Arrangement of the Multicopy H⁺-translocating Subunit c in the Membrane Sector of the *Escherichia coli* F₁F₀ ATP Synthase* 

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The multicopy subunit *c* of the H⁺-transporting F₁F₀ ATP synthase of *Escherichia coli* is thought to fold across the membrane as a hairpin of two hydrophobic α-helices. The conserved Asp⁶¹, centered in the second transmembrane helix, is essential for H⁺ transport. In this study, we have made sequential Cys substitutions across both transmembrane helices and used disulfide cross-link formation to determine the oligomeric arrangement of the c subunits. Cross-link formation between single Cys substitutions in helix 1 provided initial limitations on how the subunits could be arranged. Double Cys substitutions at positions 14/16, 16/18, and 21/23 in helix 1 and 70/72 in helix 2 led to the formation of cross-linking multimers upon oxidation. Double Cys substitutions in helix 1 and helix 2, at residues 14/72, 21/65, and 20/66, respectively, also formed cross-linked multimers. These results indicate that at least 10 and probably 12 subunits interact in a front-to-back fashion to form a ring-like arrangement in F₀. Helix 1 packs at the interior and helix 2 at the periphery of the ring. The model indicates that the Asp⁶¹ carboxylate is centered between the helical faces of adjacent subunit c at the center of a four-helix bundle.

Several recent studies now show that subunit γ rotates within the core of the hexagonally arranged αβγɛδ complex to presumably drive the binding changes in each β-subunit (5–7). Both subunit γ and ε appear to rotate as a unit (8). The mechanism of coupling H⁺ translocation through F₀ to γ subunit rotation is unknown.

The *E. coli* F₀ is the simplest type found in nature. It consists of three subunits with a stoichiometry of a₁b₂c₁₀ (9). Electron microscopic studies suggest that the a and b subunits pack at the periphery of a complex of subunit c (10). Subunit b, with a single transmembrane helix and larger cytoplasmic domain, is proposed to associate with subunit δ to make up a stator that binds and fixes the F₁ αβγɛδ catalytic head group to F₀ (11, 12). Subunit a is thought to fold through the membrane with five or six transmembrane helices and play a key role in the H⁺ transport (2, 13). Structural and genetic studies indicate that subunit c folds in the membrane as a hairpin of two hydrophobic α-helices connected by a polar loop on the F₁ binding side of the membrane (2, 14). A conserved Asp or Glu (Asp⁶¹ in *E. coli*) centered in the second transmembrane helix is essential for H⁺ transport (2, 15). The most compelling evidence for a direct role of the side chain carboxyl in cation binding has come from work on the related Na⁺-translocating enzyme of *Propionigenium modestum* (16, 17) and on a modified *E. coli* enzyme that binds Li⁺ (18). The coupling of proton movements to binding changes in F₁ appears to occur by an interaction between the loop region of subunit c with subunits ε and γ (15, 19, 20). Several models have been proposed whereby ATP synthesis in the F₁ domain is coupled to proton movements through F₀ via movements of subunit c relative to the multicopy subunit a (21–23).

Information on the structural organization of F₀ subunits is essential if we are to understand how H⁺ transport is coupled to ATP synthesis. In this study, Cys was substituted into subunit c in order to determine the arrangement of subunits by disulfide cross-linking. The cross-linking data support the structural model of monomeric subunit c as determined by NMR (24).¹ A ringlike arrangement of 12 subunits c with helix 1 in the center and helix 2 at the periphery is indicated. The subunits interact with the front face of one subunit packed against the back face of the next subunit with the Asp⁶¹ carboxylate centered within a four-helix bundle between adjacent subunits c. The model provides insights and limitations into how H⁺ translocation can be coupled to the rotation and synthesis of ATP within F₁.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotide-directed Mutagenesis and Plasmid Construction—**

The plasmids used in this study are derivatives of plasmid pDF163, which contains the wild type uncBEFH genes (bases 870–3216)² (25).

¹ Girvin, M. E., Rastogi, V. K., Abildgaard, P., Markley, J. L., and Fillingame, R. H. (1998) Biochemistry, in press.

² The unc DNA numbering system corresponds to that used by Walker et al. (52).

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This paper is dedicated to Dr. E. Brown.

