Chromatographic Purification of the Chloroplast ATP Synthase (CF₀-CF₁) and the Role of CF₀ Subunit IV in Proton Conduction*

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Chromatographic procedures were developed to purify chloroplast ATP synthase (CF₀-CF₁) in large amounts and to resolve subunits from this enzyme. The ATP synthase thus obtained has high ATP-Pᵢ exchange and Mg²⁺-ATPase activities upon incorporation into asloelctin liposomes. The purity of this preparation was about 95%. By modifications of this chromatographic procedure, we purified subunit IV-deficient CF₀-CF₁, subunit IV-deficient CF₀, and subunit IV. Both ATP-Pᵢ exchange and Mg²⁺-ATPase activities were impaired by depletion of subunit IV from CF₀-CF₁. Partial restoration of these activities was obtained by reconstituting subunit IV-deficient CF₀-CF₁ with subunit IV. The impairment of these activities was likely caused by a loss in proton conductivity of CF₀ upon removal of subunit IV. The dicyclohexylcarbodiimide-sensitive Mg²⁺-ATPase of subunit IV-deficient CF₀-CF₁ was not as sensitive to the depletion of subunit IV as ATP-Pᵢ exchange. Nearly 90% of subunit IV could be removed, but Mg²⁺-ATPase activity was inhibited by only 40-60%. Thus subunit IV of CF₀-CF₁ may not participate directly in proton transfer but may have a role in organizing and/or stabilizing CF₀ structure.

The thylakoid membrane-associated ATP synthase of chloroplasts couples the proton motive force generated by electron transport to ATP synthesis. The ATP synthase of chloroplasts is quite similar to that in Escherichia coli and thermophilic bacterial and mitochondrial membranes. It is composed of two structurally distinct components in chloroplasts termed coupling factor 1 (CF₁) and CF₀. CF₀ is a peripheral membrane protein that contains five different subunits (α, β, γ, δ, and ε) and exhibits catalytic activity. CF₀ is an integral membrane protein complex which mediates proton translocation across the thylakoid membranes and, in conjunction with CF₁, couples proton flow to ATP synthesis and hydrolysis. CF₀ contains four subunits, designated I, II, III, and IV (1-3). We have purified four-subunit CF₀ and demonstrated that the four subunits were sufficient to form an active CF₀ which was capable of conducting protons and coupling proton flow to ATP synthesis and hydrolysis (4). Nevertheless, the roles of the individual subunits are poorly understood.

The counterpart of CF₀ in E. coli (F₀) is so far the best studied complex among the F₉-F₁ type ATP synthases. It consists of three subunits: a, b, and c. Both biochemical and genetic results suggested that all three subunits of F₀ are required for proton translocation (5-8). The c subunit, and probably the a subunit as well, directly participate in proton translocation (9-13). The b subunit has an extensive hydrophilic domain which has been suggested to be exposed to cytoplasm and to associate with F₁ (14, 15). Subunit III of CF₀, which is similar to subunit c, is a DCCD-binding protein and is directly involved in proton translocation. Subunit III itself is capable of forming active proton channels (16, 17).

Based on DNA sequences and the derived secondary polypeptide structures, subunit I is proposed to be analogous to subunit b (18, 19), and subunit IV is similar to subunit a (2, 20). Subunit II of CF₀ has no analog in E. coli F₀.

Direct information concerning the role of subunit IV of CF₀ was obtained in this study by selective removal and reconstitution of subunit IV. Subunit IV was shown to be required for DCCD-sensitive proton translocation by CF₀ and for stimulation of ATP Pᵢ exchange and DCCD-sensitive Mg²⁺-ATPase activities in asloelctin liposomes reconstituted with CF₀ deficient in subunit IV. To our knowledge, this is the first successful reconstitution of energy coupling by a CF₀ polypeptide.

