it was critical to wash the membranes to remove RuBP carboxylase which proved to be an overwhelming contaminant in crude F_1 extracts from unwashed membranes. The pyrophosphate wash method of Strotmann *et al.* (15) not only effectively extracted RuBP carboxylase but also accomplished removal of most of the phycocyanin and therefore was especially beneficial in the F_1 purification procedure.

Purification of the solubilized *Spirulina* coupling factor followed the general protocols established for CF_1 (16, 29). However, since initial attempts at F_1 purification performed at room temperature resulted in a reconstitutively inactive preparation (11), all procedures were instead carried out at 4°C in the presence of 10% glycerol. Crude extracts were first subjected to ion-exchange chromatography. The ATPase eluted as a sharp peak between 270 and 320 mM NaCl with linear 100–500 mM NaCl gradients, and this was sufficient, in some instances, to purify the ATPase from chloroform extracts. More frequently, a sucrose gradient was required in addition to chromatography. In the case of low-ionic-strength extracts, it was more convenient to elute the enzyme with a step gradient in place of a linear gradient. Purification of low-ionic-strength extracts to homogeneity required two sucrose gradients following the ion-exchange step. Figure 1 shows the protein and ATPase profiles of the first sucrose gradient after chromatography; the small protein peak near the top of the gradient is a residual phycocyanin contamination remaining after ion-exchange chromatography. Table I details the yields and purification of a typical procedure used to purify low-ionic-strength extracts.

The purity of isolated *Spirulina* F_1 was estimated by native polyacrylamide gel electrophoresis. The purified protein produced a single band by Coomassie blue staining (Fig. 2); the correlation of this band with CaATPase activity was demonstrated by enzymatic staining (19) (not shown). Nondenaturing polyacrylamide gradient gel electrophoresis was used to estimate the molecular weight of the purified *Spirulina* F_1 holoenzyme. An example of a standard curve from a polyacrylamide gradient gel is shown in Fig. 3. As shown in Table II, the estimated molecular weight is about 320,000. By this procedure the estimated molecular weight of spinach CF_1 is 392,000, a value in close agreement with the molecular weight determined for spinach CF_1 by Moroney *et al.* (30) using two independent methods, light scattering and

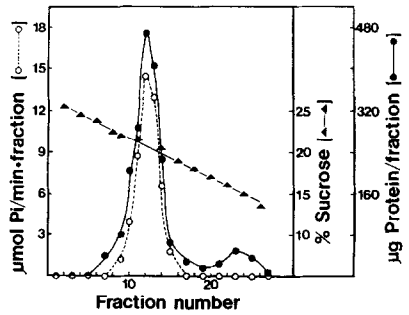

FIG. 1. Sucrose density gradient centrifugation of *Spirulina* F_1. *Spirulina* F_1, after ion-exchange chromatography, was centrifuged on a 12–28% sucrose gradient as described under Materials and Methods and 30 drop fractions were collected from the bottom of the gradient.

TABLE I

PURIFICATION OF *Spirulina* F_1

Step	Protein[a]	Activity[b]	Specific activity[c]	Yield
(1) Tris-Tricine extract	203.5	203.5	1.0	100%
(2) DEAE-Sephadex A-25 step gradient	20.7	118.1	5.7	58
(3) First sucrose gradient	3.6	91.7	25.6	45
(4) Second sucrose gradient	1.5	53.4	36.0	26

Note. *Spirulina* F_1 was purified from Tris-Tricine extracts of *Spirulina* sonic vesicles as described under Materials and Methods (F_1 was extracted from vesicles containing 33 mg chl *a*).

[a] mg protein.

[b] μmol P_i min^{-1}.

[c] μmol P_i min^{-1} mg $protein^{-1}$.

analytical ultracentrifugation. A smaller molecular weight for *Spirulina* F_1 was also indicated by the fact that *Spirulina* F_1 migrated more slowly during sucrose gradient centrifugation that did spinach CF_1 (data not shown). A molecular weight of 320,000 is inconsistent with a stoichiometry of 3:3:1:1:1 for α, β, γ, δ, and ϵ, respectively, deduced for spinach CF_1 (30) from its holoenzyme molecular weight and indicated for *Escherichia coli* F_1 from labeling experiments (31). Whether *Spirulina* F_1 has a subunit stoichiometry different from that of CF_1 or whether it exhibits anomalous migration on polyacrylamide gels can only be determined by a combination of amino acid labeling experiments for estimation of subunit stoichiometry and alternative procedures for estimation of molecular

FIG. 2. Native polyacrylamide gel electrophoresis of purified *Spirulina* F_1 and spinach CF_1. *Spirulina* F_1 (lane 2) and spinach CF_1 (lane 1) were electrophoresed on native 3-22% polyacrylamide gradient gels as described under Materials and Methods. The protein concentrations were 10 and 15 μg, respectively.

