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# Aspects of Subunit Interactions in the Chloroplast ATP Synthase[1]

## I. Isolation of a Chloroplast Coupling Factor 1-Subunit III Complex from Spinach Thylakoids

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**A chloroplast ATP synthase complex ($CF_1$ [chloroplast-coupling factor 1]-$CF_0$ [membrane-spanning portion of chloroplast ATP synthase]) depleted of all $CF_0$ subunits except subunit III (also known as the proteolipid subunit) was purified to study the interaction between $CF_1$ and subunit III. Subunit III has a putative role in proton translocation across the thylakoid membrane during photophosphorylation; therefore, an accurate model of subunit interactions involving subunit III will be valuable for elucidating the mechanism and regulation of energy coupling. Purification of the complex from a crude $CF_1$-$CF_0$ preparation from spinach (*Spinacia oleracea*) thylakoids was accomplished by detergent treatment during anion-exchange chromatography. Subunit III in the complex was positively identified by amino acid analysis and N-terminal sequencing. The association of subunit III with $CF_1$ was verified by linear sucrose gradient centrifugation, immunoprecipitation, and incorporation of the complex into asolectin liposomes. After incorporation into liposomes, $CF_1$ was removed from the $CF_1$-III complex by ethylenediaminetetracetate treatment. The subunit III-proteoliposomes were competent to rebind purified $CF_1$. These results indicate that subunit III directly interacts with $CF_1$ in spinach thylakoids.**

Membrane-associated ATP synthesis in chloroplasts, many bacteria, and mitochondria is accomplished by $F_1$-$F_0$ ATP synthases. The chloroplast ATP synthase is composed of two distinct components, $CF_1$ and $CF_0$. $CF_1$ is an extrinsic membrane protein that contains the sites of nucleotide binding, catalytic activity, and regulation of the enzyme. $CF_0$ is an integral membrane protein complex with the function of translocating protons across the thylakoid to a specifically attached $CF_1$ moiety (for reviews, see McCarty and Carmeli, 1982; Hammes, 1983; Strotmann and Bickel-Sandkötter, 1984; Futai et al., 1989; Junge, 1989). $CF_1$ contains five different subunits, denoted $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$ in order of decreasing molecular mass. $CF_1$ has a molecular mass of 400,000 D (Moroney et al., 1983) and a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Süss and Schmidt, 1982; McCarty and Moroney, 1985). $CF_0$ contains four different subunits, with a probable stoichiometry of $I_1II_2III_{6-12}IV_1$ and molecular mass (for $III_{12}$) of 171,000 D (Süss and Schmidt, 1982; Fromme et al., 1987a, 1987b; Grotjohann and Gräber, 1990). Based on hydropathy plot analysis of the amino acid sequences of $CF_0$ subunits I, III, and IV, each appears to contain at least one membrane-spanning region in addition to hydrophilic regions (Hudson et al., 1987). A clear understanding of the association of the two components of the ATP synthase will be invaluable for determining the role of proton flow in enzyme function.

Subunit interactions between $CF_1$ and $CF_0$ have been studied by a variety of methods, but the connections are still not clearly understood. It has been suggested that the $\alpha$ subunit of $CF_1$ is associated with $CF_0$, because the $\alpha$ subunit of $CF_1$ in thylakoids is much more resistant to proteolysis than that of the solubilized enzyme (Moroney and McCarty, 1982a, 1982b). The proximity of the $\alpha$ subunit of thermophilic bacterial $F_1$ to the membrane has been indicated by labeling of $\alpha$ with a photoreactive phospholipid (Gao and Bäuerlein, 1987). $\delta$ and $\epsilon$ are not required for attachment of $CF_1$ to $CF_0$, but membranes reconstituted with the enzyme lacking either subunit are proton leaky and unable to synthesize ATP (Patrie and McCarty, 1984; Richter et al., 1984). Chemical cross-linking of *Vicia faba* $CF_1$-$CF_0$ subunits revealed possible associations among $\alpha$-II, $\beta$-I, $\beta$-II, $\gamma$-II, $\delta$-I, and $\epsilon$-III (Süss, 1986). Rott and Nelson (1983) also carried out cross-linking studies of spinach $CF_1$-$CF_0$. Both of these studies were carried out before the full complement of $CF_0$ subunits was identified; consequently, their results are difficult to interpret.

Analysis of *Chlamydomonas reinhardtii* photophosphorylation mutants lacking various $F_0$ subunits suggests that, in *C. reinhardtii*, subunit III is required for binding of $CF_1$ to $CF_0$ and that subunits I and IV may function to stabilize the association (Lemaire and Wollman, 1989a, 1989b). In the

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Abbreviations: $CF_0$, membrane-spanning portion of the chloroplast ATP synthase; $CF_1$, chloroplast-coupling factor 1; $CF_1$-$CF_0$, chloroplast ATP synthase, holoenzyme; $CF_1$-III, chloroplast-coupling factor 1 associated with subunit III of $CF_0$; IgG, immunoglobulin G.

structurally similar *Escherichia coli* $F_1$-$F_0$, incubation with antibodies against subunit c (equivalent to subunit III) prevented binding of $F_1$ to $F_0$ in membrane vesicles. The antibodies were also able to displace $F_1$ from $F_0$ when added to nonstripped membranes (Deckers-Hebestreit and Altendorf, 1992). The binding of subunit III to $CF_1$ has not been positively demonstrated in higher plant chloroplasts. Chloroplast subunit III, isolated by organic extraction, is unable to bind $CF_1$ when incorporated into liposomes, although it is capable of slow proton translocation across the membrane (Nelson et al., 1977; Sigrist-Nelson and Azzi, 1980).

