Defining the Domain of Binding of $F_1$ Subunit $\epsilon$ with the Polar Loop of $F_0$ Subunit $c$ in the *Escherichia coli* ATP Synthase

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We have previously shown that the E31C-substituted $\epsilon$ subunit of $F_1$ can be cross-linked by disulfide bond formation to the Q42C-substituted $c$ subunit of $F_0$ in the *Escherichia coli* $F_1F_0$-ATP synthase complex (Zhang, Y., and Fillingame, R. H. (1995) *J. Biol. Chem.* 270, 24609–24614). The interactions of subunits $\epsilon$ and $c$ are thought to be central to the coupling of $H^+$ transport through $F_0$ to ATP synthesis in $F_1$. To further define the domains of interaction, we have introduced additional Cys into subunit $\epsilon$ and subunit $c$ and tested for cross-link formation following sulfhydryl oxidation. The results show that Cys, in a continuous stretch of residues 26–33 in subunit $\epsilon$, can be cross-linked to Cys at positions 40, 42, and 44 in the polar loop region of subunit $c$. The results are interpreted, and the subunit interaction is modeled using the NMR and x-ray diffraction structures of the monomeric subunits together with information on the packing arrangement of subunit $c$ in a ring of 12 subunits. In the model, residues 28–33 form a turn of antiparallel $\beta$-sheet which packs between the polar loop regions of adjacent subunit $c$ at the cytoplasmic surface of the $c_{12}$ oligomer.

The $H^+$-transporting, $F_0F_1$-ATP synthase of *Escherichia coli* utilizes an $H^+$ electrochemical gradient to drive ATP synthesis during oxidative phosphorylation (1). Similar enzymes are found in mitochondria, chloroplasts, and other bacteria. The enzymes are composed of two sectors, termed $F_1$ and $F_0$. The $F_1$ sector contains the catalytic sites for ATP synthesis, and when released from membrane, it shows ATPase activity. The $F_0$ sector traverses the membrane and functions as the $H^+$ transporter. When $F_1$ is bound to $F_0$, the complex acts as a reversible, $H^+$-transporting ATP synthase or ATPase. In *E. coli*, $F_1$ is composed of five types of subunits in an $\alpha_2\beta_2\gamma_2\delta e$ stoichiometry, and $F_0$ is composed of three types of subunits in an $\alpha_3\beta_3\gamma_2$ stoichiometry (2–4). The structure of much of the $\alpha_\beta\gamma$ portion of $F_1$ has been solved by x-ray diffraction analysis and shows subunit $\gamma$ extending through the center of a hexamer of the larger, alternating $\alpha$ and $\beta$ subunits (5, 6). During catalysis, the $\gamma$ and $\epsilon$ subunits have been shown to rotate in 120° steps between the three alternating catalytic sites in the $\beta$ subunits (7–13). Subunits $\gamma$ and $\epsilon$ are thought to rotate as a unit because they can be cross-linked to each other with minimal inhibitory effects on ATPase activity (14, 15).

The relation of structure and mechanism in $F_0$ is less thoroughly understood. The largely hydrophobic subunit $\alpha$ folds in the membrane with five transmembrane helices (16, 17), at least two of which likely interact with subunit $c$ during proton transport (18–20). Subunit $b$ is anchored in the membrane via a single transmembrane helix that is connected to a polar, elongated cytoplasmic domain that is thought to play a key role in fixing $F_1$ to $F_0$ (21). Subunit $c$ is a protein of 79-amino acid residues that folds in the membrane in a hairpin-like structure. The two hydrophobic transmembrane $\alpha$-helices are joined by a more polar loop region that is exposed to the $F_1$ binding side of the membrane. Aspartyl 61, lying at the center of transmembrane helix-2, is thought to be the site of $H^+$ binding during transport (22). The polar loop was proposed to play a key role in coupling $H^+$ transport to ATP synthesis or hydrolysis based upon the uncoupled phenotypes of mutants with substitutions in the conserved Arg$^{11}$-Gln$^{12}$-Pro$^{13}$ sequence of the polar loop (22, 23). The “uncoupled” phenotype of the cQ42E mutant proved to be suppressed by second site substitutions in Glu$^{39}$ of $F_1$ subunit $\epsilon$ (24), and this led to cross-linking studies demonstrating a physical proximity between the polar loop and subunits $\gamma$ and $\epsilon$ of $F_1$ (14, 25, 26). A recently determined NMR structure of monomeric subunit $c$ conforms well with folding predictions made from biochemical and genetic analysis (27).