MATERIALS AND METHODS

Purification of CF₀-CF₁. Subunit IV-deficient CF₀-CF₁. Subunit IV-deficient CF₀ and Subunit IV

Purification of CF₀-CF₁. The methods developed for the selective removal of CF₀ subunits are based on a chromatographic purification of CF₀-CF₁ that has not been described previously. For this reason and since intact CF₀-CF₁ is used as the standard for comparison of the activities of subunit IV-deficient enzyme, this method is described here. Thylakoids were prepared from market spinach leaves, and CF₀-CF₁ was solubilized from thylakoid membranes with 30 mM octyl glucoside and 0.4% (w/v) sodium cholate buffer according to Picket and Racker (1). Two methods were used to purify CF₀-CF₁ from the detergent extract. (a) The detergent extract was fractionated by adding a saturated ammonium sulfate (pH 8.0) solution. The precipitate between 37.5 and 45% saturation of ammonium sulfate was subjected to sucrose gradient centrifugation in a Beckman SW 41 rotor at 35,000 rpm for 18 h as described by Nelson (21). The fractions containing CF₀-CF₁, as revealed by SDS-polyacrylamide gel electrophoresis were collected. (b) The detergent extract was precipitated at 48% saturation of ammonium sulfate by adding saturated ammonium sulfate solution. The precipitate was dissolved in a minimal volume of 12474
of 3 mM MgCl₂, 10% (v/v) glycerol, 50 mM sodium phosphate buffer (pH 8.0) and was stored under liquid nitrogen. The 48% saturation of ammonium sulfate fraction containing 70–100 mg of protein was dialyzed against 1 liter of buffer A containing 10% glycerol, 1 mM MgCl₂, 1 mM DTT, and 50 mM sodium phosphate buffer (pH 7.0) for 8 h. Triton X-100 was added to the dialyzed sample from a 10% (v/v) solution to give a final concentration of 0.5%. The crude CF₆ CF₃ subunit IV was dialyzed against 1 liter of buffer B (pH 7.0) equilibrated with buffer B containing the ingredients of buffer A with the addition of 0.5% Triton X-100 and 0.01% (w/v) asolectin. The column was washed with 3 column volumes of buffer B, followed by 3 column volumes of buffer C containing 10% glycerol, 1 mM MgCl₂, 1 mM EDTA, 0.1 mM DTT, 0.01% asolectin, and 50 mM sodium phosphate buffer (pH 7.0). Then the column was washed with a 20–190 mM sodium phosphate buffer gradient (2 × 2.5 column volumes) (pH 7.0) in buffer C. Finally, CF₆ CF₃ subunit IV was eluted by 2 column volumes of buffer D containing 10% glycerol, 0.5% sodium cholate, 1 mM MgCl₂, 1 mM DTT, 0.01% asolectin, 0.5 M ammonium sulfate, and 100 mM sodium phosphate buffer (pH 7.0).

If high purity was desired, the peak fractions containing greater than 0.5 mg of protein/ml were pooled and dialyzed against 2 liters of buffer A for 8 h. Triton X-100 was added to the dialyzed sample from a 10% solution to give a concentration of 0.5%. The chromatographic procedure described above was repeated.

**Purification of Subunit IV-deficient CF₆ CF₃**—The crude CF₆ CF₃ was applied to the DEAE-Trisacryl column as described in the purification of CF₆ CF₃ (method b). After being washed with 3 column volumes of buffer B, the column was washed with 3 column volumes of buffer E containing 10% glycerol, 1 mM MgCl₂, 1 mM DTT, 0.06% (w/v) Zwittergent 3-12, 0.01% asolectin, and 50 mM sodium phosphate buffer (pH 7.0) to remove subunit (2). Then the column was washed with buffer C and the phosphate buffer gradient as described in the purification of CF₆ CF₃. Finally, subunit IV-deficient CF₆ CF₃ was eluted from the column by buffer D.

For storage of the purified protein, asolectin and ATP were added from 4% asolectin and 0.1 mM ATP (method b) solutions to the ATP synthase peak fractions obtained by the DEAE-Trisacryl chromatography to give final concentrations of 0.1% asolectin and 0.1 mM ATP. The purified CF₆ CF₃ and subunit IV-deficient CF₆ CF₃ were kept under liquid nitrogen.