The aim of this study was to isolate an enzyme complex that was depleted of all $CF_0$ subunits except III, for the purpose of examining interactions between subunit III and $CF_1$. Subunit III, also historically known as the proteolipid, is a very hydrophobic membrane-spanning polypeptide (Hudson et al., 1987) that is present in six to 12 copies per enzyme (Süss and Schmidt, 1982; Fromme et al., 1987a, 1987b; Grotjohann and Gräber, 1990). It is part of the putative proton channel (Nelson et al., 1977; Sigrist-Nelson and Azzi, 1980) and can be blocked by covalent modification with *N,N''*-dicyclohexylcarbodiimide (Sigrist-Nelson et al., 1978). Its role in proton translocation indicates a close association with $CF_1$. However, past evidence for direct interactions is ambiguous. Feng and McCarty (1990a, 1990b) introduced a chromatographic method for purifying $CF_1$-$CF_0$ and for obtaining the complex lacking either subunit IV or subunits I and IV. Their work suggested that it might be possible to purify a $CF_1$-subunit III complex. In this paper, we describe a chromatographic method for purification of a $CF_1$-subunit III complex, positive identification of subunit III, and verification of the association between $CF_1$ and subunit III. Finally, we give evidence that $CF_1$ can rebind to isolated subunit III in liposomes. In the accompanying paper (Wetzel and McCarty, 1993), we describe the characterization of enzymic activity of the purified complex.

## MATERIALS AND METHODS

### Preparation of $CF_1$, Crude $CF_1$-$CF_0$, Purified $CF_1$-$CF_0$, and $CF_1$-III from *Spinacia oleracea*

$CF_1$ was purified according to the method of Shapiro and McCarty (1990). Crude $CF_1$-$CF_0$ was isolated by cholate/octylglucoside extraction of thylakoids (Pick and Racker, 1979; Feng and McCarty, 1990a). Purified $CF_1$-$CF_0$ was prepared according to the method of Feng and McCarty (1990a).

$CF_1$-III was prepared as follows. All steps were carried out at 4°C. Crude $CF_1$-$CF_0$ (100–120 mg of protein) was dialyzed for 8 to 12 h against 1 L of 50 mM $NaH_2PO_4$-NaOH (pH 7.0), 10% (v/v) glycerol, 1 mM $MgCl_2$, and 1 mM DTT. Triton X-100 was added to the dialyzed protein from a 10% stock to a final concentration of 0.5%. Note: All of the following buffers contained 10% (v/v) glycerol, 1 mM $MgCl_2$, and 1 mM DTT with additional components as described for each step and were titrated to pH 7.0 with either NaOH or HCl. The crude $CF_1$-$CF_0$ was applied to a 2.5- × 9-cm DEAE-Trisacryl-M column that was equilibrated with a buffer containing 50 mM $NaH_2PO_4$-NaOH, 0.5% Triton X-100, and 0.01% (w/v) asolectin. The column was washed with 4 bed volumes of the same buffer as used for equilibration; most of the pigmented impurities eluted. The flow rate was 1 mL $min^{-1}$ during protein application and until the pigmented front reached the bottom of the column, and then the flow was adjusted to 2 mL $min^{-1}$ for the rest of the procedure.

Next, the column was washed with 1 bed volume of 50 mM $NaH_2PO_4$-NaOH, 2 mM Zwittergent 3–12, and 0.02% asolectin and then by a gradient of 2 bed volumes of this same buffer to 2 bed volumes of 5 mM $NaH_2PO_4$-NaOH, 20 mM Zwittergent 3–12, and 0.02% asolectin, followed by 1 bed volume of the gradient end-point buffer. This removes subunits I and IV. To remove subunit II and most of the Rubisco, the column was washed with 3 bed volumes of 5 mM $NaH_2PO_4$-NaOH, 0.5% (w/v) Na-cholate, and 0.02% asolectin, followed by a gradient (2 bed volumes of each buffer) of this same buffer to 190 mM $NaH_2PO_4$-NaOH, 0.5% Na-cholate, and 0.02% asolectin.

The $CF_1$-III complex was then eluted from the column by 2 bed volumes of 100 mM $NaH_2PO_4$-NaOH, 0.05% Na-cholate, 0.01% asolectin, and 0.5 M $(NH_4)_2SO_4$. Peak protein fractions were combined, and ATP and asolectin were added to final concentrations of 0.1 mM and 0.1% (w/v), respectively, before the purified $CF_1$-III was stored in small aliquots under liquid nitrogen. Purity of the complex was determined by SDS-PAGE. The yield and purification of $CF_1$-III from crude $CF_1$-$CF_0$ were calculated based on total and specific sulfite-stimulated $Mg^{2+}$-ATPase activities of the complexes (Richter et al., 1987; Wetzel and McCarty, 1993).

### Identification of Subunit III

The identity of subunit III was verified by amino acid analysis and N-terminal amino acid sequencing, based on the reported sequence for spinach subunit III (Deno et al., 1984). A sample of $CF_1$-III was electrophoretically separated into subunits using SDS-PAGE and then electroblotted onto a polyvinylidene fluoride membrane (Problot, Applied Biosystems) according to the manufacturer's instructions. The protein was visualized by Coomassie blue staining, and the band corresponding to subunit III (apparent molecular mass 8 kD) was excised from the surrounding material and dried. The sample was divided, with one-half used for amino acid analysis (Cohen et al., 1984) and the other half subjected to gas-phase N-terminal sequencing (Shively, 1986). Both analyses included blanks from nonprotein-staining regions of the electroblot.

### Association of $CF_1$-III

The association of subunit III with $CF_1$ was analyzed using Suc density gradient ultracentrifugation, by immunoprecipitation of the complex with anti-$\beta$ antibodies, and by incorporation into asolectin liposomes.

