Subunit Interactions within the Chloroplast ATP Synthase (CF₀-CF₁)
as Deduced by Specific Depletion of CF₀ Polypeptides*

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The proton-linked ATP synthase (CF₁-CF₀) of chloroplasts consists of a catalytic component (CF₁) and a membrane-embedded part (CF₀) that interacts with CF₁ and contains a proton channel. The subunits of CF₀ which are involved in binding of CF₁ were studied by examining the effect of selective depletion of subunits I, II, and IV of CF₀ from the chloroplast ATP synthase on the assembly of the remaining CF₀ subunits with CF₁. Dissociated CF₀ subunits were identified by sucrose density gradient centrifugation. Removal of subunit IV alone from CF₀-CF₁ did not cause dissociation of the other CF₀ subunits from CF₁. Upon removal of both subunits I and IV from CF₀-CF₁, subunit II also dissociated, but subunit III was still bound to CF₁. Thus, at least two subunits of CF₀, I and III, directly associate with CF₁. Subunit II is unlikely to bind CF₁ directly and may associate with subunit I. Although depletion of subunit IV does not cause dissociation of CF₀ from CF₁, its interaction with CF₁ subunits is uncertain.

The interactions between CF₀, and CF₁ of the chloroplast ATP synthase may involve several subunits both in CF₁ and CF₀. In CF₁, at least two subunits, α and δ, probably associate with CF₀ subunits (1-4). Reconstitution of subunit δ deficient CF₁ with CF₀-depleted thylakoid membranes indicates that subunit δ is not absolutely required for the binding of CF₀ to CF₁ (1-4), but in the absence of the δ subunit, CF₁ binding is reduced (5) and the CF₀ proton channel is not blocked (1-4). Subunit α of CF₁ may be protected from proteolytic cleavage when CF₁ is bound to the thylakoid membranes (2). Two subunits of CF₀, I and II, have large hydrophilic domains that are proposed to protrude into the chloroplast stroma and possibly interact with CF₁ (6-8). Evidence from the studies of the biogenesis of chloroplast ATP synthase in Chlamydomonas reinhardtii, a green algae, shows that subunit III may also be involved in the binding of CF₁. Chloroplast ATP synthase in C. reinhardtii contains nine subunits, five in CF₁ and four in CF₀, similar to the subunit composition of chloroplast ATP synthase in higher plants (9). Lemarie and Wollman (10) reported that in a mutant lacking subunit IV, a significant amount of CF₁ in the cells was membrane-bound (5-10% of that in the wild type), but in a mutant lacking both subunits III and IV, binding of CF₁ to the membranes was abolished. Their studies suggest that subunit III may be involved in the binding of CF₁ to the thylakoid membranes while subunit IV may not be absolutely required. However, depletion of any CF₀ subunits in these mutants could prevent the assembly of CF₀ or alter CF₀ structure. It is difficult, therefore, to draw unambiguous conclusions about the subunit interactions in CF₀-CF₁ from these experiments. Another approach to studying the subunit interactions in CF₀-CF₁ employed chemical cross-linking. By this method, cross-links of α-II, β-I, β-II, γ-II, δ-I, and ε-III were reported (11). Similar experiments were done by Rott and Nelson (12). However, the yield of cross-linked product was low, and nonspecific cross-links and aggregation cannot be excluded.

In this paper, we report a different approach to studying the subunit interactions between CF₁ and CF₀. By examining the association of CF₀ subunits with CF₁ after selective removal of particular CF₀ subunits by procedures previously reported (10), we were able to determine more specifically which subunits of CF₀ directly interact with CF₁.