Recent experiments now indicate that the $c_{12}$ oligomer of $F_0$ is organized in a ring with transmembrane helix-1 on the inside and transmembrane helix-2 on the outside (2, 4, 28) and with the $a$ and $b$ subunits associating at the periphery of the ring (2, 20). Such an arrangement is consistent with low resolution electron and atomic force microscopic images (29–31). The structural data fit well with rotary models where $H^+$ transport at the $a-c$ interface is proposed to drive rotation of the oligomeric $c$ ring as the Asp$^{83}$ carboxylate is protonated and deprotonated from alternate access channels on each side of the membrane (10, 32–34). The rotation of subunit $c$ is proposed to drive the rotation of the $\gamma\epsilon$ unit in $F_1$ via a fixed linkage between subunit $c$ and the $\gamma$ and $\epsilon$ subunits (14) although other explanations have been proposed (22, 25). The elongated cytoplasmic domain of subunit $b$ is thought to extend from the membrane surface to the top of $F_1$ as a “second stalk”, or stator, to hold $F_1$ fixed as subunit $\gamma$ rotates at the center of the molecule (21, 35–37).

In this study, the interacting regions of the polar loop of subunits $c$ and subunit $\epsilon$ are more thoroughly defined by disulfide cross-linking of Cys introduced into the two subunits. The experimental design and interpretation was aided by a structural model for subunit $\epsilon$ derived by NMR (38) and x-ray crystallography (39). The Cys residues of subunit $\epsilon$ that form cross-links with subunit $c$ localize to a span of residues 26–33 which fold as two strands of antiparallel $\beta$-sheet connected by a loop. A structural model for the subunit-subunit interaction is developed from the structures of the monomeric subunits and information on the organization of the $c$-oligomer, using distance constraints from the cross-linking data reported here. The model indicates that the segment of antiparallel $\beta$-sheet...
encompassing residues 26–33 of subunit $\epsilon$ packs in a space between neighboring polar loops of the subunit $\epsilon$ oligomer.

**EXPERIMENTAL PROCEDURES**

**Mutant Construction and Expression**—The plasmids constructed in this study are derivatives of plasmid pY2201 (25), which carries the eight structural genes of *unc* operon coding $F_1F_0$ (bases 870–10172; Ref. 40). The Cys substitutions in subunit $c$ and subunit $\epsilon$ were introduced by oligonucleotide-directed mutagenesis using the strategy described previously (25). The plasmids were expressed in strain OM204 (41), a strain in which the *unc* operon is deleted from the chromosome.

**Membrane Preparations and Cross-Link Analysis**—Cells were grown and membranes prepared by the methods described (25). To catalyze disulfide bond formation, aliquots of 200-μl membrane vesicles at 10 mg/ml in TMG buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 10% (v/v) glycerol) were treated with 20 μl of a mixture of 15 mM CuSO$_4$ and 45 mM 1,10-phenanthroline in 50% ethanol. After a 1-h incubation at room temperature, the reaction was stopped by addition of 20 μl of 0.5 M Na$_2$EDTA and 20 μl of 0.5 M N-ethylmaleimide (NEM) in dimethyl sulfoxide, and the sample was incubated for a further 60 min. The sample was then diluted in 250 μl of 2× SDS sample buffer (125 mM Tris-H$_2$SO$_4$, pH 8.6, 4% SDS, 20% glycerol, and 0.02% bromophenol blue) and incubated at 30 °C for 1 h. The solubilized membrane proteins were separated by SDS-polyacrylamide gel electrophoresis using a 7.5–15% acrylamide gradient and the Tris-Tricine buffer described by Schägger and von Jagow (42). After electrophoretic transfer to nitrocellulose paper (43), immunostaining was carried out using the Enhanced Chemiluminescence System (Amersham Pharmacia Biotech). The rabbit antiserum to subunit $c$ used was that described by Girvin et al. (44). Antibodies that nonspecifically cross-reacted with *E. coli* membrane proteins were removed by preabsorption with membranes prepared from a mutant strain with a deleted *unc* operon (44). The mouse monoclonal antibody to subunit $\epsilon$ (13-A7, eH1, Ref. 45) was a gift from Dr. R. Capaldi (University of Oregon, Eugene, OR).