For Suc density gradient ultracentrifugation, solid $(NH_4)_2SO_4$ was added to approximately 3 mg of $CF_1$-III in 10% glycerol, 100 mM $NaH_2PO_4$-NaOH (pH 7.0), 1 mM $MgCl_2$, 1 mM DTT, and 0.5 M $(NH_4)_2SO_4$ (adjusted to pH 7.0) to a final level of 45% saturation at 0°C, and the mixture was allowed to sit at 4°C for 1 h. The protein precipitate was pelleted, resuspended in 0.4 mL of 30 mM Tris-succinate (pH

6.5), 0.5 mM EDTA (pH 8.0), 0.1 mM ATP, 0.1% asolectin, and either 0.4% (w/v) Na-cholate or 0.2% (v/v) Triton X-100, and then layered onto a 4.4-mL Suc gradient. The gradients contained the same components as the resuspension buffer, with a linear Suc concentration gradient of 7 to 40% for the Na-cholate buffer samples and 7 to 30% for the Triton X-100 buffer samples. Two Na-cholate gradients were run, one containing 100 mM $Na_2SO_3$ and one lacking it. The Suc gradients were centrifuged at 164,000*g*, 4°C, for 16 to 18 h. Fractions (0.3–0.4 mL) were collected by puncturing the bottom of the tube, and aliquots of each fraction were added to double-strength gel sample buffer for analysis by SDS-PAGE.

$CF_1$-III was precipitated by anti-$\beta$ antibodies as another means of verifying the association of the complex. IgGs were isolated from rabbit anti-$\beta$ antiserum according to the procedure of Livingston (1974). Antibody titer was determined by the ELISA, with the activity of peroxidase conjugated to goat anti-rabbit antibody used for detection. Specificity of the IgGs was verified by immunoblot analysis (Towbin et al., 1979), using the same second antibody as described above in conjunction with the Amersham ECL Western Detection System.

Immunoprecipitation of $CF_1$, purified $CF_1$-$CF_0$, and $CF_1$-III by anti-$\beta$ IgGs was carried out by the following procedure. Each of the complexes (700 $\mu$g) was precipitated with solid $(NH_4)_2SO_4$, pelleted, and resuspended in 300 $\mu$L of buffer containing 10% glycerol, 50 mM $NaH_2PO_4$-NaOH (pH 7.0), and 1 mM $MgCl_2$. Each sample was dialyzed in 6000 to 8000 mol wt cutoff dialysis tubing against 1 L of the same buffer, with one change, for 15 h at 4°C. Na-cholate was added to the dialyzed material from a 20% stock to a final concentration of 0.5% to solubilize the protein. The protein concentration of each sample was measured by the Pierce BCA protein assay, and cholate buffer (dialysis buffer supplemented with 0.5% Na-cholate) was added as needed to equalize each sample to 1 mg of protein $mL^{-1}$. Aliquots of each sample were removed and saved as pretreatment protein samples. Anti-$\beta$ IgG was added to 300 $\mu$L of each precipitation sample, plus a zero-protein control, and the samples were mixed end-over-end for 1 h at room temperature and overnight at 4°C.

Protein A covalently coupled to Sepharose CL-4B (4.5 mg), swollen in cholate buffer, was added to each sample and mixed end-over-end for 2.5 h at room temperature. The samples were centrifuged to pellet the Sepharose beads and bound protein. The supernatants, containing unprecipitated protein, were carefully removed. The pretreatment samples and the unprecipitated protein samples were each prepared for SDS-PAGE by adding an equal volume of double-strength gel sample buffer and heating to 37°C for 1 h. The protein A-Sepharose beads were washed four times with cholate buffer, resuspended in 90 $\mu$L of gel sample buffer, heated to 37°C for 1 h, boiled for 1.5 min, and then centrifuged to pellet the Sepharose beads. The supernatants, containing precipitated protein, were carefully removed and, along with the prepared gel samples of pretreatment and unprecipitated protein, were electrophoresed in a 15% SDS-polyacrylamide gel.

Purified $CF_1$-$CF_0$, $CF_1$-III, and $CF_1$ were each reconstituted into asolectin liposomes by two different methods. First, modifications of the procedures of Admon et al. (1985) and Krupinski and Hammes (1986) were used as follows: the enzyme complexes (0.14 mg of protein) were precipitated with solid $(NH_4)_2SO_4$ (to 50% saturation at 0°C) and resuspended in a buffer containing 0.9% (w/v) Na-cholate, 0.8% (w/v) sonicated asolectin (final asolectin:protein weight ratio of 40:1), 2 mM $MgCl_2$, 150 mM KCl, 0.5 mM EDTA (pH 8.0), and 10 mM Tricine-NaOH (pH 8.0) and incubated on ice for 10 min. To remove cholate and thus cause liposome formation, each sample was passed by centrifugation through 3 mL of Sephadex G-50 (fine) in a disposable filter column (Penefsky, 1977) that had been equilibrated with 2 mM $MgCl_2$, 150 mM KCl, 0.5 mM EDTA (pH 8.0), and 10 mM Tricine-NaOH (pH 8.0). Excess buffer was removed by an initial centrifugation step before the columns were used.

The second method used for incorporation of enzyme complexes into asolectin vesicles was that of Schmidt and Gräber (1985). Protein (0.4 mg) was mixed with 35 mg of sonicated asolectin in 2% (w/v) Na-cholate pH (8.0), 1% (w/v) Na-deoxycholate, 10 mM Tricine-NaOH (pH 8.0), 0.1 mM EDTA (pH 8.0), and 0.5 mM DTT in 4 mL of total volume. The mixture was dialyzed in 6000 to 8000 mol wt cutoff dialysis tubing against 1 L of 10 mM Tricine-NaOH (pH 8.0), 2.5 mM $MgCl_2$, 0.2 mM EDTA (pH 8.0), and 0.5 mM DTT at 30°C for 12 h. As suggested by Grotjohann and Gräber (1990), the dialyzed proteoliposomes were centrifuged (11,290*g* for 6 min) to remove large vesicles and denatured material.