MATERIALS AND METHODS
Preparation of CF₀-CF₁, Subunit-deficient CF₀-CF₁, and CF₁—Spinach chloroplast CF₀-CF₁ and subunit IV-deficient CF₀-CF₁ were prepared by the DEAE-Trisacryl chromatographic methods as previously described (13). CF₁ was prepared according to Feng and McCarty (5). For preparation of subunit I- and IV-deficient CF₀-CF₁, the 48% saturation of the ammonium sulfate fraction of crude ATP synthase was applied to a DEAE-Trisacryl column (2.5 x 9 cm) as previously described for purification of subunit IV of CF₁ (33). The column was washed with 3 column volumes of 10% glycerol, 1 mM MgCl₂, 1 mM DTT, 0.5% Triton X-100, 0.01% asolectin, and 50 mM sodium phosphate (pH 7.0) followed by 3 column volumes of 10% glycerol, 1 mM MgCl₂, 1 mM DTT, 0.5% Triton X-100, 0.01% asolectin, and 60 mM sodium phosphate (pH 7.0). Subunits I and IV were then eluted from 3 column volumes of 10% glycerol, 1 mM MgCl₂, 1 mM DTT, 20 mM Zwittergent 3-12, 0.01% asolectin, and 50 mM sodium phosphate (pH 7.0). The column was then washed with 3 column volumes of 10% glycerol, 1 mM MgCl₂, 1 mM DTT, 0.5% sodium cholate, 0.1% asolectin, and 50 mM sodium phosphate (pH 7.0). Finally, subunit I- and IV-deficient CF₁-CF₀ was eluted from the column by 10% glycerol, 1 mM MgCl₂, 1 mM DTT, 0.5% cholate, 0.01% asolectin, 0.5 mM ammonium sulfate, and 100 mM sodium phosphate (pH 7.0).

Sucrose Density Gradient Centrifugation—Sodium ammonium sulfate was added to the DEAE-Trisacryl chromatographic fractions containing CF₀-CF₁, or subunit deficient CF₀-CF₁, to 46% of saturation. The mixtures were kept on ice for 30 min and centrifuged at 12,000 x g for 10 min. The precipitates were dissolved in 0.1 mM ATP, 0.5 mM EDTA, 0.1% asolectin, 0.2% Triton X-100, and 30 mM Tris succinate (pH 6.5) to a concentration of about 20 mg of protein/ml. The protein solution (0.5 ml) was layered onto a 10-ml 5-30% sucrose gradient and centrifuged at 4°C for 18 h. Fractions of 0.6-0.8 ml were collected from the bottom of each centrifuge tube. CF₀ prepared as previously described (5) was diazylated against 1

*This work was supported by Grant DMB 88-03608 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The abbreviations used are: CF₀, chloroplast FₐFₒ ATP; CF₁, chloroplast coupling factor 1; Fₒ, the hydrophobic proton conducting sector of H⁺-ATPase; DCCD, N,N'-dicyclohexylcarbodi-imide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.
liter of 0.1 mM ATP, 0.5 mM EDTA, and 30 mM Tris succinate (pH 6.5) for 6 h to remove glycerol before it was applied to the sucrose gradient and centrifuged.

Other Analytic Methods—Protein concentration was determined according to Bensadoun and Weinstein (15). SDS-polyacrylamide gel electrophoresis was carried out as described by Fling and Gregerson (16). Silver staining of SDS-polyacrylamide gels was carried out according to Oakley et al. (17).

Chemicals—DEAE-Trisacryl was purchased from Pharmacia LKB Biotechnology Inc. Asolectin was obtained from Associated Concentrates. [14]C]DDC was purchased from Amersham Corp. Acrylamide was from Bio-Rad and Zwittergent 3-12 from Calbiochem. Other chemicals were reagent grade. Cholic acid was purified by recrystallization (18).

RESULTS

We have developed methods to remove selectively subunits I and IV from spinach CFo-CF1 (13). By examining the effect of depletion of particular CFo subunits on the association of the remaining CFo subunits with CF1, we can determine which CFo subunits interact with CF1. Since the molecular masses of CFo subunits are much lower than the mass of CF1, CFo subunits dissociated from CF1 can be easily identified following sucrose density gradient centrifugation.