**Measurement of ATP-induced Proton Uptake**

The purified ATP synthase was precipitated at 45% saturation of ammonium sulfate by adding solid salt and centrifuging the mixture at 12,000 × g for 10 min. The pellets, containing 2 mg of protein, were solubilized in 1 ml of 1.4% sodium cholate (pH 8.0) and 4.0% asolectin and incubated with 50 mM DTT on ice for 30 min. Incorporation of the ATP synthase into liposomes and measurement of ATP-induced proton uptake were carried out according to Admon et al. (25), with the exception that the ΔpH-sensitive fluorescence probe 9-aminoacridine was replaced by quinacrine. Quinacrine fluorescence was monitored with a Farrand model 801 spectrofluorometer. Excitation was 426 nm, and emission was 505 nm with 10-nm slits for both. The quinacrine fluorescence was passed through an Oriel LP47 filter, which transmits wavelengths >458 nm.

**Reconstitution of Subunit IV-deficient CF₆ with Subunit IV and Measurement of Proton Conducting Activity Driven by a K⁺ Gradient**

Concentrated subunit IV (20 μg in 200 μl) was added to the concentrated subunit IV-deficient CF₆ (100 μg in 200 μl). The mixture was dialyzed against 1 liter of 10% glycerol, 1 mM MgCl₂, 0.1 mM DTT, and 50 mM sodium phosphate (pH 8.0) in 0000–8000 molecular weight cutoff dialysis tubing for 3 days. The dialysis buffer was changed every 24 h. Duplicate samples containing either subunit IV-deficient CF₆ (100 μg) or subunit IV (20 μg) were prepared. The final volume was adjusted to 400 μl by adding the dialysis buffer. Reconstitution of CF₆ or subunit IV into asolectin liposomes was carried out as described by Okamoto et al. (26) with the following modifications. The weight ratio of CF₆ or subunit IV-deficient CF₆ to asolectin was about 1:1 and subunit IV to asolectin was about 1:650. The final concentration of asolectin was 20 mg/ml in the protein/lipid-detergent mixture. The mixtures were dialyzed against 1 liter of 2.5 mM MgCl₂, 0.1 mM DTT, and 10 mM Tricine-NaOH (pH 8.0) for 18 h at 4°C. The reconstituted proteoliposomes were loaded with KCl, and the proton translocation was measured as previously described (4).

**Other Analytic Methods**

Protein was determined according to Bensadoun and Weinstein (27); 0.3% SDS was included in the protein assay to solubilize lipids. SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (28) and Gregersen and Luyten (29). SDS-polyacrylamide gels were cast according to Oakley et al. (29).
Chemicals

DEAE-Trisacryl was purchased from Pharmacia LKB Biotechnology Inc. Assolectin was obtained from Associated Concentrates; quinacrine was from Sigma. Acrylamide was from Bio-Rad; ACMA was purchased from Molecular Probes, Inc. Zwittergent 3-12 was from Calbiochem. Other biochemicals were reagent grade. Cholic acid was purified by recrystallization (30).

RESULTS

Purification of CF\textsubscript{0}-CF\textsubscript{1}—Although the sucrose gradient centrifugation procedure is a well developed method for purifying CF\textsubscript{0}-CF\textsubscript{1} (1, 21), it is not suitable for large scale preparation and subsequent isolation of CF\textsubscript{0} subunits. The chromatographic procedure described in this paper overcomes these limitations. In our procedure, the crude chloroplast ATP synthase (48% ammonium sulfate precipitate) was bound to a DEAE-Trisacryl anion exchange column in the presence of 0.5% Triton X-100. Decreasing the concentration of Triton X-100 to less than 0.5% significantly reduced the amount of the ATP synthase that bound to the column.\textsuperscript{3} The majority of the contaminating proteins and colored materials in the crude preparation was eluted from the column by 50 mM sodium phosphate buffer in the presence of Triton X-100. Subsequent washing of the column with a 20–190 mM sodium phosphate buffer gradient in the presence of 0.5% sodium cholate efficiently removed ribulose bisphosphate carboxylase/oxygenase and free CF\textsubscript{1}, without loss of the ATP synthase. In the presence of sodium cholate, the ATP synthase bound to DEAE-Trisacryl very tightly and was only eluted from the column by a sudden increase in the ionic strength of the elution buffer. The fractions containing the ATP synthase were slightly yellowish. The presence of phosphate in the buffer system is crucial for obtaining highly active ATP synthase. The purification procedure described under “Materials and Methods” is for medium scale preparation. This purification can readily be scaled up to a final yield of 100 mg of ATP synthase by starting with 400–500 mg of the 48% saturation of ammonium sulfate precipitate on a 5 x 10-cm DEAE-Trisacryl column.