To determine the extent of complex incorporation, each sample was subjected to centrifugation at $96{,}600g_{(ave)}$ for 15 min, the supernatants were removed and saved, and the pelleted liposomes were resuspended to the same volume in the buffer used for column equilibration and centrifuged again. Aliquots of the original liposome samples, the first supernatants and pellets, and second supernatants and pellets were each used to measure sulfite-stimulated $Mg^{2+}$-ATPase activity (Richter et al., 1987; Wetzel and McCarty, 1993). The percentage of total activity partitioning with the pellet was assumed to be the percentage of the whole functional complex incorporated into liposomes, with the $CF_1$ values as a control for nonspecific binding.

### Reconstitution of $CF_1$ with $CF_1$-Depleted Subunit III Proteoliposomes

The gel filtration method described above was used to prepare $CF_1$-III and $CF_1$-$CF_0$ proteoliposomes and control (zero protein) liposomes with the following modifications: approximately 200 $\mu$g of each complex type was precipitated and then incorporated into liposomes, in a final buffer (600 $\mu$L) containing 30 mM KCl, 10 mM Tricine-NaOH (pH 8.0), 0.5 mM EDTA (pH 8.0), and 2 mM $MgCl_2$ (buffer A).

Each sample was divided into three (200 $\mu$L) aliquots and centrifuged (100,000*g*, 10 min) to pellet the (proteo)liposomes. The supernatants were discarded. One aliquot of each sample ("pretreatment") was resuspended in 150 $\mu$L of buffer B (buffer A plus 50 mM DTT) and set aside on ice until assayed for enzyme activity. The second aliquot of each sample ("nonstripped") was resuspended in 200 $\mu$L of buffer A. The final aliquot of each sample ("EDTA stripped") was

resuspended in 200 μL of 0.75 mM EDTA (pH 8.0), 1 mM Tricine-NaOH (pH 8.0). Both of these treatments proceeded for 30 min at room temperature, and then the samples were centrifuged to pellet the (proteo)liposomes.

Each pellet was resuspended in 200 μL of buffer containing 30 mM KCl, 10 mM Tricine-NaOH (pH 8.0), 0.5 mM EDTA (pH 8.0), and 1 mg $mL^{-1}$ of BSA. Each sample was then divided into two, 100-μL aliquots (12 total). One of each set of aliquots ("minus-$CF_1$") was mixed with 100 μL of buffer B, and the others ("plus-$CF_1$") were mixed with 100 μL of (1 mg $mL^{-1}$) DTT-reduced $CF_1$ suspended in buffer B. Finally, $MgCl_2$ (0.5 M stock) was added to all aliquots to 5 mM. The reconstitution was allowed to proceed for 20 min at room temperature before centrifugation to pellet the (proteo)liposomes. Each pellet was resuspended in 200 μL of buffer B, centrifuged again, and resuspended in 150 μL of buffer B. All samples were then placed on ice until assayed for sulfite-stimulated $Mg^{2+}$-ATPase activity (Richter et al., 1987; Wetzel and McCarty, 1993).

### Miscellaneous Procedures

SDS-PAGE was performed according to the method of Fling and Gregerson (1986), with gel sample buffer modified from that of Grotjohann and Gräber (1990) to contain 10% glycerol, 4% (w/v) SDS, 10% (w/v) β-mercaptoethanol, and 125 mM Tris-HCl (pH 6.8), with the final pH adjusted to 7.1 with NaOH. Gels were stained either with Coomassie brilliant blue R or by the silver staining method of Wray et al. (1981).

Protein was determined either by the method of Lowry et al. (1951), modifications of the Lowry method (Bensadoun and Weinstein, 1976; Markwell et al., 1978), or the Pierce BCA protein assay system, depending on the presence of interfering compounds. Apparent protein concentration values were adjusted to account for the presence of asolectin, based on standard curves. Asolectin interferes with the BCA protein assay system in an inconsistent manner (Kessler and Fanestil, 1986). With the method of Lowry et al. (1951), asolectin (final sample concentrations up to 0.05%) results in an increase of the intercept (zero protein) of the standard curves of up to 0.02 *A* unit but does not change the slope of the standard curves. BSA was used for all of the standard curves.

### Chemicals

DEAE-Trisacryl-M (IBF Biotechnics) was obtained from Serva Biochemicals (Paramus, NJ). Zwittergent 3–12 was purchased from Calbiochem Corp. (San Diego, CA). Asolectin was obtained from Associated Concentrates (Woodside, NY). Cholate was purified by treatment with activated charcoal, followed by repeated recrystallization (Kagawa and Racker, 1971). Protein A covalently coupled to Sepharose CL-4B was purchased from Sigma Chemical Co. Other reagents were of normal laboratory grade and were purchased from routine sources.

## RESULTS

### Preparation of $CF_1$-III

The procedure described for the purification of $CF_1$-III from crude $CF_1$-$CF_0$ is a modification of that used by Feng and McCarty (1990b) to prepare other subunit-deficient forms of $CF_1$-$CF_0$. Most of the impurities in the crude mixture are eluted from the DEAE-Trisacryl-M column by the initial 0.5% Triton X-100, 50 mM sodium phosphate buffer wash, while the $CF_1$-$CF_0$ complex remains bound to the matrix. Subsequent washes with staggered, inverse gradients of Zwittergent 3–12 and sodium phosphate remove subunits I and IV, and then the potentially destabilized subunit II (Feng and McCarty, 1990b) is eluted by a sodium phosphate gradient in the presence of 0.5% Na-cholate.

