The Subunit δ-Subunit b Domain of the Escherichia coli F₁F₀ ATPase

THE b SUBUNITS INTERACT WITH F₁ AS A DIMER AND THROUGH THE δ SUBUNIT* (Received for publication, August 6, 1997, and in revised form, September 8, 1997)

Andrew J. W. Rodgers‡*, Stephan Wilkens‡, Robert A. Capaldi‡, Michael B. Morris§, Susan M. Howitt†, and Roderick A. Capaldi‡‡

From the ‡Institute of Molecular Biology, University of Oregon, Eugene Oregon 97403-1229, §Department of Pharmacy, University of Sydney, New South Wales 2006, Australia, and †Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, Canberra ACT 2601, Australia

The δ and subunits are both involved in binding the F₁ to the F₀ part in the Escherichia coli ATP synthase (ECF₁F₀). The interaction of the purified δ subunit and the isolated hydrophilic domain of the b subunit (b₄sol) has been studied here. Purified δ binds to b₄sol weakly in solution, as indicated by NMR studies and protease protection experiments. On F₁, i.e., in the presence of ECF₁-δ, δ, and b₄sol interact strongly, and a complex of ECF₁-b₄sol can be isolated by native gel electrophoresis. Both δ subunit and b₄sol are protected from trypsin cleavage in this complex. In contrast, the δ subunit is rapidly degraded by the protease when bound to ECF₁, when b₄sol is absent. The interaction of b₄sol with ECF₁ involves the C-terminal domain of δ as δ₁-134, cannot replace intact δ in the binding experiments.

As purified, b₄sol is a stable dimer with 80% α helix. A monomeric form of b₄sol can be obtained by introducing the mutation A128D (Howitt, S. M., Rodgers, A. J., W., Jeffrey, P. D., and Cox, G. B. (1996) J. Biol. Chem. 271, 7038–7042). Monomeric b₄sol has less α helix, i.e., only 58%, is much more sensitive to trypsin cleavage than F₁, and unfolds at much lower temperatures than the dimer in circular dichroism melting studies, indicating a less stable structure. The b₄sol dimer, but not monomer, binds to δ in ECF₁.

To examine whether subunit b is a monomer or dimer is a C-terminal fusion with glutathione S-transferase, and the equivalent polypeptides in the mitochondrial enzyme, are characterized by an N-terminal membrane intercalated region and a large hydrophilic C-terminal domain (12). Based on trypsin digestion studies (7, 13, 14), it is this cytoplasmic domain of the b subunit that is involved in the binding of F₁ to F₀. A truncated form of the b subunit containing the C-terminal domain has been obtained genetically and purified (15, 16). The construct generated by Dunn (15) included residues 25–156 and an N-terminal octapeptide extension derived from the vector pUC8. The polypeptide produced by Howitt et al. (16), purified initially as a C-terminal fusion with glutathione S-transferase, includes residues 29–156, with Gly-Ser introduced at the N terminus to allow cleavage of the b subunit cytoplasmic domain from the fusion by thrombin. Both forms of the C-terminal domain, here called b₄sol as proposed by Dunn (15), are highly soluble in aqueous buffer. Sedimentation velocity centrifugation and circular dichroism spectroscopy studies show that b₄sol is an elongated dimer with a high α-helical content (15, 16).

Recent mutational analysis has established that residues Val¹²⁴, Ala¹²⁸, and Gly¹³¹ lie on one face of an α helix formed by F₁, F₀ ATP synthase; ECF₁F₀ ATP synthase; MOPS, 3-[N-morpholino]propanesulfonic acid; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate; MRW, mean residue weight.
a conserved hydrophobic region (Val124 to Ala132) near the C terminus of the b subunit (16). The mutation bG131D had previously been shown to allow assembly of the b subunit into the membrane but to prevent assembly of the whole F,F,ATPase complex (17). Replacement of Ala128 by Asp is now known to disrupt dimer formation: bmut with the mutation A128D is a stable monomer (16). Whether the dimeric structure seen in bmut is representative of the structural organization of the b subunit in the F,F complex is not known.