The SDS-polyacrylamide gel electrophoresis pattern of the purified ATP synthase is shown in Fig. 1. Like the ATP synthase purified by the sucrose gradient centrifugation procedure, the chromatographically prepared protein complex contains five CF\textsubscript{1} polypeptides and four CF\textsubscript{0} subunits, I, II, III, and IV, with apparent molecular masses of 18, 16, 8, and 20 kDa, respectively. Subunit III did not stain well with Coomassie Blue, and its presence was confirmed by \textsuperscript{14}C DCCD labeling (data not shown). The purity of the chromatographic preparation was 95% as judged by densitometric scanning of Coomassie Blue-stained gels. The purity of our preparation was very slightly lower than that of the sucrose gradient centrifugation preparation (97% for the ATP synthase peak fraction). This preparation is suitable for most experiments that require high activity. If higher purity is desired, repeating the anion exchange chromatographic procedure can increase the purity above 98%, but the specific ATP-P\textsubscript{i} exchange activity will be reduced by about 50%.

Table I shows a comparison of the purification of CF\textsubscript{0}-CF\textsubscript{1} by sucrose gradient centrifugation and anion exchange chromatography. Recovery of the ATP-P\textsubscript{i} exchange activity by our chromatographic purification procedure is usually more than 50%, which is more than four times that obtained by the sucrose gradient centrifugation procedure. The typical specific ATP-P\textsubscript{i} exchange activity of the ATP synthase purified by our chromatographic method was in the range of 100–250 nmol/mg ATP synthase/min, which was equivalent to or often higher than that purified by the centrifugation method. The protein yield of the chromatographic purification was typically 2–3 times that of the sucrose gradient preparation.

The chromatographically purified preparation of chloroplast ATP synthase, when reconstituted into liposomes, catalyzes ATP hydrolysis. The DCCD-sensitive ATPase activity was about 200 nmol of ATP hydrolyzed/mg of ATP synthase/min. ATP-induced ΔpH formation in reconstituted CF\textsubscript{0}-CF\textsubscript{1}-containing liposomes can be measured by determining the quenching of quinacrine fluorescence (Fig. 2). Typically, for such reconstituted proteoliposomes, 40–50% quenching of the quinacrine fluorescence can be obtained when ATP is added. The ATP-induced quenching was abolished by the ionophore gramicidin and completely inhibited by preincubation of the proteoliposomes with DCCD (Fig. 2).

Preparation of Subunit IV-deficient CF\textsubscript{0}-CF\textsubscript{1}, Subunit IV-deficient CF\textsubscript{0}, and Subunit IV—By modification of the chromatographic method for purifying CF\textsubscript{0}-CF\textsubscript{1}, subunit IV-deficient CF\textsubscript{0}-CF\textsubscript{1} can be obtained. We found that subunit IV dissociates from CF\textsubscript{0}-CF\textsubscript{1} at a very low concentration (0.08%) of Zwittergent 3-12, which has a critical micellar concentration of 0.12%. The dissociated subunit IV can be eluted from the DEAE-Trisacryl column by 50 mM phosphate buffer in the presence of Zwittergent 3-12 while subunit IV-deficient CF\textsubscript{0}-CF\textsubscript{1} remains on the column and can be collected separately. All remaining subunits in subunit IV-deficient CF\textsubscript{0}-CF\textsubscript{1} appeared to be associated together as judged by sucrose density gradient centrifugation (31). The SDS-polyacrylamide gel electrophoresis pattern of subunit IV-deficient CF\textsubscript{0}-CF\textsubscript{1} is shown in Fig. 3. Subunit IV stained much better with silver

\footnote{Y. Feng and R. E. McCarty, unpublished observation.}
TABLE I
Comparison of chloroplast ATP synthase purification by sucrose gradient centrifugation and by DEAE-Trisacryl chromatography