**Structural Modeling of Subunit-c-Subunit Interaction**—A model for subunit $c$ interaction in the $c_6$ oligomer has been derived from the NMR model (27) using distance constraints derived from the cross-linking data of Jones et al. (28). A subunit $c$ dimer, taken from the oligomer model, was manually docked to the N-terminal domain of subunit $\epsilon$ (residues 1–57) so that the distances between the $\alpha$-carbons of cross-linked residues were <12 Å. The range for $\alpha$-carbon distances in naturally occurring disulfide bonds in proteins is 4–7.5 Å (46). A somewhat wider distance constraint range of 4–11 Å was used in the molecular mechanics calculations done here to allow for possible thermal motions and distortions of structure on cross-link formation. The shortest of the two distances between the Cys $\alpha$-carbon in each of the two subunits $c$ and the Cys $\alpha$-carbon in $\epsilon$ in the manually docked structure was used to impose a distance constraint. The positions of all the atoms of the $c$ subunits were fixed except for residues 39–45, which were left unrestrained. The backbone angles in subunit $c$ were restrained to their value in the x-ray structure (39) using quadratic constraints. Energy minimized was performed with CVFF (constant valence force field) using the steepest descents and then conjugated gradient methods as implemented in DISCOVER 3.0 (Molecular Simulations Inc.) until the maximum derivative was below 0.1 kcal mol$^{-1}$ Å$^{-1}$.

**RESULTS**

**Properties of Cys-substituted Double Mutants**—All of the mutants constructed grew on succinate minimal medium, which indicates formation of a functional ATP synthase (Table I). Two of the $\epsilon$-substituted, cQ42C mutants showed very little membrane ATPase activity and nondetectable amounts of $\epsilon$ subunit on immunoblotting (Table I). In these two cases, eV25C/cQ42C and eG27C/cQ42C, we conclude that the F$_1$–F$_0$ complex is probably stable under in vivo conditions but that the F$_1$–ATPase and $\epsilon$ subunit dissociate from the membrane during membrane preparation.

**Cross-linking of cysteine-substituted $\epsilon$ subunits to Q42C subunit $c$**

| Substitution in subunit $\epsilon$ | Growth on succinate* | ATPase activity* | Presence of subunit $\epsilon$ in membrane | Formation of $\epsilon$–c cross-link |
|----------------------------------|----------------------|------------------|------------------------------------------|----------------------------------|
| None                             | 1.8–2.2              | 0.88 ± 0.06      | +                                        |                                  |
| S10C                             | ±2.0                 | 0.18             | +                                        | 0                                |
| Q24C                            | ±1.8                 | 0.41 ± 0.06      | +                                        | 0                                |
| V25C                            | ±1.4                 | 0.12 ± 0.03      | 0                                        | +                                |
| T26C                            | ±1.8                 | 0.51 ± 0.22      | +                                        | +                                |
| G27C                            | ±1.6                 | 0.06 ± 0.05      | 0                                        | +                                |
| S28C                            | ±1.8                 | 0.44 ± 0.09      | +                                        | +                                |
| E29C                            | ±1.6                 | 0.33 ± 0.09      | +                                        | +                                |
| G30C                            | ±1.8                 | 0.64 ± 0.17      | +                                        | +                                |
| E31C                            | ±1.8                 | 0.44 ± 0.13      | +                                        | +                                |
| L32C                            | ±1.8                 | 0.99 ± 0.88      | +                                        | +                                |
| G33C                            | ±1.8–2.2             | 0.62 ± 0.13      | +                                        | +                                |
| E34C                            | ±1.8                 | 0.37 ± 0.11      | +                                        | +                                |
| H33C                            | ±1.4                 | 0.19 ± 0.03      | +                                        | +                                |

* Membrane ATPase activity was measured in 50 mM Tris-H$_2$SO$_4$, pH 7.8, 0.2 mM MgSO$_4$, 0.4 mM [14CP]ATP. Activities shown are the average of at least two sets of assays (done in triplicate with S.D. <10%) on one or more membrane preparations.