Here, we have explored the arrangement of δ and the b subunits in ECF,F in some detail. Clear evidence that the b subunit binds to F₁ via the δ subunit is reported, and it is shown that the dimer is both required for, and represents, the arrangement of subunit b in the complex.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids Containing Mutations in the uncF Gene—** Site-directed mutagenesis was carried out on an M13 mp18 clone containing the unc genes B, E, F and H on a 2356-base pair HindIII/EcoRI fragment, according to the method of Kunkel et al. (18). Residues Ser60, Gln104, Ala128, Gly131, and Ser146 were replaced by Cys using the following oligonucleotides (sequence changes underlined): GCAAAGGCGTGCGGACCGAC (bS60C), ATCGTGGCCTGTGCGCAGGCG (bQ104C), GCTATCCTGCTGTGCTGACGG (bA128C), GGGGCCGCAAGG (bG131C) and GCTGGCTGATCGAACATCGTG (bS146C). 909-base pair BsrGI/BesHI fragments carrying the mutated uncF genes were excised from the M13 mp18 replicative form and ligated into the vector pRA100 (described in Agrigaler et al. (19)), creating plasmids pRA165 (bS60C), pRA166 (bQ104C), pRA167 (bA128C), pRA168 (bG131C), and pRA169 (bS146C). These plasmids were used to transform the unc E. coli strain AN888 (20). Strains XLI-Blue (Stratagene) and CJ236 (New England Biolabs) were used in cloning and mutagenesis procedures.

**Media and Growth of Organisms—** Bacterial strains used in the overexpression of glutathione S-transferase fusion proteins (16) were grown in Luria broth supplemented with 33 mM glycerol. Wild-type and mutant (A128D) forms of bsol were purified as described previously (16), using glutathione-linked agarose resin and thrombin obtained from Sigma. Strains used for the preparation of F,F-ATPase were grown in minimal medium with supplements (21).

**CD Spectroscopy—** Circular dichroism spectra were obtained using a Jasco J-720 spectropolarimeter. For the determination of the relative percentages of secondary structure, wild-type b subunit cytoplasmic domain (1–134) and the corresponding mutant A128D (10–255) g/liter) were dissolved in 5 mM MgSO₄, 10 mM sodium phosphate, pH 7.0. The concentration of protein was determined using amino acid analysis. The samples were placed in 0.1-mm path length cells at 20 °C, and spectra were acquired using a scan speed of 200 nm/min, response time of 1 s, bandwidth of 1 nm, and resolution of 0.5 nm. For each sample, 10 acquisitions were collected between 190 and 250 nm and co-added, and 50 acquisitions were collected between 200 and 182 nm and co-added. The two sets were concatenated and the spectrum of the buffer, collected in the same way, was subtracted. The raw data were converted to mean residue ellipticity (θ) using a mean residue weight of 109.5 calculated from the amino acid sequence. Data were analyzed using the computer program VARIAN (22) to obtain estimates of the percentages of α helix, parallel and antiparallel β sheet, turns, and “other” structure.

For the temperature dependence of unfolding, both wild-type cytoplasmic domain (0.21 g/liter) and the mutant A128D (0.14 g/liter) were placed in 1-mm path length cells. The cells were placed in the jacketed cell holder of the spectropolarimeter. The temperature in the cell was controlled through the Jasco software and a Neslab RTE-111 water bath. The temperature in the cell was controlled through the Jasco software and a Neslab RTE-111 water bath. The temperatures were measured with and without prior incubation of the samples with 20 mM dithiothreitol for 2 h at 22 °C.