The ATP synthase was incubated with 50 mM DTT for 30 min on ice and then was incorporated into asolectin vesicles by gel filtration as described by Krupinski and Hammes (22). Reconstituted proteoliposomes containing 5–20 μg of protein were assayed for ATP-Pi exchange as described by Pick (23).

| Preparation recovery | Protein | ATP-Pi exchange | Total activity | % |
|----------------------|---------|----------------|---------------|---|
| Detergent extract    | 280     | 25             | 7000          | 100 |
| Sucrose gradient centrifugation* | 37.5–45% (NH₄)₂SO₄ fraction | 32 | 96 | 3072 | 44 |
| After sucrose gradient centrifugation | 8 | 100 | 800 | 11 |
| DEAE-Trisacryl chromatography* | 48% (NH₄)₂SO₄ fraction | 80 | 60 | 4800 | 68 |
| After DEAE-Trisacryl column | 20 | 183 | 3660 | 52 |

* The detergent extract of chloroplast thylakoid membranes was fractionated by ammonium sulfate. Precipitate between 37.5 and 45% saturation of ammonium sulfate was centrifuged on a sucrose gradient in the presence of 0.2% Triton X-100 according to Nelson (21).

The detergent extract was precipitated at 48% saturation of ammonium sulfate. The precipitate was fractionated on a DEAE-Trisacryl column as described under "Materials and Methods."

![Fig. 2. ATP-dependent pH gradient formation in ATP synthase-containing liposomes.](image)

A, ATP-dependent quenching of quinacrine fluorescence in ATP synthase-containing liposomes. B, as in A except that ATP synthase-containing liposomes were incubated with 50 μM DCCD for 30 min on ice before assay.

![Fig. 3. SDS-polyacrylamide gel electrophoresis of subunit IV-deficient CFo-CF₁.](image)

Electrophoresis was carried out on a 15% polyacrylamide gel. Samples in lanes 1 and 2, containing 60 μg of protein, were run on the gel stained by Coomassie Blue, and samples in lanes 3 and 4 containing 30 μg of protein were run on the gel stained by silver nitrate. Lanes 1 and 3, CF₀-CF₁ purified by the chromatographic method; lanes 2 and 4, subunit IV-deficient CF₀-CF₁.
constant (about 30 nmol/mg protein/min) and, therefore, to a great extent the percentage of the residual activity in the subunit IV-deficient CFo-CF1 preparation depends on the activity of the corresponding CFo-CF1 preparations, which varied from 100 to 250 nmol/mg protein/min. Strong inhibition of ATP-Pi exchange which is consistent with the deficiency of subunit IV is always observed only when the activity of the corresponding CFo-CF1 preparation is over 150 nmol/mg protein/min. Although the DCCD-sensitive Mg2+-ATPase is also impaired upon removal of subunit IV, the percentage of residual DCCD-sensitive Mg2+-ATPase activity is always higher than that of the ATP-Pi exchange. Removal of 80–90% of subunit IV caused only a 40–55% inhibition of Mg2+-ATPase.

Reconstitution of subunit IV with subunit IV-deficient CFo-CF1 by a dialysis procedure partially restored the ATP-Pi exchange activity (Fig. 6). However, when the amount of subunit IV was increased above a certain level during reconstitution, the ATP-Pi exchange was inhibited. The reason for inhibition of exchange at higher subunit IV levels is unclear but may be the result of traces of detergent in the preparation. Residual Zwittergent appears not only to induce proton leakiness of the membrane but also to prevent correct reconstitution of subunit IV with subunit IV-deficient CFo-CF1. The direct addition of subunit IV to subunit IV-deficient CFo-CF1, only caused further inhibition of the residual activity in the subunit IV-deficient CFo-CF1 preparation (data not shown). It is necessary to reconstitute subunit IV with subunit IV-deficient CFo-CF1 by the dialysis procedure to reduce the Zwittergent concentration. Like the ATP-Pi exchange reaction, the Mg2+-ATPase activity was also restored by dialysis of subunit IV-deficient CFo-CF1 with subunit IV (Fig. 7). In contrast to ATP-Pi exchange, Mg2+-ATPase was not inhibited at high concentrations of subunit IV. This is understandable because Mg2+-ATPase activity is much less sensitive to the proton leak caused by the residual detergent than ATP-Pi exchange. The restoration of ATP-Pi exchange and Mg2+-ATPase activities to subunit IV-deficient CFo-CF1 by the addition of subunit IV provides strong evidence that the impairment of the activities in the subunit IV-deficient CFo-CF1 preparation is the direct consequence of the loss of subunit IV.