To obtain subunit IV-deficient CFo-CF1, CFo-CF1 was bound to a DEAE-Trisacryl column. Subunit IV was dissociated from the complex and eluted from the column by 0.08% Zwittergent 3-12 in the presence of 50 mM sodium phosphate buffer (13). Subunit IV-deficient CFo-CF1 recovered from the column was applied to a sucrose density gradient. Triton X-100 (0.2%) was included in the sucrose gradient buffer solution to prevent aggregation of polypeptides. Triton X-100 was used to keep CFo-CF1 soluble during purification by the sucrose gradient centrifugation procedure developed by Pick and Racker (14). This detergent does not cause dissociation of CFo subunits from CF1. Fig. 1 shows the SDS-polyacrylamide electrophoresis pattern of the sucrose gradient fractions containing either subunit IV-deficient CFo-CF1 or CF1. Because of its lower molecular mass, CF1 sediments slower than CFo when subjected to radial acceleration in an ultracentrifuge and moved to a sucrose gradient zone containing 8-11% sucrose (Fig. 1B), whereas subunit IV-deficient CFo-CF1 sediments at a zone containing 20-22% sucrose (Fig. 1A). In the absence of subunit IV, the other CFo subunits remained associated with CF1. The sucrose density gradient peak fraction of subunit IV-deficient CFo-CF1 was electrophoresed on a SDS-polyacrylamide gel (Fig. 2). The gel was lightly stained with silver and scanned with a densitometer to quantitate the residual subunit IV in subunit IV-deficient CFo-CF1. In comparison with control CFo-CF1, there was only 18% residual subunit IV in subunit IV-deficient CFo-CF1. These experiments clearly demonstrate that depletion of subunit IV does not cause dissociation of the other CFo subunits from CF1.

Subunits I and IV can be depleted together from CFo-CF1 on a DEAE-Trisacryl column by elution of the column with 20 mM Zwittergent 3-12 at a low concentration of sodium phosphate (5 mM) (13). After removal of the Zwittergent from the column, subunit I- and IV-deficient CFo-CF1 was eluted with a high salt buffer. The subunit-deficient CFo-CF1 thus obtained was partially purified. In addition to the subunit-deficient CFo-CF1, ribulose bisphosphate carboxylase/oxygenase and some other minor contaminants were present (Fig. 3). This preparation was applied onto a sucrose density gra-
Subunit Interactions between CF<sub>0</sub> and CF<sub>1</sub>

Samples obtained by the DEAE-Trisacryl chromatography followed by sucrose density gradient centrifugation were analyzed on 15% acrylamide gels as described under "Materials and Methods." The amounts of subunits I and II were determined by scanning the Coomassie Blue-stained gels with a densitometer. Subunit IV was quantitated on silver-stained gels by densitometric scanning. The amount of subunit III was determined by [<sup>14</sup>C]DCCD labeling. Amounts of CF<sub>0</sub> subunits in control CF<sub>0</sub>-CF<sub>1</sub> were assumed to be 100%.

| CF<sub>0</sub> subunits depleted | Subunits that remain associated with CF<sub>1</sub> |
|-------------------------------|-----------------------------------------------|
| IV                           | I     | II   | III | IV   |
| I + II + IV                  | 102   | 96   | >90 | 18   |

Table I

Quantitation of the remaining CF<sub>0</sub> subunits associated with CF<sub>1</sub> in subunit-deficient CF<sub>0</sub>-CF<sub>1</sub>

Samples obtained by the DEAE-Trisacryl chromatography followed by sucrose density gradient centrifugation were analyzed on 15% acrylamide gels as described under "Materials and Methods." The amounts of subunits I and II were determined by scanning the Coomassie Blue-stained gels with a densitometer. Subunit IV was quantitated on silver-stained gels by densitometric scanning. The amount of subunit III was determined by [<sup>14</sup>C]DCCD labeling. Amounts of CF<sub>0</sub> subunits in control CF<sub>0</sub>-CF<sub>1</sub> were assumed to be 100%.