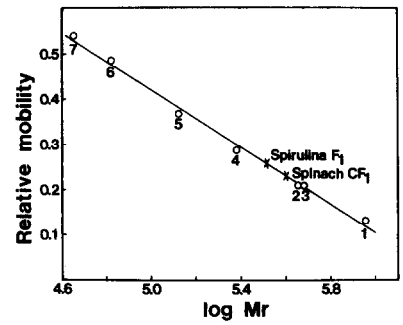

FIG. 3. Molecular weight determination of *Spirulina* F_1 holoenzyme by native 3-22% polyacrylamide gradient gel electrophoresis. Purified *Spirulina* F_1 and spinach CF_1 were electrophoresed on native polyacrylamide gradient gels along with molecular weight standards: (1) apoferritin dimer (900,000), (2) apoferritin monomer (450,000), (3) urease tetramer (480,000), (4) urease dimer (240,000), (5) bovine serum albumin dimer (132,000), (6) bovine serum albumin monomer (66,000), and (7) chicken egg albumin (45,000). The standard curve was determined by linear regression and the correlation coefficient was 0.997.

TABLE II

MOLECULAR WEIGHTS OF THE HOLOENZYME AND SUBUNITS OF *Spirulina* F_1 AND SPINACH CF_1

	Spirulina F_1	Spinach CF_1
	(n = 5)	(n = 5)
Holoenzyme	320 ± 9	392 ± 13
	(n = 7)	(n = 9)
Subunits	53.4 ± 1.4	56.9 ± 1.8
	51.6 ± 1.4	51.4 ± 1.6[a]
	36.0 ± 0.8	36.9 ± 0.8
	21.1 ± 0.5	21.3 ± 0.6
	14.7 ± 0.5	16.0 ± 0.3[b]

Note. *Spirulina* F_1 and spinach CF_1 were electrophoresed on native polyacrylamide 3-22% gradient gels and denaturing SDS polyacrylamide gradient gels along with molecular weight standards as described under Materials and Methods. The ± indicates the SE. Molecular weights should be multiplied by 1000.

[a] 53,874 from DNA sequence (33).

[b] 14,702 from DNA sequence (33).

weight such as analytical ultracentrifugation.

The subunit composition of *Spirulina* F_1 was examined by denaturing SDS-polyacrylamide gel electrophoresis (Fig. 4). Under most electrophoretic conditions, such as constant percentage gels, the two large subunits of *Spirulina* F_1 were not resolved unless electrophoresis was performed on small amounts of protein. In order to separate the α and β subunits, a number of different polyacrylamide gradient gels were tested; a 9-15% gradient gel optimally resolved the two subunits. Although the migration of the α and β as a single band under most electrophoretic conditions could have resulted from proteolytic modification of one or both of the subunits, we feel this is unlikely for the following reasons. First, protease inhibitors were present, as described under Materials and Methods, at each step in the procedure. Second, *Spirulina* membranes subjected to denaturing electrophoresis show a single band in the α, β region which exactly comigrates with the α, β band of purified *Spirulina* F_1. Third, when *Spirulina* F_1 is reacted with antibodies against individual subunits of CF_1 (protein blotting), the enzyme shows a single band reacting with anti-α and a single band reacting with anti-β, indicating that no proteolysis of the α and β subunits of *Spirulina* F_1 had taken place (N. Nelson, personal communication). Figure 4 shows different amounts of *Spirulina* F_1 and spinach CF_1 separated on an SDS 9-15% gradient gel. Although we reported earlier that a sixth polypeptide with a molecular weight of 28,500 copurified with F_1 (32), we now believe that this polypeptide was a contaminant, since it is absent in highly purified preparations of *Spirulina* F_1. The estimated molecular weights of the subunits of *Spirulina* F_1, in order of decreasing size, are 53,400, 51,600, 36,000, 21,100, and 14,700 (Table II). For purposes of comparison Table II also shows the estimated molecular weights of the subunits of spinach CF_1 coelectrophoresed with *Spirulina* F_1, as well as the molecular weights of the β and ϵ