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Plasmid pNOC, a derivative of plasmid pDF163 with a C21S substitution in subunit c, was used as a template for the introduction of all the Cys substitutions described. All three of the F_0 subunits coded by plasmid pNOC lack Cys. Plasmid pNOC was constructed by a rapid site-directed mutagenesis procedure (26). An antisense oligonucleotide, 5′-GGGCGCCATACGCTTCTCATCGGAGCAAGC-3′, corresponding to positions Leu19–Pro27 of subunit c was synthesized to incorporate to a single base change (underlined) to create a Ser codon at position 21 and to overlap the nearby SnaBI restriction enzyme site (italics). The polymerase chain reaction (PCR) was then performed using this primer and a sense oligonucleotide primer designed to the coding strand (bases 1540–1560), upstream of the SnaBI restriction enzyme site (1561–1563), using plasmid pDF163 DNA as the template. The PCR product was then digested with PstI and SnaBI restriction enzymes and ligated to the equivalent sites of plasmid pDF163 to generate plasmid pNOC. The substitution was verified by sequencing the entire fragment.

PCR mutagenesis procedures were used to generate other Cys substitutions. Where possible a one-step PCR strategy was employed by taking advantage of restriction enzyme sites in the vicinity of the desired substitution. The majority of helix 1 substitutions were constructed in this fashion using BsrGI (bases 1911–1916, overlapping BsrGI site) and AvaI restriction enzymes. Amplification was achieved with an antisense primer to bases 2162–2167 (bases 1911–1916, overlapping BsrGI site). Amplification was carried out using the BsrGI site. Amplification was achieved with an antisense primer to bases 2162–2167. This enabled cloning of the product into the BsrGI and HpaI sites of plasmid pNOC. For the remaining substitutions and those in helix 2, a two-stage PCR mutagenesis procedure was used (26). This procedure requires a specific mutagenic primer and two wild type primers, 5′ and 3′ to the region of interest. In this case, the sense primer 5′ to the region was designed to bases 1540–1560 so that the PstI site was incorporated into the PCR product. The antisense primer 3′ to the region was designed to bases 2303–2319, so that the HpaI and SnaBI (bases 2256–2262) sites were incorporated into the PCR product. The first PCR step involves use of the mutagenic primer with one of the wild type primers. This first product then serves as a megaprimer for the second round of PCR with the second wild type primer. The product was then digested with PstI and HpaI or SnaBI and ligated into the respective sites of plasmid pNOC. Double Cys substitutions were introduced in combination in helix 1 and helix 2 by subcloning. The PstI/HpaI fragment from a plasmid carrying a single Cys substitution in helix 1 was ligated into the respective sites of a plasmid plasmid derivative carrying a helix 2 Cys substitution. Correct subcloning was confirmed by DNA sequencing. To generate double Cys substitutions in helix 1, or double Cys substitutions in helix 2, the PCR mutagenesis procedures described above were employed using one of the single Cys mutant DNA as the template with a primer generated to create the second Cys substitution. All substitutions were verified by sequencing the entire subcloned fragment.

**Expression and Cross-link Analysis—**A chromosomal uncBEFH deleted strain, JWP109 (pyrE41, entA403, ArgH1, rpsLS109, supE44, ΔuncBEFH), was transformed with plasmid pNOC and its Cys-substituted derivatives. Complementation was tested by transferring transformant colonies to minimal medium 63 plates (27) containing 22 mM succinate, 2 mg/liter thiamine, 0.2 mM uracil, 0.2 mM L-arginine, 0.02 mM dihydroxybenzoic acid, and 100 mg/liter ampicillin. Control plates contained 0.2% glucose instead of succinate.