Results from the purification procedure are displayed in the SDS-PAGE gel of Figure 1. Lane 1 shows the subunit composition of the crude $CF_1$-$CF_0$, and lanes 2 through 4 show purified $CF_1$-$CF_0$, $CF_1$-III, and $CF_1$, respectively. The most persistent contaminants are the small and large subunits of Rubisco and subunits of the Cyt $b_6/f$ complex. Cyt-containing protein was identified spectrophotometrically from the difference spectrum of reduced versus oxidized protein (Hurt and Hauska, 1981), and the Rubisco subunits were identified by their positions on the SDS gel relative to a standard. Greater purity can be obtained by repeating the DEAE-Trisacryl-M chromatography step and/or passing the protein complex through a Rubisco immunoaffinity column, but the overall yield and specific activity of the enzyme will decrease. For the purposes of our investigation, one chromatography step was judged to result in an acceptable level of purification.

Table I shows typical values for recovery of $CF_1$-III during purification from crude $CF_1$-$CF_0$. Activity was measured as sulfite-stimulated $Mg^{2+}$-ATPase after reduction with DTT. The yield and degree of purification were somewhat variable.

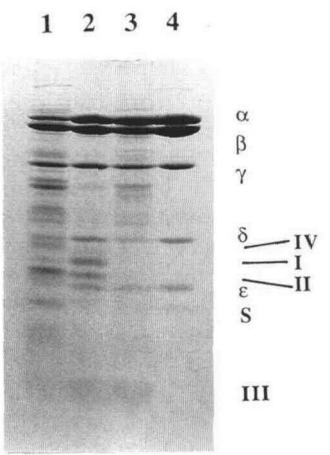


**Figure 1.** SDS-PAGE of crude $CF_1$-$CF_0$, purified $CF_1$-$CF_0$, $CF_1$-III, and $CF_1$. Protein (35 μg per lane) was prepared as described in "Materials and Methods," run on a 15% SDS-polyacrylamide gel, and stained with Coomassie blue. Lane 1, Crude $CF_1$-$CF_0$; lane 2, purified $CF_1$-$CF_0$; lane 3, $CF_1$-III; lane 4, $CF_1$. S indicates location of the small subunit of Rubisco.

**Table I.** *Recovery of $CF_1$-III after purification from crude $CF_1$-$CF_0$*

Sulfite-stimulated $Mg^{2+}$-ATPase activity (5–15 μg of protein) was measured in 4 mM ATP, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), and 100 mM $Na_2SO_3$ at 37°C for 10 min.

| | Crude $CF_1$-$CF_0$ | $CF_1$-III |
|---|---|---|
| Total protein (mg) | 100 | 6.7 |
| Total activity (μmol of Pi $min^{-1}$) | 283 | 60 |
| Specific activity (μmol of Pi $mg^{-1}$ $min^{-1}$) | 2.8 | 9.0 |
| Purification (fold) | 1 | 3.2 |
| Yield (% by activity) | 100 | 21 |

The purity of the crude $CF_1$-$CF_0$ starting material was a major source of the variation because preparations from a common crude $CF_1$-$CF_0$ stock showed similar yield and purity (not shown). Specific activity of $CF_1$-III is considerably higher than for the crude material, with a calculated 3.2-fold purification, whereas a comparison of total activity values indicates a yield of 21%. The range of yields was 14 to 21%. Because of the uncertainty about the subunit stoichiometry and mol wt of $CF_0$, and of the subunit III complex, specific activity values must be viewed with caution. Some $CF_1$ elutes from the column during the final sodium phosphate gradient, contributing to a decrease in recovery.

### Identification of Subunit III

The putative subunit III band electroblotted onto a polyvinylidene fluoride membrane from a 15% SDS-PAGE gel was subjected to amino acid analysis and gas-phase N-terminal sequencing. The observed amino acid composition (molar percent) and N-terminal sequence closely agree with predicted values based on published sequence information (Table II). This agreement indicates positive identification of subunit III in the purified complex. It was reported by Howe et al. (1982) that sequencing required removal of a putative blocked N terminus in subunit III, but this was not necessary in our analysis.

### Association of $CF_1$-III

The presence of subunit III in the same fractions as $CF_1$ does not evince their physical association; therefore, the association of the complex was tested in several ways. Figure 2 shows Suc density centrifugation fraction profiles of $CF_1$-III in the presence of Na-cholate plus $Na_2SO_3$ and Triton X-100, respectively. In Na-cholate plus $Na_2SO_3$, subunit III sediments with $CF_1$ to a level of 27% Suc, indicating an association that remains stable during the centrifugation. Similar results were obtained in a gradient containing Na-cholate without $Na_2SO_3$, but the equilibrated protein band was more diffuse (not shown). It appears that most of the subunit III sedimented with only half of the $CF_1$ (Fig. 2A, fraction 4 versus 5); this is probably an artifact due to the prolonged centrifugation and should not be taken as an indication of a large population of $CF_1$ lacking subunit III in the $CF_1$-III complex (see accompanying paper, Wetzel and McCarty, 1993). When in the presence of Triton X-100, conversely, subunit III dissociates from $CF_1$ and sediments at much lighter densities of 14 to 16% Suc (Fig. 2B). The $CF_1$ continues to sediment to 22 to 24% Suc. The purified $CF_1$-$CF_0$ holoenzyme remains associated during Triton X-100 Suc density centrifugation (Feng and McCarty, 1990b), which suggests that subunits I, II, and/or IV are necessary for stabilizing the complex in the presence of the detergent.

$CF_1$-III complex association was further tested by immunoprecipitation with anti-β IgGs. Figure 3 shows the SDS-PAGE-separated subunit profiles of $CF_1$, $CF_1$-III, purified $CF_1$-$CF_0$, and a zero-protein control. Pretreatment profiles (left panel, lanes 1–4) show the complete subunit compositions of the complexes. Precipitated profiles (center panel, lanes 1–4) show the subunits that fractionated with the pellet after binding of anti-β IgG and protein A-Sepharose beads. $CF_1$ is precipitated by the antibodies (center panel, lane 3). The $CF_1$-III lane (center panel, lane 4) clearly shows that subunit III remains associated with $CF_1$ throughout the precipitation procedure. Purified $CF_1$-$CF_0$ was not effectively precipitated under these conditions (center panel, lane 2); it is possible that $CF_0$ subunits I, II, and/or IV partially occlude antigenic sites on β. The right panel (lanes 1–4) shows the unprecipitated material from each complex. Lane 1 of each panel contains the IgG and buffer control. Results of the precipitation experiments support the hypothesis of $CF_1$-subunit III association.