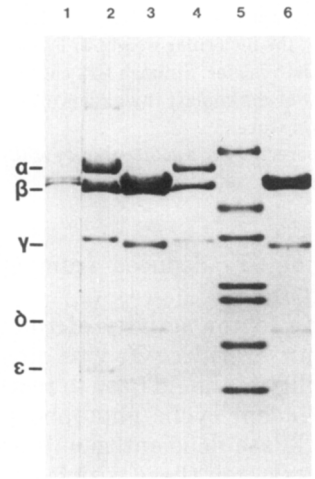

FIG. 4. SDS 9-15% polyacrylamide gel electrophoresis of *Spirulina* F_1 and spinach CF_1. SDS-polyacrylamide gel electrophoresis was performed as described under Materials and Methods. Lane 5, molecular weight standards; lanes 1, 3, and 6, *Spirulina* F_1 (6, 34, and 20 μg, respectively); lanes 2 and 4, spinach CF_1 (20 and 8 μg, respectively). The subunits of CF_1 are labeled on the left margin of the figure. The molecular weight standards are bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), and α-lactalbumin (14,200).

TABLE III

THE STOICHIOMETRY OF *Spirulina* F_1 TO CHLOROPHYLL AND P700 CONTENT OF *Spirulina* SONIC VESICLES COMPARED TO SPINACH CF_1 CONTENT OF CHLOROPLAST THYLAKOIDS

Specific CaATPase activity of *Spirulina* sonic vesicles	14.5 ± 1.4 μmol P_i min^{-1} mg chl^{-1} (n = 7)
Specific CaATPase activity of purified *Spirulina* F_1	33.7 ± 3.4 μmol P_i min^{-1} mg $protein^{-1}$ (n = 6)
P700 content of sonic vesicles	1/96 ± 8 chlorophylls (n = 5)

	Stoichiometry	
	Spirulina	Spinach
mol F_1/mol chl	1 F_1/830 chl	1 CF_1/1060 chl[a]
mol P-700/mol chl	1/96	1/508
mol F_1/mol P700	0.12 F_1/P700	0.48 CF_1/P700

Note. The stoichiometry of *Spirulina* F_1 was estimated based on the specific CaATPase activity of the sonic vesicles, specific CaATPase activity of purified *Spirulina* F_1, the molecular weight of F_1, and the P700 content of the vesicles. Spinach CF_1 content (15) and P700 content of chloroplast thylakoids (37) were taken from the literature.

[a] Value corrected for a molecular weight of 400,000 for CF_1.

subunits of CF_1 deduced from DNA sequences (33).

Coupling factor activity of the purified five-subunit *Spirulina* F_1 was determined by the ability of the ATPase to reconstitute PMS-dependent cyclic photophosphorylation in ATPase-deficient membranes. Before F_1 extraction these vesicles exhibited rates of cyclic phosphorylation of 150–200 μmol ATP h^{-1} mg chl^{-1}. It was previously shown that the partially purified *Spirulina* F_1 reconstituted ATP synthesis in EDTA-treated vesicles; however these vesicles retained one-third of their ATPase activity (11). In order to more completely resolve the vesicles and thus permit us to assign a catalytic function to the exogenously added F_1, the Nelson and Eytan (34) procedure using 2 M NaBr treatment was employed with sonic vesicles. This treatment removes more than 90% of the ATPase from thylakoid membranes and has been successfully employed by Andreo *et al.* (35) to investigate coupling factor activity of different preparations of CF_1. As found with chloroplast thylakoids, 2 M NaBr treatment of *Spirulina* membrane vesicles also leaves less than 10% of the ATPase activity intact on the membrane. A densitometer scan of purified *Spirulina* F_1 control (untreated) membrane vesicles (CaATPase activity, 13.5 μmol P_i min^{-1} mg chl^{-1}) and NaBr-treated vesicles (CaATPase activity, 0.9 μmol P_i min^{-1} mg chl^{-1}) electrophoresed on SDS-10-20% polyacrylamide gradient gels indicated that the bands comigrating with $\alpha + \beta$ and γ subunits were strongly depleted in the NaBr vesicles, with little to no change in the rest of its polypeptide composition (data not shown). The treated membranes exhibited rates of photophosphorylation between 0 and 1.6 μmol ATP h^{-1} mg chl^{-1}. When the soluble, purified *Spirulina* coupling factor is added to the resolved membranes, ATP synthesis is restored up to 40–50 μmol ATP h^{-1} mg chl^{-1}, or 20–33% of the levels observed in pyrophosphate-washed membranes (data not shown). Figure 5 illustrates the dependence of reconstitution on the amount of *Spirulina* F_1 added to the depleted membranes. Saturating rates of ATP synthesis were achieved at about 2 μg F_1/μg chl, or ap-