**Colonies size on succinate minimal medium after 64 h of growth.

**Single set of assays.**

1 The *unc* DNA numbering system corresponds to that used by Walker et al. (40).
2 The abbreviations used are: NEM, N-ethylmaleimide; CuP, Cu(II) (phenanthroline)$_2$; Tricine, N-tris(hydroxymethyl)methylglycine.
3 O. Y. Dmitriev, M. E. Girvin, P. C. Jones, and R. H. Fillingame, submitted for publication.
4 A coordinate file of the final model is available by E-mail at dmitriev@iris.chem.wisc.edu.

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3 A number of minor bands were also detected with the anti-$\epsilon$ antibody.5 Comigration of the product on the two types of blots was confirmed using several types of gels of varying acrylamide concentration. The $\epsilon$-c product seen in untreated membranes is presumed to form by autoxidation as the membranes are isolated from the cell. The extent of cross-link formation was enhanced by Cu(II)(phenanthroline)$_2$ (CuP) catalyzed oxidation. Cross-linking was largely reversed by subsequent treatment with 0.5 mM dithiothreitol.

The intensity of the $\epsilon$-immunostained product that is detected with anti-$\epsilon$ antibody does prove to be misleading. Beginning with the anti-$\epsilon$ blot shown in Fig. 1, note the much greater intensity of staining of the $\epsilon$-c product in cQ42C/eQ29C versus cQ42C/eE31C membranes despite the loading of equal amounts of membrane protein in all lanes. Note also for the eE29C membranes that the changes in intensity of $\epsilon$-c are considerably greater than the changes in intensity of monomeric $\epsilon$, i.e. the changes are not in the inverse proportions expected in a precursor-product relationship. We interpret this to mean that the $\epsilon$-c heterodimer of the eE29C mutant protein binds antibody better than the eE29C monomer and also better than the $\epsilon$-c heterodimer of the eE31C mutant. This interpretation is
products stain much more intensely with anti-e shown to not form a cross-link (Table I). Most of the mutants. No cross-linking was observed with Cys substituted linking also occurred in the absence of CuP in each of these mutants. No cross-linking was observed with Cys substituted in the various membranes shown in Fig. 3 was reduced by treatment with dithiothreitol. The results from several similar experiments are summarized in Table I. Qualitatively confirmed by the relative intensities of the bands on the anti-c blot. In the untreated membrane samples, the intensity of the anti-c immunostained e-c product is much greater for e31C than for e29C, i.e., just the reverse of the pattern seen with anti-e antibody. We have concluded that the best way to approximate the extent of e-c formation may be to compare the amounts of monomeric e remaining after various treatments. Using this criteria, the e31C mutant would appear to form at least as much and possibly more e-c product by either autoxidation or CuP-catalyzed oxidation.

Other mutants also show e-c products whose staining intensities differ considerably with the two antibodies. In the experiment shown in Fig. 2, membranes were washed with 1 mM Tris-HCl, pH 8.0, 0.5 mM Na2EDTA, a procedure which removes F1, and uncross-linked subunit e and other non specific immunoreactive proteins. The eS28C, eE29C and eG30C e-c products stain much more intensely with anti-e antibody than with anti-c antibody. Conversely, the e-c product in the eG30C mutant stains much less intensely with anti-e body than with anti-c antibody. Other e-c products stain with nearly equivalent intensities in the two blots. In summary, the blots do not provide a good quantitative means of distinguishing the extent of cross-link formation in different mutant membranes.