The partitioning of sulfite-stimulated $Mg^{2+}$-ATPase activity of DTT-reduced enzyme complexes upon incorporation into

**Table II.** *Comparison of published (expected) amino acid composition (molar percent) and N-terminal sequence[a] for spinach subunit III (Deno et al., 1984) with those observed for subunit III isolated from the purified $CF_1$-III complex*

See "Materials and Methods" for procedural details.

| Amino Acid | Amino Acid Analysis | |
|---|---|---|
| | Expected | Observed |
| | M% | |
| Ala | 21.0 | 21.7 |
| Leu | 14.8 | 15.8 |
| Gly | 13.6 | 13.8 |
| Glu + Gln | 8.6 | 9.0 |
| Val | 8.6 | 8.6 |
| Ile | 7.4 | 7.3 |
| Pro | 4.9 | 5.5 |
| Ser | 3.7 | 3.1 |
| Thr | 3.7 | 3.4 |
| Phe | 3.7 | 3.4 |
| Asp + Asn | 2.5 | 2.3 |
| Arg | 2.5 | 2.7 |
| Met | 2.5 | 1.3 |
| Tyr | 1.2 | 1.3 |
| Lys | 1.2 | 0.5 |
| His | 0 | 0 |
| Cys | 0 | 0.6 |

[a] N-terminal sequence: Expected, (N terminal) Met-Asn-Pro-Leu-Ile-Ala-Ala-Ala-Ser-Val; observed, (N terminal) Met-Asn-Pro-Leu-Ile-Ala-Ala-Ala-Glu-Val/Gly.

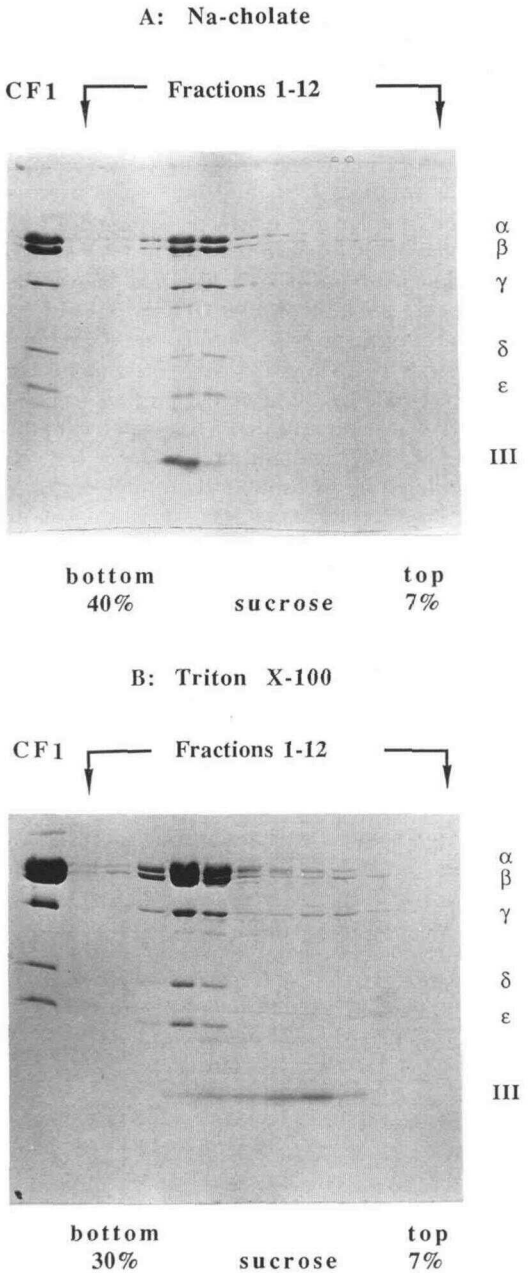


**Figure 2.** Suc density gradient centrifugation of $CF_1$-III in the presence of Na-cholate or Triton X-100. $CF_1$-III was centrifugally equilibrated in a 4.4-mL linear Suc gradient containing 30 mM Tris-succinate (pH 6.5), 0.5 mM EDTA, 0.1 mM ATP, 0.1% asolectin, and either 0.4% Na-cholate, 100 mM $Na_2SO_3$, and 7 to 40% Suc (A) or 0.2% Triton X-100 and 7 to 30% Suc (B). Fractions were collected, and aliquots were prepared and run on 15% SDS-polyacrylamide gels. The gels were stained with Coomassie blue. A shows subunit III remaining associated with $CF_1$ in the presence of Na-cholate, and B shows that Triton X-100 causes dissociation of subunit III. The first lane of each gel is a $CF_1$ standard.

asolectin liposomes is described in Table III. The activity that partitioned with the pelleted liposomes was assumed to represent enzyme complexes incorporated in liposomes. The results from the gel filtration method of liposome incorporation are displayed in Table III. For $CF_1$-III, there was 18% incorporation, and for purified $CF_1$-$CF_0$ there was 25% incorporation. The $CF_1$ sample showed 4% of total activity in the pelleted liposomes, which is a measure of nonspecific binding of $CF_1$ to the liposomes. Washing the pelleted liposomes resulted in some changes in the total activity ratios, but these changes are difficult to assess because there was overall loss or gain of total activity for some of the samples. The nonadditivity of the fractions can be attributed to experimental error: volumes and total amounts of protein were typically very small, making handling and quantitation difficult. However, the pellets of $CF_1$-$CF_0$ and $CF_1$-III proteoliposomes retained an appreciably greater percentage of total activity than that of the $CF_1$ liposomes even after washing. Similar results were obtained each time this experiment was repeated (data not shown).