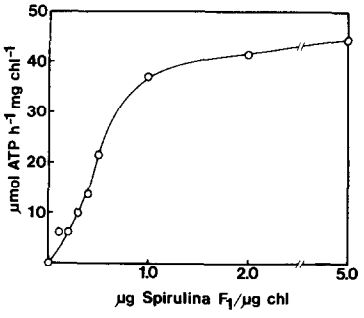

FIG. 5. Reconstitution of cyclic photophosphorylation in NaBr-treated *Spirulina* membrane vesicles by purified *Spirulina* F_1. The indicated amounts of purified *Spirulina* F_1 were incubated with NaBr-treated *Spirulina* vesicles (33 μg chl) for 15 min and aliquots (13 μg chl) were assayed for PMS-dependent photophosphorylation as described under Materials and Methods.

proximately five times the estimated concentration of coupling factor in isolated sonic vesicles (see Discussion).

DISCUSSION

The data from this report and the accompanying paper support the conclusion that a single molecular species, i.e., *Spirulina* F_1, is responsible for ATP hydrolytic and synthetic activities of the vesicles. As the photosynthetic electron transport chain of cyanobacteria is largely the same as found in chloroplasts, it would be of interest to compare the stoichiometry of coupling factor content per electron transport chain in the two systems. For all of these studies, membranes and F_1 were isolated from cultures of *Spirulina platensis* grown under relatively high light intensity and harvested at late log phase. Since literature values exist for CF_1 and P700 content of spinach thylakoids (15, 36, 37), we have related the *Spirulina* F_1 content to the chlorophyll and P700 concentration found in *Spirulina* photosynthetic membranes. This was done by comparing the specific ATPase activities of trypsin-activated membranes and purified F_1, based on a molecular weight estimation of 320,000 for *Spirulina* F_1. A somewhat similar approach was used by Strotmann *et al.* (15) to estimate spinach CF_1 content. Several lines of evidence indicated that this was a reasonable approach in *S. platensis*. One, it was found that the extent of solubilization of ATPase activity by low-ionic-strength treatment resulted in a commensurate decrease in membrane ATPase activity (data not shown), and thus the membrane ATPase activity is a reflection of membrane-bound F_1 activity. Two, the phosphorylation efficiency or $P/2e$ of isolated *Spirulina* membrane vesicles is 0.9 with methyl viologen as electron acceptor (38). This $P/2e$ is in the range of published figures for this reaction in cyanobacteria [e.g., (2, 3)] and indicates that the membranes are intact and functional with respect to electron transport and photophosphorylation. It was shown in (39) that release of less than 20% of CF_1 from spinach thylakoids caused a 50% decrease in the rate of cyclic photophosphorylation. Thus, it is reasonable to expect that detachment of a small percentage of F_1 molecules from *Spirulina* membranes should result in substantial uncoupling as evidenced by low $P/2e$ values and, since this is not observed, the *Spirulina* membranes as isolated probably contain almost all if not all of their endogenous complement of F_1 molecules.