**Fig. 3.** SDS-polyacrylamide gel electrophoresis pattern of sucrose density gradient fractions of subunit I- and IV-deficient CF<sub>0</sub>-CF<sub>1</sub>. The subunit I- and IV-deficient CF<sub>0</sub>-CF<sub>1</sub> preparation was obtained by the chromatographic method described under "Materials and Methods" and fractionated on a sucrose density gradient. After centrifugation, 18 fractions were collected from the sucrose gradient, and samples were electrophoresed on a 15% acrylamide gel. Lane I, CF<sub>0</sub>-CF<sub>1</sub> (30 µg); lane II, subunit I- and IV-deficient CF<sub>0</sub>-CF<sub>1</sub> (30 µg) obtained by DEAE-Trisacryl chromatography; lanes 1-11, sucrose density gradient fractions (fractions 1-11) of subunit I- and IV-deficient CF<sub>0</sub>-CF<sub>1</sub>. The gel was stained by Coomassie Blue. SW, sucrose gradient fractions (fractions 1-11) of subunit I- and IV-deficient CF<sub>0</sub>-CF<sub>1</sub>.

**Fig. 4.** Identification of [<sup>14</sup>C]DCCD-binding proteins in the sucrose gradient fractions containing subunit II or CF<sub>1</sub>. Sucrose gradient fraction 2 (the sample shown in Fig. 3, lane 2) (150 µg of protein) containing subunit II, fraction 10 (the sample shown in Fig. 3, lane 10) (110 µg of protein) containing CF<sub>1</sub>, and control CF<sub>0</sub>-CF<sub>1</sub> (50 µg) were treated with 50 µM [<sup>14</sup>C]DCCD and applied onto a 15% acrylamide gel. Electrophoresis and staining were carried out as described in the legend to Fig. 3. The gel was sliced and the radioactivity of the slices was determined as described under "Materials and Methods." A, densitometric scan of a Coomassie Blue-stained CF<sub>0</sub>-CF<sub>1</sub>; B, [<sup>14</sup>C]DCCD labeling pattern of control CF<sub>0</sub>-CF<sub>1</sub>; C, [<sup>14</sup>C]DCCD labeling pattern of sucrose gradient fraction 2; D, [<sup>14</sup>C]DCCD labeling pattern of sucrose gradient fraction 10.

Zwittergent, however (Fig. 3), so it is unlikely that subunit II binds to CF<sub>1</sub> by itself.

The sucrose density gradient fractions of the subunit I- and IV-deficient CF<sub>0</sub>-CF<sub>1</sub> preparation were treated with [<sup>14</sup>C]DCCD to determine whether subunit III in this preparation was associated with CF<sub>1</sub>. The labeled fractions were then run on a SDS-polyacrylamide gel. Fig. 4 shows the [<sup>14</sup>C]DCCD labeling patterns of the sucrose density gradient fractions containing subunit II and CF<sub>1</sub>. Both fractions contained a [<sup>14</sup>C]DCCD-labeled polypeptide with a molecular mass of 8 kDa. The distribution of the 8-kDa DCCD-binding polypeptide in the sucrose density gradient is shown in Fig. 5. The majority of radioactivity was present in the fractions contain-
Wollman’s experiments (10) in which C. reinhardtii mutants were used to study the subunit interactions between CFo and CF1. This approach is advantageous because it allows for the study of subunit interactions in assembly without the confounding effects of subunit depletion on assembly of the complex.

Our results show that removal of subunit IV by treatment of CFo-CF1 with 0.08% Zwittergent does not cause dissociation of other CFo subunits from CF1. All remaining CFo subunits sediment with CF1. Although we do not know at this stage whether subunit IV directly interacts with CF1, it is clear that association of the other CFo subunits with CF1 is not dependent on the binding of subunit IV. Subunit III appears to interact directly with CF1. It associates with CF1, even in the absence of other CFo subunits. Our results regarding the requirement for subunits III and IV in the binding of CF1 are in agreement with the conclusions made by Lemaire and Wollman (10), who studied the assembly of CFo-CF1 in C. reinhardtii mutants in which subunit IV or both subunits III and IV were depleted. An opposite observation in regard to the interaction of subunit III and CF1 came from the study of binding of CF1 to butanol-extracted chloroplast subunit III (23). Subunit III thus obtained did not bind CF1, when incorporated into liposomes. This could be due to improper orientation or organization of subunit III in the liposomes or possible denaturation of subunit III upon butanol extraction. The subunit interactions involved in the binding of subunits I and II to CF1 seem more complicated than that of subunit III. These two subunits form a stable association with CF1 in the absence of subunit IV, but in the absence of both subunits I and IV, subunit II by itself can be dissociated from CF1. This indicates that there is likely to be a direct interaction between subunits I and II. Association of subunit II with CF1 could be mainly through subunit I which is proposed to have a CF1-binding site at its large hydrophilic domain and to have a hydrophobic domain inserted into the membrane (6, 7, 24).