$CF_1$-$CF_0$ and $CF_1$-III were also incorporated into liposomes by a cholate/deoxycholate dialysis procedure (Table III). $CF_1$-$CF_0$ showed 23% incorporation, with 1% lost after washing, and $CF_1$-III incorporated 19% of the total activity in the liposome fraction with a loss of 2% due to washing. There was no $CF_1$ control run in this experiment. The ability to become incorporated into liposomes indicates that $CF_1$ is associated with a lipophilic moiety, either $CF_0$ or subunit III.

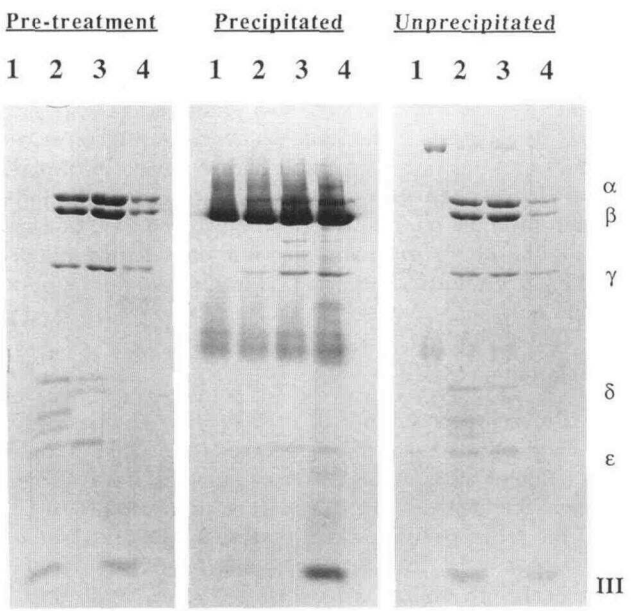


**Figure 3.** Immunoprecipitation of $CF_1$, purified $CF_1$-$CF_0$, and $CF_1$-III with anti-$\beta$ IgGs. Protein complexes were precipitated with antibody plus protein A covalently bound to Sepharose. Unprecipitated material was separated from the precipitated by centrifugation. Samples of pretreatment, precipitated, and unprecipitated material were prepared and subjected to SDS-PAGE. Lane 1, IgG, zero-enzyme control; lane 2, IgG plus purified $CF_1$-$CF_0$; lane 3, IgG plus $CF_1$; lane 4, IgG plus $CF_1$-III. The gel was stained with Coomassie blue.

**Table III.** *Partitioning of ATPase activity upon incorporation of enzyme complexes in asolectin liposomes*

$CF_1$, purified $CF_1$-$CF_0$, and $CF_1$-III were each incorporated in asolectin liposomes as described in "Materials and Methods." Cholate removal was either by Sephadex column or by dialysis. The liposome preparations were pelleted by centrifugation, and the pellets were washed and resuspended. Samples from precentrifugation, first pellet and supernatant, and washed pellet and supernatant were each assayed for sulfite-stimulated $Mg^{2+}$-ATPase activity (37°C, 10 min). N.D., Not detectable. The initial total activity values (nmol of Pi $min^{-1}$) were $CF_1$-$CF_0$ = 59, $CF_1$-III = 22, and $CF_1$ = 55 when cholate was removed by Sephadex column and $CF_1$-$CF_0$ = 153 and $CF_1$-III = 73 when cholate was removed by dialysis.

| Fraction | Percentage of Initial Total Activity | | |
|---|---|---|---|
| | $CF_1$-$CF_0$ | $CF_1$-III | $CF_1$ |
| Cholate removal by Sephadex column | | | |
| Pellet | 25 | 18 | 4 |
| Supernatant | 83 | 81 | 87 |
| Washed pellet | 16 | 6 | 1 |
| Supernatant | 10 | 7 | 3 |
| Cholate removal by dialysis | | | |
| Pellet | 23 | 102 | |
| Supernatant | 78 | 102 | |
| Washed pellet | 22 | 17 | |
| Supernatant | 6 | N.D. | |

### Reconstitution of $CF_1$ with $CF_1$-Depleted Subunit III Proteoliposomes

$CF_1$ will bind to isolated subunit III or $CF_0$ in proteoliposomes but not to zero-protein liposomes. Table IV shows the results of sulfite-stimulated $Mg^{2+}$-ATPase activity measurements of EDTA-stripped, nonstripped, $CF_1$-reconstituted, and -unreconstituted liposomes. The amount of total activity reflects the amount of $CF_1$ bound to the proteoliposomes. Clearly, treatment with EDTA under low salt conditions removes $CF_1$ from both $CF_1$-$CF_0$ and $CF_1$-III proteoliposomes. Reconstitution of $CF_1$ with the stripped vesicles results in restoration of activity. $CF_1$ will bind to nonstripped proteoliposomes, indicating that some $CF_0$ or subunit III sites are vacant in these samples. The zero-protein liposomes bound an undetectable amount of $CF_1$, supporting the hypothesis that the binding of $CF_1$ to $CF_0$ or subunit III in proteoliposomes is specific.

## DISCUSSION

Evidence presented in this paper supports a direct association between $CF_1$ and subunit III. The enzyme purified as described is clearly deficient in all $CF_0$ subunits except subunit III, although it may be slightly contaminated with Rubisco and Cyt $b_6/f$ complex subunits. The presence of non-ATPase complex proteins does not affect the conclusions reached from the presented data.