Reconstitution of Proton Translocating Activity—We have shown that purified CFo is capable of translocating protons when incorporated into asolectin liposomes (4). Proton translocation was measured after loading CFo-containing liposomes with potassium and induction of a potassium diffusion potential by valinomycin. The proton uptake due to a negative inside membrane potential was monitored by ACMA, a fluorescent pH indicator. When the same experiments were performed with the proteoliposomes containing either subunit IV-deficient CFo or subunit IV, there was almost no DCCD-sensitive proton translocation (Fig. 8, B and C). Reconstituting subunit IV and subunit IV-deficient CFo restored proton conducting activity, which was inhibited by DCCD (Fig. 8A). The slow rate of proton transfer by the reconstituted CFo is probably partially due to loss of some subunit III during purification.

DISCUSSION

The chromatographic procedure described in this paper is a convenient way to purify CFo-CF1 in large quantity with high purity. By modifications of this procedure, we obtained subunit IV-deficient CFo-CF1, CFo (4), subunit IV-deficient CFo, and subunit IV. The CFo-CF1 purified by this procedure has high activity in comparison with that purified by the
Subunit IV of Chloroplast $F_0$

**TABLE II**

Impairment of ATP-P$_\text{i}$ exchange and Mg$^{2+}$-ATPase activities upon removal of subunit IV from CF$_0$-CF$_1$

Freshly prepared CF$_0$-CF$_1$ and subunit IV-deficient CF$_0$-CF$_1$ were incorporated into asolectin liposomes by gel filtration. Aliquots of proteoliposomes were assayed for the activities as described in Table I and under "Materials and Methods." The residual subunit IV in the subunit IV-deficient CF$_0$-CF$_1$ preparation was 12%. For determination of DCCD-sensitive Mg$^{2+}$-ATPase activity, the mean activity of duplicate samples treated with 50 $\mu$M DCCD was subtracted from the total activity of DCCD-untreated samples.

| Preparation | ATP-P$_\text{i}$ exchange | DCCD-sensitive Mg$^{2+}$-ATPase |
|-------------|---------------------------|-------------------------------|
| CF$_0$-CF$_1$ | 206                        | 100 206 95 45               |
| Subunit IV-deficient CF$_0$-CF$_1$ | 31                         | 15 208 45                 |

**Fig. 6.** ATP-P$_\text{i}$ exchange activity upon reconstitution of subunit IV with subunit IV-deficient CF$_0$-CF$_1$. Subunit IV (0, 1, 5, 10, and 20 $\mu$g) was reconstituted with 0.5 mg of subunit IV-deficient CF$_0$-CF$_1$ by dialysis as described under "Materials and Methods." Then the reconstituted CF$_0$-CF$_1$ was incorporated into asolectin liposomes by the gel filtration procedure. Aliquots of CF$_0$-CF$_1$-containing liposomes were assayed for ATP-P$_\text{i}$ exchange activity as described in Table I.

**Fig. 7.** Mg$^{2+}$-ATPase activity upon reconstitution of subunit IV with subunit IV-deficient CF$_0$-CF$_1$. Reconstitution of subunit IV with subunit IV-deficient CF$_0$-CF$_1$ and subsequent reconstitution of CF$_0$-CF$_1$ with asolectin liposomes was carried out as described in Fig. 6. Aliquots of CF$_0$-CF$_1$-containing liposomes were assayed for Mg$^{2+}$-ATPase activity as described in Table II. Duplicate samples were treated with 50 $\mu$M DCCD prior to the assay. $\square$, subunit IV-deficient CF$_0$-CF$_1$+ subunit IV; $\blacksquare$, subunit IV-deficient CF$_0$-CF$_1$+ subunit IV + DCCD. The DCCD-insensitive Mg$^{2+}$-ATPase activity was subtracted from the total activity in DCCD-untreated samples; the DCCD-sensitive Mg$^{2+}$-ATPase activity is shown in the inset.