A survey of e-c cross-linking in a series of double-Cys-substituted pairs is shown in Fig. 3. In the presence of CuP, most or all of the Cys-substituted subunit e was cross-linked with Cys42-substituted subunit c when Cys was substituted as positions 26, 28, 29, 30, 32, and 33 in subunit e. Detectable cross-linking also occurred in the absence of CuP in each of these mutants. No cross-linking was observed with Cys substituted at positions 24, 25, 27, 34 and 38 of subunit e. In the case of the eV25C mutant, subunit e was not incorporated into the membrane. This was also the case for the g27C mutant (not shown). In other experiments, the eQ42C/eS10C pair was also shown to not form a cross-link (Table I). Most of the e-c-cross-linked product formed in the various membranes shown in Fig. 3 was reduced by treatment with dithiothreitol. The results from several similar experiments are summarized in Table I.

DISCUSSION

The surface of subunit e lying proximal to subunit c has been mapped by cross-linking experiments to a region encompassing residues 26–33, which in the NMR and x-ray diffraction structures of subunit e (38, 39) reside in a loop of antiparallel β-sheet (Fig. 5A). The NMR and x-ray diffraction structures agree closely and show subunit e to be a protein of two distinct domains. The N-terminal domain of 86 residues folds in a...
10-stranded β-sandwich and the C-terminal domain of 45 residues is formed from two α-helices arranged in an antiparallel coiled coil. Much of the C-terminal domain appears to be non-essential because it can be deleted without effect on ATP synthase function (47). Cross-linking and chemical modification experiments indicate that the surface of the β-sandwich, including residues His38 and Ser10, neighbor the γ subunit and that His38 lies close to the surface of F0 (14, 38, 39, 48). Residue 31 of ε must also lie proximal to the surface of F0 because the εE31C-substituted protein can be cross-linked to Cys at positions 40, 42, and 43 of subunit c (25). The loop including residues 26–33 protrudes from the “bottom” of subunit ε as a well defined lobe (Fig. 5A), and most Cys replacements in this loop were cross-linked to Q42C subunit c in the experiments described here. In addition, Cys at positions 28, 31, and 32 in subunit ε were shown to cross-link to Cys lying on opposing flattened faces of the polar loop of subunit c in the NMR structure (Ref. 27; Fig. 5B), i.e. at either position 42 or at positions 40 and 44 in the loop.

The interacting surfaces of subunit ε and subunit c have been modeled beginning with a model for the c subunit oligomer described elsewhere.5 In the modeling, equivalent distance constraints were imposed for each cross-link formed because of difficulties in quantitatively distinguishing the extent of cross-link formation. In the model, the loop of antiparallel β-sheet that is centered around εGlu31 packs between the polar loops of two c subunits (Fig. 6A). The model also depicts the εGlu31 residue lying close enough to the conserved and essential cArg41 residue to interact electrostatically and also close to cGln42 (Fig. 6B). The positioning of these side chains in the model should be interpreted with caution because the model is derived without use of side chain distance constraints. However, with these precautions, the general proximity of residues in the model does provide a reasonable explanation for the uncoupling effects of the εGlu31 mutation (23) in that charge-charge repulsion would be expected between the εGlu42 and εGlu31 carboxylates. The charge-charge repulsion explanation is supported by the differences in pH dependence of neutral versus positively charged ε31 suppressor substitutions in restoring function to the εGlu31 mutant (24). The smaller uncoupling effects of some substitutions in εGlu31 (49), versus the εQ42E mutation, are not as easily rationalized by the model.
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In the model shown in Fig. 6A, it is notable that the space between loops of subunit c is essentially filled by the packing with subunit e. Because residue 205 of subunit γ is also known to cross-link with residues 42, 43, and 44 of subunit c (14, 26), it seems likely that the region around γ-205 packs between a different set of c subunits than those interacting with e. The shielding of two separate pairs of subunit c by the binding of subunits γ and e, respectively, may explain the observations of Watts and Capaldi (50) on the functional effects of NEM modification of Cys42 in cQ42C mutant membranes. Function was retained during the initial phase of modification of approximately 60% of the subunits and then lost during modification of the last 40%. The inhibitory phase of NEM modification may correspond to reaction with Cys42 at the c-c or c-e interfaces.

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