**Trpase Cleavage Experiments—** In experiments comparing cleavage patterns of wild-type and mutant bsol, these proteins (1 mg/ml) were prepared in 50 mM Tris-HCl, pH 7.0, 1 mM EDTA, and 2 mM CuCl₂. All cross-linking reactions were stopped by the addition of 5 mM EDTA, and ATPase activities (24) were measured with and without prior incubation of the samples with 20 mM dithiothreitol for 2 h at 22 °C.

**CuCl₂ Induced Cross-linking of ECF,F—** ECF,F,F was isolated and reconstituted into egg-lecithin vesicles as described in Agrigaler et al. (23). ATP synthase-containing vesicles collected from the Sephadex G-50 column were pelleted by centrifugation for 1 h at 45,000 rpm at 4 °C in a Beckman Ti60 rotor. The pellets were resuspended in 50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 2 mM dithiothreitol, and 10% glycerol and stored in liquid nitrogen. Prior to cross-linking experiments with CuCl₂, the reducing agent was removed by pelleting the enzyme at 175,000 × g for 30 min at 4 °C in a Beckman TLA100.2 rotor. The sample was then washed twice by successive resuspension and centrifugation steps in 50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, and 10% glycerol. Final resuspension was in the same buffer at a protein concentration of 1 mg/ml.

**Analysis of binding of the b subunit cytoplasmic domain (bmut) to ECF,F—** Mixtures of polypeptides were first analyzed by native agarose gel electrophoresis (A) through a gel containing 100 mM glycerol, 10 mM Tris acetate, pH 7.0, 86 glitner sucrose, and 1% agarose. Lanes from left to right: I, ECF; II, δ; III, δ + δ; IV, δ + δ + δ; V, δ + δ + δ + δ; VI, δ + δ + δ + δ + δ. Bands were excised from the agarose gel and separated on a 10–18% gradient of polyacrylamide in SDS (B). The individual lanes of the SDS-polyacrylamide gel are for the bands indicated in the agarose gel.

where the parameters θ₀ and θ₁ are the starting and ending ellipticity values, respectively, a and b are the slopes of the initial and final linear parts of the plots, respectively, d is the dispersion of the data, and t is the temperature at a given ellipticity, θ₁. The fitting program returned estimates of the values of the parameters (including θ₀) ± the standard errors.

**CuCl₂ Induced Cross-linking of ECF,F—** ECF,F,F was isolated and reconstituted into egg-lecithin vesicles as described in Agrigaler et al. (23). ATP synthase-containing vesicles collected from the Sephadex G-50 column were pelleted by centrifugation for 1 h at 45,000 rpm at 4 °C in a Beckman Ti60 rotor. The pellets were resuspended in 50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, and 10% glycerol and stored in liquid nitrogen. Prior to cross-linking experiments with CuCl₂, the reducing agent was removed by pelleting the enzyme at 175,000 × g for 30 min at 4 °C in a Beckman TLA100.2 rotor. The sample was then washed twice by successive resuspension and centrifugation steps in 50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, and 10% glycerol. Final resuspension was in the same buffer at a protein concentration of 1 mg/ml. Cross-linking was carried out at 22 °C for 2 h using concentrations of between 5 µM and 200 µM CuCl₂. All cross-linking reactions were stopped by the addition of 5 mM EDTA, and ATPase activities (24) were measured with and without prior incubation of the samples with 20 mM dithiothreitol for 2 h at 22 °C.

**Yeast Extract Experiments—** In experiments comparing cleavage patterns of wild-type and mutant bsol, these proteins (1 mg/ml) were prepared in 50 mM Tris-HCl, pH 7.0, 1 mM EDTA, and 2 µM CuCl₂. The ratio of trp to trp (w/w) was 1:3000. The reaction, conducted at 13 °C, was stopped by adding 4 mM phenylmethylsulfonyl fluoride from a freshly prepared stock solution. Trpase cleavage products were analyzed using 16% SDS-polyacrylamide gel electrophoresis gels prepared and run according to the method of Schagger and von Jagow (25).