Although we have shown that binding of subunits I and II to CF1 does not require subunit IV, possible interactions between subunit IV and subunits I and II cannot be excluded, and such interactions may stabilize the whole complex.

**Escherichia coli** FO, contains three subunits called a, b, and c that correspond to CFo IV, I, and III, respectively. The b subunit has a large hydrophilic domain likely involved in F1 binding and a hydrophobic domain interacting with other F0 subunits (25–28). There are two copies of subunit b in each F0-F1 molecule (27). From chemical cross-linking evidence, these two b subunits may form a dimer in *E. coli* ATP synthase (27). Our experiment shows that, similar to the subunit b dimer in *E. coli* FO, subunits I and II of CFo also interact with each other. This structural similarity in b-b and I-II interaction is particularly interesting. Based on the DNA sequences, subunits I (or b) and II (or b’) of the ATP synthase from the cyanobacterium *Synechococcus* have a similar predicted secondary polypeptide structure featuring a hydrophilic domain and a NH2-terminal hydrophobic domain (29). Cozens and Walker (29) suggested that the cyanobacterial ATP synthase could contain single copies of subunits I and II instead of two identical b subunits as in the *E. coli* ATP synthase. The 32 NH2-terminal residues of CFo subunit II of spinach show 41% homology to that of the cyanobacteria (30). Immunoneutralization evidence also indicates that the topography of subunits I and II is similar and that both have a large hydrophilic carboxyl-terminal portion protruding into the stroma (7). It is possible that subunits I and II of CFo perform the function that the b dimer of *E. coli* FO does.

**Fig. 5. Distribution of the 8-kDa DCCD-binding protein in the sucrose density gradient.** The sucrose gradient fractions (100 μl) of subunit I- and IV-deficient CFo-CF1 (fractions 1-11 shown in Fig. 3) were treated with [14C]DCCD. Samples were electrophoresed on a 15% polyacrylamide gel and stained with Coomassie Blue as described under “Materials and Methods.” The 8-kDa region (1.5 cm) of each lane was cut out, and radioactivity was determined. The protein content of 100-μl aliquots of each sucrose density gradient fraction was measured.
stromed in our experiments. This interaction could be a common feature in F₀, F₁ type ATP synthases. In E. coli, subunit c is postulated to fold in the membrane like a hairpin with two nonpolar α-helices separated by a polar loop region which is accessible to antibodies (8, 31). The Gln⁵ residue in the putative polar loop region of subunit c when substituted with certain amino acids results in altered F₁ binding and uncoupling (32, 33).

In the accompanying paper (13) we reported that DCCD-sensitive ATPase activity of F₀F₁ after incorporation into asolectin liposomes was relatively insensitive to removal of subunit IV. Subunit IV may thus not directly participate in the results that we described here show that at least two subunits of F₀F₁, I and III, are involved in F₁ binding, but the CF₁ subunits with which they interact are unclear. The α and δ subunits of CF₁ may be involved in binding to F₀ (1, 2, 4, 34). We have depleted the δ subunit from subunit III-associated CF₁,² It will be of interest to examine the association of subunit III with this CF₁ (⁻β). Resolution of F₀ into isolated component subunits followed by reconstitution of proton translocation activity by recombination of isolated subunits has so far been most successful with the E. coli F₀ (35). We have purified subunit IV of F₀F₁ (13), and other CF₁ subunits may be resolved by Zwittergent 3-12 on DEAE-Trisacryl columns by the procedures reported here and in the accompanying paper (13).

Acknowledgments—We thank John Telford for photographing the SDS-polyacrylamide gels and Adam B. Shapiro, Xenia Young, and Jianping Xiao for a critical revision of the manuscript.

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