Association of the complex was confirmed by several methods. Cosedimentation of subunit III with $CF_1$ during Suc density centrifugation in the presence of cholate indicates association. Immunoprecipitation of the $CF_1$ by an antibody against the $\beta$ subunit results in concomitant precipitation of subunit III, again indicating association. Finally, the association of subunit III with $CF_1$ confers to $CF_1$ the ability to be incorporated into asolectin vesicles. Specificity of the binding of subunit III to $CF_1$ is explored in the accompanying paper (Wetzel and McCarty, 1993).

The incorporation of both $CF_1$-III and $CF_1$-$CF_0$ into liposomes could be improved. Reconstitution of protein complexes into artificial membranes is still as much art as science. Although the activity that partitioned with the vesicles is an indication of successfully incorporated complexes, the activity that remained in the soluble fraction does not necessarily indicate that the complexes were unable to become reconstituted. With further experimentation, especially with protein:lipid:detergent ratios in the liposome formation mixture, a much higher level of enzyme incorporation may be achieved.

Evidence supporting (C)$F_1$-proteolipid interaction has been obtained for bacteria and for lower plant chloroplasts but is ambiguous in the case of higher plant chloroplasts. The primary argument against the association is based on the observation that isolated subunit III will not bind purified $CF_1$ (Nelson et al., 1977; Sigrist-Nelson and Azzi, 1980). In these experiments, organic solvent extraction was used to prepare subunit III before it was incorporated into liposomes. Our results clearly demonstrate that subunit III is competent to bind $CF_1$ in the presence of $MgCl_2$. The asolectin membrane environment may stabilize a subunit III complex structure that is potentially disrupted during organic solvent extraction.

The stoichiometry of subunit III remains an open question. Lacking a clean antibody against subunit III, immunological

**Table IV.** *Reconstitution of $CF_1$ with subunit III or $CF_0$ in liposomes*

$CF_1$-III and $CF_1$-$CF_0$ were each incorporated into asolectin liposomes. The preparations were either treated with EDTA under low-salt conditions to strip $CF_1$ from the membranes (Stripped) or left in buffer (Nonstripped). Following centrifugation to remove liberated $CF_1$ from the proteoliposomes, fresh DTT-reduced $CF_1$ was added for reconstitution. Buffer containing an equivalent amount of DTT was added to control samples. Unbound $CF_1$ was removed, and all samples were assayed for sulfite-stimulated $Mg^{2+}$-ATPase activity (990 $\mu$L of 4 mM ATP, 2 mM $MgCl_2$, 10 mM Tricine-NaOH [pH 8.0] 100 mM $Na_2SO_3$ and 10 $\mu$L of proteoliposomes, 37°C, 10 min). Liposomes prepared in the absence of the protein complexes did not bind a significant amount of $CF_1$ either before or after EDTA treatment. Total activity is given as nmol of Pi formed per min.

| Liposomes | Reconstitution | Total ATPase Activity Bound | |
|---|---|---|---|
| | | Nonstripped | Stripped |
| $CF_1$-III | Buffer | 0.21 | <0.02 |
| | $CF_1$ | 0.54 | 0.16 |
| $CF_1$-$CF_0$ | Buffer | 0.33 | <0.02 |
| | $CF_1$ | 0.64 | 0.44 |

techniques could not be used. Dye binding and silver staining of subunit III are inconsistent from gel to gel, precluding densitometry as a quantitative approach. Fromme et al. (1987a) suggest a model of a complex of 12 subunits of subunit III, based on the presence of a high molecular mass (100 kD) protein band that correlates with the absence of the typical 8-kD band for subunit III under certain SDS-PAGE conditions. The same phenomenon was observed by us in some of the gels run for this study (unpublished observation).

Solubilization by cholate versus Triton X-100 and the resultant association or dissociation of subunit III, respectively, provides a useful method for obtaining isolated subunit III for further study. One obstacle faced in using subunit III isolated by this method is removal of Triton X-100 from the preparation, because the low critical micellar concentration of Triton X-100 makes it difficult to extract. Previously published methods for purifying subunit III require harsh or denaturing treatments, including organic solvents (Fillingame, 1976; Nelson et al., 1977; Sigrist et al., 1977) or preparative SDS-PAGE (Fromme et al., 1987a). Removal of $CF_1$ from the $CF_1$-III complex in solution by Triton X-100 (this paper) or by EDTA (this paper) or NaBr treatment (Grotjohann and Gräber, 1990) of $CF_1$-III proteoliposomes provides milder conditions for obtaining a potentially more physiologically intact complex for the study of proton translocation.

That $CF_1$ remains bound to subunit III and can be incorporated into liposomes in the absence of other $CF_0$ subunits does not preclude other $CF_1$-$CF_0$ connections. In addition to the evidence described in the introduction to this paper, results of other studies have supported the idea of multiple subunit interactions. Apley and Wagner (1990) found that, when $CF_1$-$CF_0$ is treated with eosin-5-isothiocyanate in artificial membranes, subunit III is specifically labeled and dissociates from the ATP synthase complex into monomers. $CF_1$ remains associated with the membrane, presumably bound to the other $CF_0$ subunits. Feng and McCarty (1990a) determined that subunit IV was required for ATP synthesis in the reconstituted enzyme complex. In *E. coli*, purified subunit c (equivalent to subunit III) is capable of limited binding of $F_1$ (Schneider and Altendorf, 1985). Also in *E. coli*, anti-b antibodies (b is equivalent to subunit I) disrupt binding of $F_1$ to $F_0$ (Deckers-Hebestreit et al., 1992). The cumulative evidence from studies of $CF_1$-$CF_0$ interactions indicates a model with several points of contact. It seems likely that there are different functions for each region of association: regulation of proton flow, transduction of electrochemical membrane potential, and conformational changes associated with activation or deactivation of the enzyme complex are possibilities. Now that methods exist for depletion and isolation of each $CF_0$ subunit, reconstitution of enzymically active ATP synthase complexes should begin to reveal more information about the influence of the subunits on $CF_1$ function.

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