**RESULTS**

**Analysis of binding of the b subunit cytoplasmic domain (bmut) to ECF,F—** Mixtures of polypeptides were first analyzed by native agarose gel electrophoresis (A) through a gel containing 100 mM glycerol, 10 mM Tris acetate, pH 7.0, 86 glitner sucrose, and 1% agarose. Lanes from left to right: I, ECF, δ; II, δ; III, δ + δ; IV, δ + δ + δ; V, δ + δ + δ + δ; VI, δ + δ + δ + δ + δ. Bands were excised from the agarose gel and separated on a 10–18% gradient of polyacrylamide in SDS (B). The individual lanes of the SDS-polyacrylamide gel are for the bands indicated in the agarose gel.
mM EDTA, 10% glycerol. In experiments comparing cleavage of bsol and ECF1 in the presence and absence of bsol, the ratio of trypsin to bsol (w/w) was 1:5000. In experiments comparing cleavage of bsol and E1 in the presence and absence of bsol, the ratio of trypsin to bsol (w/w) was 1:300. These reactions were conducted at 22 °C and were stopped by adding 4 mM phenylmethylsulfonyl fluoride.

**NMR Studies**—The δ subunit and δ1–134 were produced from pJCI kindly provided by Dr. Stanley Dunn (University of Western Ontario). Polypeptides were purified, and NMR spectra were obtained as described previously (9).

**Other Methods**—Atebrin fluorescence quenching activities were assayed as described by Hatch et al. (26). Protein concentrations were determined using the BCA protein assay from Pierce, with bovine serum albumin as a standard. F1-ATPase was prepared from membranes of strain AN1460 (27) as described by Cox et al. (28). Stripped membranes were prepared from strain AN2840 (29) using the method described by Wise et al. (30).

**RESULTS**

The bsol used here was obtained by thrombin release from a fusion protein that includes glutathione S-transferase at the N terminus (16). It contains residues 25–156 with a Gly-Ser N-terminal extension. Both intact δ and δ1–134 were obtained as described previously (9). All of the isolated subunits and subunit fragments were pure, based on SDS-polyacrylamide gel electrophoresis (Fig. 1).

**F1-δ-bsol Interactions Determined by Native Gel Electrophoresis**—Interactions between F1-δ, δ, δ1–134, and bsol were examined first by native gel electrophoresis. In a typical experiment, such as shown in Fig. 1A, fractions were mixed and then electrophoresed through agarose. Protein was detected on the native gel by Coomassie Brilliant Blue staining. Bands containing protein were excised, dissolved in SDS, and then subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1B). Moreover, a complex could be formed between F1-δ, F1 + δ, and δ1–134, and even F1 + δ + bsol migrated similarly.

No complex formation was observed between bsol and either pure δ or δ1–134 in native gel electrophoresis (Fig. 1A, lane IV). However, both δ and δ1–134 bound to F1-δ to form a complex that was retained through the electrophoresis step (Fig. 1A, lanes VII and IX). Moreover, a complex could be formed be-
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Fig. 3. SDS-polyacrylamide gel electrophoresis of ECF₁, (3.5 mg/ml), bₗ₀ (300 µg/ml), and δ subunit (200 µg/ml), alone or in combination, treated with trypsin at 22 °C for the times indicated. Proteins were dissolved in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol. Lane sets A–C, trypsin was added to final concentration 0.7 µg/ml. Lane sets D–F, trypsin was added to final concentration 1.0 µg/ml. Lane A, bₗ₀ alone (20 µg); B, bₗ₀ + ECF₁ (250 µg); C, ECF₁ alone; D, bₗ₀ alone; E, bₗ₀ + δ (14 µg); F, δ alone. In all cases, the reactions were stopped by adding 4 mM phenylmethylsulfonyl fluoride.

between F₁-δ, δ, and bₗ₀ (Fig. 1A, lane VIII), but not between F₁-δ and bₗ₀ (Fig. 1A, lane VII), or between F₁ + δ₁₁–134 + bₗ₀ (Fig. 1A, lane X). These results indicate that bₗ₀ binds tightly only to F₁, when δ is present, and that the C-terminal 43 residues are important for this interaction.