The results of this analysis, compiled in Table III, show that on a chlorophyll basis, the F_1 content of *Spirulina* membranes (1 F_1 per 830 chl) is similar to spinach CF_1 content (1 CF_1 per 1060 chl). A more recent report by Roos and Berzborn (40) quantified by electroimmunodiffusion the CF_1 content of thylakoids isolated from spinach plants grown under different light intensities. Their calculation, adjusted for a size of M_r 400,000 for CF_1, is 1 CF_1 per 860–1480 Chl depending on the light regime, and does not significantly alter the discussion below. A different picture emerges, however, when coupling factor content is related to P700 content. A number of cyanobacteria have been found to be substantially enriched in P700 content relative to spinach chloroplasts [see, e.g., discussion in (41)], and *Spirulina* is no exception. The P700 concentration determined for *Spirulina* (1/96 chl) is slightly higher than other reports (41), but the reported values (about 1/125 chl) do not affect these calculations to any great extent. The coupling factor/P700 ratio of *Spirulina* photosynthetic membranes is only one-quarter that found in spinach thylakoids, 0.12 mol F_1 mol $P700^{-1}$ compared to 0.48 mol CF_1 mol $P700^{-1}$.

The functional significance of different F_1/P700 ratios is uncertain, although Roos and Berzborn (40) have suggested that photophosphorylation rates in spinach are limited by CF_1 content. From a structural point of view it is worth noting that the protein:chl ratio found in cyanobacterial photosynthetic membranes from *Anacystis nidulans* (42) and *S. platensis* (Hicks and Yocum, unpublished) is about 20:1, which is three to four times greater than found in spinach thylakoids. Accordingly, the polypeptide composition of cyanobacterial membranes analyzed by polyacrylamide gel electrophoresis (42) is much more com-

plex than that of chloroplast thylakoids; this may reflect, in part, the presence of a respiratory electron transport chain on the cyanobacterial thylakoid (43). It is possible that there may be a structural limitation on the number of ATPase complexes that can reside on a cyanobacterial thylakoid.

In summary, a five-subunit, reconstitutively active F_1 ATPase was purified from the cyanobacterium *Spirulina platensis*. In terms of its latent ATPase activity and the procedures that elicit its activity, described in the following paper, *Spirulina* F_1 more closely resembles the spinach chloroplast coupling factor than respiratory coupling factors isolated from bacteria and mitochondria. We have also found that *Spirulina* F_1 and spinach CF_1 are closely related according to functional and immunological tests (Hicks, Nelson, and Yocum, manuscript in preparation). However, the differing coupling factor/P700 stoichiometries of the two enzymes suggest that the environments in which they function are different.