The results presented here show that removal of subunit IV from the CF$_0$-CF$_1$ impaired both ATP-P$_\text{i}$ exchange and Mg$^{2+}$-ATPase activities. The impaired activities were partially restored by adding back purified subunit IV to subunit IV-deficient CF$_0$-CF$_1$. Therefore, there is little doubt that the impairment in the activities is directly related to the loss of subunit IV from CF$_0$-CF$_1$. The measurements of proton conductivity of subunit IV-deficient CF$_0$ and the reconstituted CF$_0$ indicate that CF$_1$ loses its DCCD-sensitive proton conductivity when subunit IV is depleted. These results suggest that loss of ATP-P$_\text{i}$ exchange and Mg$^{2+}$-ATPase activities is caused by impairment of proton conductivity in CF$_0$ upon removal of subunit IV. After dissociation of CF$_1$, CF$_0$ in thylakoid membranes gradually converted into an inactive form and lost its proton conductivity (32-34). The mechanism underlying this conversion is not clear. Nelson and Eytan (32) suggested that CF$_1$ might play an important role in assembly and stabilization of functional CF$_0$. Our experiments show that the inactivation of CF$_0$ upon removal of CF$_1$ may be caused by dissociation of subunit IV from CF$_0$. Subunit IV can be removed from the intact CF$_0$-CF$_1$ complex by 0.08% Zwittergent 3-12, a concentration lower than the critical micellar concentration (0.12%), while the other subunits remain associated (31). It is conceivable that once CF$_1$ is dissociated from CF$_0$, the interaction between subunit IV and the other subunits of CF$_0$ becomes very weak. Thus subunit IV more easily dissociates from the other CF$_0$ subunits than in CF$_1$-associated CF$_0$. CF$_0$ may undergo further conformational change when subunit IV is dissociated. A consequence of dissociation of subunit IV is loss of proton conductivity by CF$_0$, as demonstrated in our experiments.

Subunit IV of CF$_0$ has similar secondary and tertiary structural features to the $a$ subunit of F$_0$ in E. coli (19). The role of subunit $a$ in proton translocation is still under investigation in regard to whether subunit $a$ directly participates in proton translocation or only stabilizes the conformation of the proton channel built up by oligomers of subunit $c$ (9, 35). Recently, Cain and Simoni (10-12) reported that several missense mutations in the $ucB$ (subunit $a$) gene resulted in the substitution of certain polar amino acids with nonpolar amino acids in the carboxyl-terminal region of subunit $a$. These substitutions impaired proton conductivity but did not alter $F_0$ binding. Based on these studies, it was proposed that these polar amino acid residues might directly participate in proton transport (10-13). However, this may not be an unequivocal explanation. A single amino acid change in one subunit of the complex may lead to major alterations in biogenesis, subunit assembly, conformation, and activity of the complex.
sensitive ATP hydrolysis, in contrast, protons only need to be involved in the ATP-Pi exchange activity. The partial inhibition of DCCD-sensitive Mg2+-ATPase activity by DCCD, Mg2+-ATP hydrolysis is not tightly coupled to proton transport. The subunit of *Rhodospirillum rubrum* F0F1 may be selectively removed from chromatophore membranes and high rates of ATPase activity reconstituted by the addition of either E. coli (37) or spinach chloroplast (38) subunit. Although reconstitution with *R. rubrum* subunit restores both ATPase activity and ATP-driven proton translocation, little proton translocation is linked to ATP hydrolysis by the hybrid enzymes. The hydrolysis of CTP-ATP by *R. rubrum* F0F1, also does not drive proton transport. Similar results were reported for *E. coli* ATP synthase (10–12). While proton translocation was impaired upon substitution of certain amino acids in subunit α of F0, the DCCD-sensitive ATPase activity of the membrane-bound F0F1 was much less affected.

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