**Fig. 4.** Reconstitution of stripped membranes from *E. coli* strain AN2840 by F₁-ATPase preincubated with wild-type and mutant forms of bₗ₀. Atebrin fluorescence quenching was measured as described previously (21). Atebrin was added to give a final concentration of 4 µM, NADH to 2 mM, NaCN to 2.5 mM, ATP to 1 mM, and carbonylcyanide m-chlorophenylhydrazone (CCCP) to 2 µM. 100 µl of F₁-ATPase preparation (16 mg/ml) was used for each reconstitution. A, 500 µl of wild-type form of bₗ₀ (1.5 mg/ml) was incubated with F₁-ATPase prior to the assay. B, 500 µl of the mutant (A128D) form of bₗ₀ (1.5 mg/ml) were incubated with F₁-ATPase prior to the assay. C, control in which 500 µl of buffer alone was incubated with F₁-ATPase prior to the reconstitution assay.

**Interaction of δ and bₗ₀ on F₁ Protects Both Polypeptides from Protease Digestion**—Both the δ subunit and bₗ₀ are highly unit spectrum was monitored. Many of the individual resonances of the δ subunit progressively disappeared as the ratio of bₗ₀ to δ was increased (Fig. 2) as clearly evident for Ser₁⁵², Gly₇¹, Gly₇⁵, and Asn₁⁵⁶, four residues that are widely distributed in the N-terminal domain. These results indicate that an interaction occurs between the two polypeptides with a slow to intermediate rate of exchange between a free δ subunit and δ that is bound to bₗ₀. The binding constant between δ and bₗ₀ from these NMR studies must be larger than 2 µM. This is consistent with the interaction between the two polypeptides being weaker in solution than on F₁, where a binding affinity of 2 µM was estimated by Dunn (15). The disappearance of resonances, then, represents the lowering of the tumbling rate of the δ subunit by binding of bₗ₀. The approach does not pinpoint the site of interaction, except that when the experiment was repeated with δ₁₁–134, there was significantly less broadening of resonances of the N-terminal part, as would be expected if the interaction between δ and bₗ₀ is mainly through the C-terminal 42 residues of the δ subunit. Other individual subunits of ECF₁ failed to alter the NMR spectrum of the δ.
protease sensitive in pure form (shown for bsol in Fig. 3A). Moreover, the δ subunit is still highly protease-sensitive when bound to the core ECF1 (Fig. 3C). However, as shown in Fig. 3B, when bsol is reacted with ECF1, both the δ subunit and bsol are protected from trypsin cleavage. These results confirm the role of the δ subunit in binding bsol. Fig. 3E presents data for the trypsin cleavage of the δ-bsol complex formed in solution. It is evident that there is some protection from cleavage of both δ and bsol by complex formation, but this interaction is clearly weak as proteolysis still proceeds quite rapidly.

**Dimer of bsol Is Required for Its Binding to F1**—To examine if bsol was binding to F1 at its functionally important site, the ability of the polypeptide to block the reconstitution of coupled F1F0 ATPase activity was measured by atebrin fluorescence quenching assays. In the absence of added bsol, rebinding of F1 to the membrane preparation gave ATP-dependent quenching that was 53% of the original fluorescence level (Fig. 4C) compared with 51% after preincubation of ECF1 with monomeric bsol at the same concentration (Fig. 4B). When bsol was incubated with F1 and then the complex reconstituted, ATP-dependent quenching was only 12% of the original fluorescence level (Fig. 4A). Thus, the binding of bsol blocks the functional recombination of F1 to F0.