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REFERENCES

1. PADAN, E., AND SCHULDINER, S. (1978) *J. Biol. Chem.* **253**, 3281-3286.
2. WAX, E., AND LOCKAU, W. (1980) *Z. Naturforsch. C* **35**, 98-105.
3. SPILLER, H. (1980) *Plant Physiol.* **66**, 446-450.
4. ONO, T., AND MURATA, N. (1978) *Biochim. Biophys. Acta* **502**, 477-485.
5. BOHLER, M.-C., AND BINDER, A. (1980) *Arch. Microbiol.* **124**, 155-160.
6. BINDER, A., BOHLER, M.-C., WOLF, M., AND BACHOFEN, R. (1981) *in* Proceedings of the 5th International Congress on Photosynthesis, Vol. II, pp. 771-778, Balaban International, Philadelphia.
7. LUBBERDING, H. J., ZIMMER, G., VAN WALRAVEN, S. H., SCHRICKX, J., AND KRAAYENHOF, R. (1983) *Eur. J. Biochem.* **137**, 95-99.
8. VAN WALRAVEN, H. S., KOPPENAAL, E., MARVIN, H. J., HAGENDOORN, M. J., AND KRAAYENHOF, R. (1984) *Eur. J. Biochem.* **144**, 563-569.
9. BINDER, A., AND BACHOFEN, R. (1979) *FEBS Lett.* **104**, 66-70.
10. BOHLER, M.-C., BINDER, A., AND BACHOFEN, R. (1982) *FEMS Microbiol. Lett.* **15**, 115-118.
11. OWERS-NARHI, L., ROBINSON, S. J., SELVIUS DEROO, C., AND YOCUM, C. F. (1979) *Biochem. Biophys. Res. Commun.* **90**, 1025-1031.
12. ROBINSON, S. J., SELVIUS DEROO, C., AND YOCUM, C. F. (1982) *Plant Physiol.* **70**, 154-161.
13. NELSON, N. (1980) *in* Methods in Enzymology (San Pietro, A., ed.), Vol. 69, pp. 301-313, Academic Press, New York.
14. PICCIONI, R. G., BENNOUN, P., AND CHUA, N.-H. (1981) *Eur. J. Biochem.* **117**, 93-102.
15. STROTMANN, H., HESSE, H., AND EDELMANN, K. (1973) *Biochim. Biophys. Acta* **314**, 202-210.
16. JAGENDORF, A. T. (1982) *in* Methods in Chloroplast Molecular Biology (Edelman, M., Hallick, R. B., and Chua, N.-H., eds.), pp. 881-898, Elsevier, Amsterdam.
17. PICCIONI, R., BELLEMARE, G., AND CHUA, N.-H. (1982) *in* Methods in Chloroplast Molecular Biology (Edelman, M., Hallick, R. B., and Chua, N.-H., eds.), pp. 985-1014, Elsevier, Amsterdam.
18. NEVILLE, D. M. (1977) *J. Biol. Chem.* **246**, 6328-6334.
19. HORAK, A., AND HILL, R. D. (1972) *Plant Physiol.* **49**, 365-370.
20. LEBEL, D., POIRIER, G. G., AND BEAUDOIN, A. R. (1978) *Anal. Biochem.* **85**, 86-89.
21. LANZETTA, P. A., ALVAREZ, L. J., REINACH, P. S., AND CANDIA, O. A. (1979) *Anal. Biochem.* **100**, 95-97.
22. AVRON, M. (1960) *Biochim. Biophys. Acta* **40**, 257-272.
23. GOULD, J. M., CATHER, R., AND WINGET, G. D. (1972) *Anal. Biochem.* **50**, 540-548.
24. MARSHO, T. V., AND KOK, B. (1980) *in* Methods in Enzymology (San Pietro, A., ed.), Vol. 69, pp. 280-289, Academic Press, New York.
25. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
26. ARNON, D. I. (1949) *Plant Physiol.* **24**, 1-15.
27. YOUNIS, H. M., WINGET, G. D., AND RACKER, E. (1977) *J. Biol. Chem.* **252**, 1814-1818.
28. SELMAN-REIMER, S., MERCHANT, S., AND SELMAN, B. R. (1981) *Biochemistry* **20**, 5476-5482.
29. LIEN, S., AND RACKER, E. (1971) *in* Methods in Enzymology (San Pietro, A., ed.), Vol. 24A, pp. 547-555, Academic Press, New York.
30. MORONEY, J. V., LOPRESTI, L., MCEWAN, B. F., MCCARTY, R. E., AND HAMMES, G. G. (1983) *FEBS Lett.* **158**, 58-62.
31. FOSTER, D. L., AND FILLINGAME, R. H. (1982) *J. Biol. Chem.* **257**, 2009-2015.
32. HICKS, D. B., AND YOCUM, C. F. (1984) *in* Advances

in Photosynthesis Research, Vol. II, pp. 599-602, Martinus Nijhoff/Dr. W. Junk, The Hague.

33. ZURAWSKI, G., BOTTOMLEY, W., AND WHITFIELD, P. R. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6260-6264.
34. NELSON, N., AND EYTAN, E. (1979) *in* Cation Flux across Biomembranes (Mukohata, Y., and Packer, L., eds.), pp. 409-415, Academic Press, New York.
35. ANDREO, C. S., PATRIE, W. J., AND MCCARTY, R. E. (1982) *J. Biol. Chem.* **257,** 9968-9975.
36. FRASCH, W. D., DELUCA, C. R., KULZICK, M. J., AND SELMAN, B. R. (1980) *FEBS Lett.* **122,** 125-128.
37. MELIS, A., AND BROWN, J. S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4712-4716.
38. SELVIUS DEROO, C. (1983) Ph.D. dissertation, University of Michigan, Ann Arbor.
39. BERZBORN, R. J., AND SCHROER, P. (1976) *FEBS Lett.* **70,** 271-275.
40. ROOS, P., AND BERZBORN, R. J. (1983) *Z. Naturforsch. C* **38,** 799-805.
41. LUNDELL, D. J., GLAZER, A. N., MELIS, A., AND MALKIN, R. (1985) *J. Biol. Chem.* **260,** 646-654.
42. GUIKEMA, J., AND SHERMAN, L. (1982) *Biochim. Biophys. Acta* **681,** 440-450.
43. PESCHEK, G. A. (1984) *Subcell. Biochem.* **10,** 85-191.
44. HICKS, D. B., AND YOCUM, C. F. (1986) *Arch. Biochem. Biophys.* **245,** 230-237.