**Dimerization Stabilizes the Structure of bsol**—A CD spectrum of the wild-type (dimeric) form of bsol in 10 mM sodium phosphate, pH 7.0, 5 mM MgSO4. A, wavelength scans over the range 182–260 nm at 20 °C. The raw data in millidegrees were converted to mean residue ellipticity, [θ]MRW, using a mean residue weight of 109.5. B, [θ]MRW at 222 nm plotted as a function of temperature over the range 6–82 °C. The temperature was raised at a rate of ~1 °C/min. The melting temperature, Tm, for each protein was estimated by fitting the equation described under “Experimental Procedures” using nonlinear regression. The returned values were 39.8 ± 0.1 °C for the wild-type protein and 32.2 ± 0.2 °C for A128D.

80% ± 3% (SD) α helix. The CD spectrum of the mutant (A128D) form of bsol is also shown in Fig. 5A, from which 58 ± 2% α-helix was estimated. The loss of α-helical structure observed for the mutant protein is compensated largely by an increase in “other” (random coil) structure and parallel β sheet.

The trypsin digestion patterns of the dimeric and monomeric forms of bsol are additional evidence of loss of secondary structure in the monomer. Thus, with trypsin to bsol at a ratio of 1:5000 (cf. 1:3000 in Fig. 3) as well as with 2 M glucose present to stabilize the protein, there was only a single cut of bsol in 30 min, the product of which had the N-terminal sequence QKEIAD (cutting after residue Arg36). Under equivalent conditions, the monomeric protein was cleaved at Arg 36, Arg 49, Lys 58, Arg 83, and Lys 100, indicating increased accessibility of the protease, not only in the vicinity of the mutation but at a number of points throughout the protein (results not shown).

The increased susceptibility of monomeric bsol to proteolytic cleavage correlates with decreased thermal stability as shown by CD melting studies. CD scans at a fixed wavelength of 222 nm over the temperature range 6–82 °C (Fig. 5B) showed a clear secondary structure transition with midpoint at 40 °C for the wild-type dimeric bsol. In this transition the polypeptide goes from a folded conformation to an unstructured state. The unfolding of the mutant monomeric bsol is centered at a significantly lower temperature (32 °C).
Arrangement of b Subunits in the ECF F₀ Complex Based on Disulfide Cross-link Formation by Mutant b Subunits—The stability of the dimer, and importance of the dimer in binding to F₁, supports the idea that b is a dimer in the ECF F₀ complex. To test this more directly, mutants were constructed in which a cysteine was introduced in place of the following b subunit residues, Ser^{60}, Gln^{104}, Ala^{128}, Gly^{131}, and Ser^{146}. With the exception of the mutant bS60C, which grew poorly, all of the strains grew well on solid succinate minimal medium. All of the strains exhibited growth yields in limiting (5 mM) glucose similar to the wild-type control (data not shown). The specific activities of ECF F₀ purified from each of the mutants were similar to the wild-type enzyme, i.e., in the range 22–27 μmol of ATP hydrolyzed/min/mg.

Mutant enzyme preparations were treated with CuCl₂ to induce formation of disulfide bonds. Experiments were carried out under both ATP conditions (following preincubation with AMP-PNP) and ADP conditions (addition of ATP + Mg²⁺ followed by enzyme turnover), over a range of CuCl₂ concentrations (5–200 μM). A cross-linked product in the range 32–35 kDa was observed with ECF F₀ from mutants Q104C, A128C, G131C, and S146C. No cross linking was obtained with mutant S60C. Data for the mutant bQ104C under ATP conditions are shown in Fig. 6A in the form of a concentration dependence of CuCl₂ on cross-linking yield. The maximal yields obtained with this mutant were in the range of 70%. Fig. 6B shows data for all mutants using the minimum CuCl₂ concentration required for maximum cross-link formation under ATP conditions.

The appearance of cross-linked product seen in Fig. 6, A and B, was in each case accompanied by a commensurate loss of the b subunit band. That this new band was a b subunit dimer was confirmed by antibody blotting. It reacted with anti-b subunit mAbs but not with antibodies to any of the other subunits of the complex (data not shown). Note the altered migration of both the monomeric and cross-linked dimeric forms of the mutant S146C b subunit. There was no nucleotide dependence of the yield of cross-linking for any of the mutants examined.

Subunit b dimer formation had only a small effect on ATPase activity with any of the mutants, e.g., for bQ104C, ATPase activity was 25.1 μmol/min/mg before and 19.2 μmol/min/mg after cross-linking in 70% yield. This is the same loss of ATPase activity obtained by treating wild-type enzyme with CuCl₂.

DISCUSSION

There is emerging evidence that the F₁ and F₀ parts of the ATP synthase are joined not only by the narrow and 40–45-Å long stalk seen in electron micrographs (5), and now known to be made up of the γ and ε subunits (3), but also by a second stalk provided by the δ and b subunits (9). There is clear evidence from cross-linking experiments that the δ subunit is bound at the outside of the αβ₃ subunit barrel near the top of the F₁ and away from the F₀ (10, 11). The δ subunit is a two-domain protein, with an N-terminal domain of around 105 residues, which is roughly globular and contains a six α-helical bundle (9) and a less ordered C-terminal domain. The C-terminal domain must be in close contact with the N-terminal domain in the complex as a cross link is readily formed between intrinsic Cys^{64} and Cys^{140} (31). A number of studies have shown that the ε subunit and its equivalent in the mitochondrial enzyme, OSCP, are involved in the interaction of F₁ with the F₀ part. For example, ECF F₁ does not bind to F₀ in the absence of ε. The key sites for this interaction appear to be in the C terminus of δ. C-terminal truncations of as few as 4–6 residues from either δ or OSCP prevent the rebinding of ECF F₁ or MF₁, respectively, to F₀ (32, 33).

The studies presented here show conclusively that the δ subunit binds to δ subunits, and that this interaction involves mainly the C-terminal domain of δ. This arrangement has been speculated upon based on cross-linking of δ to subunit CF₀₁ in the chloroplast enzyme (34). However, the data reported here are the first direct binding experiments. It is shown that δ subunit is required for the interaction of the cytoplasmic domain of subunit b with F₁. This binding requires the C-terminal domain of δ, as the interaction was lost when δ^{1–134} was used in the reconstitution experiments. A complex was obtained between F₁ + δ + b<sub>sol</sub> which was stable to native gel electrophoresis. No such stable complex was formed between δ and b<sub>sol</sub> in the absence of the F₁. It is likely that the αβ₃γ domain helps stabilize the interactions between the N- and C-terminal domains of δ, which are required for the tight bind-
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ing of b subunits. Weak interaction between δ and bδ in solution was observed by NMR and in protease digestion studies. Both the δ and bδ are protected from trypsin digestion in the ECF1 + δ + bδ complex, while the protection of δ and bδ by mixing the two in solution is much less. The binding of bδ blocks re-binding of F1 + δ to F0, consistent with the functional interaction between the cytoplasmic domain of δ and δ in the reconstitution experiments.

Purified wild-type bδ is a stable dimer, and only a dimer, not monomer, is able to block F1 + δ binding to F0. A priori, the two copies of subunit b could provide separate connections between the F1 and F0 parts. However, the results presented here argue against this. First, dimer formation appears to be much less α-helical, because it is highly protease sensitive and denatures at lower temperatures.

More direct evidence that b subunits are close in F1F0 was sought by cross-linking studies. Mutants were created with Cys residues at several sites along the C-terminal domain. On the addition of the oxidizing agent Cu2+, disulfide bonds were generated in high yield between Cys at positions 104, 128, 131, and 146, but not at 60. This could happen only if the two b subunits are paired for a significant length. As the δ subunit is near the top of the F1 (10, 11), the obvious arrangement of the b subunits is an extended one running up one side of the F1F0 structure. In several of the mutants described here, it is possible to react the introduced Cys with bulky maleimides. This should allow us to label the b subunits, e.g. with gold particles, and then visualize them in side views of F1F0 by cryoelectron microscopy.

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