Preparation of membrane vesicles, protein and ATPase assays, and analysis of cross-linked products by gel electrophoresis and immunoblotting were carried out as described previously (19). Cross-linking studies involved the addition of 1.5 mM CuP as an oxidant to catalyze disulfide bond formation. Membrane vesicles at 5 mg/ml (protein concentration) in TMG buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl_2, and 10% (v/v) glycerol) were treated with 1.5 mM CuP for 1 h at room temperature (22–24 °C). The reaction was terminated with 50 mM EDTA and 25 mM N-ethylmaleimide and incubated for an additional 10 min. The addition of EDTA and N-ethylmaleimide to the reaction mix for 10 min prior to addition prevented cross-link formation. This was also true for membrane vesicles solubilized in sample buffer. N-Ethylmaleimide alone was not sufficient. The sample was then mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, and 0.02% bromphenol blue) containing 20 mM EDTA. To reduce disulfide cross-links, 2× sample buffer was made with 5% (v/v) in β-mercaptoethanol, and samples were incubated for 1 h at room temperature. Products not reduced by these conditions could be reduced by treatment with 2× sample buffer containing 10% β-mercaptoethanol and 8 M urea for 1–12 h at 22–24 °C.

The solubilized membrane proteins were separated by SDS-polyacrylamide gel electrophoresis with the Tris-Tricine buffer system of Schägger and von Jagow (28). After electrophoresis, proteins were transferred from the gel electrophoretically onto a polyvinylidine difluoride membrane (29). Rabbit antisera specific to subunit c (30) was pretreated as described (31) and diluted 1:10,000 prior to use. Immunostaining was carried out using the ECL system (Amersham Pharma- cia Biotech), and multiple exposures were scanned within a linear range of intensity to estimate the extent of cross-link product formation.

**RESULTS**

**Effect of Single Cys Substitutions on Function—**Single Cys substitutions were introduced at positions 8–31 of helix 1 and positions 53–75 and 78 of helix 2 (Table I). The Cys substitutions coded in plasmid pNOC, which carries the uncBEFH genes encoding subunits a, c, b, and δ of F_0 F_1 respectively, were transformed into the ΔuncBEFH strain JWP109. Growth of transformants was tested on succinate minimal medium, where growth depends on a functional oxidative phosphorylase.

**TABLE I**

| Substitution | Growth on succinate | Cross-link, c-c homodimer | Substitution | Growth on succinate | Cross-link, c-c homodimer |
|--------------|---------------------|--------------------------|--------------|---------------------|--------------------------|
| WT           | 2.5 0               |                          | pNOC         | 2.5 0               |                          |
| L8C          | ++ 0                | 0                        | L8C          | ++ 0                | 0                        |
| Y10C         | 2 +/−               | ++ 0                     | Y10C         | 2 +/−               | ++ 0                     |
| M11C         | 2 +/−               | 0                        | M11C         | 2 +/−               | 0                        |
| M12C         | 2 +/−               | 0                        | M12C         | 2 +/−               | 0                        |
| A12C         | 2 0                 | 0                        | A12C         | 2 0                 | 0                        |
| A13C         | 0 0                 | 0                        | A13C         | 0 0                 | 0                        |
| A14C         | 0 0                 | 0                        | A14C         | 0 0                 | 0                        |
| V15C         | 1.5 +/−             | 1.5/−0                   | V15C         | 1.5 +/−             | 1.5/−0                   |
| M18C         | 1.5 0               | 1.5/−0                   | M18C         | 1.5 0               | 1.5/−0                   |
| M17C         | 2 0                 | 0                        | M17C         | 2 0                 | 0                        |
| G18C         | 0 0                 | 0                        | G18C         | 0 0                 | 0                        |
| L19C         | 2 0                 | 1.5/−0                   | L19C         | 2 0                 | 1.5/−0                   |
| A20C         | 1.2 0               | 1.5/−0                   | A20C         | 1.2 0               | 1.5/−0                   |
| A21C         | 1.5 0               | 2                        | A21C         | 1.5 0               | 2                        |
| A22C         | 1.5 0               | 0.8 −/−0                 | A22C         | 1.5 0               | 0.8 −/−0                 |
| G23C         | 2 0                 | 1.5 −/−0                 | G23C         | 2 0                 | 1.5 −/−0                 |
| A24C         | 2 −/−0              | 1.5 −/−0                 | A24C         | 2 −/−0              | 1.5 −/−0                 |
| A25C         | 0 0                 | 0                        | A25C         | 0 0                 | 0                        |
| A26C         | 2 0                 | 0.5 +/−0                 | A26C         | 2 0                 | 0.5 +/−0                 |
| G27C         | 0 +/−0              | 2 +/−0                   | G27C         | 0 +/−0              | 2 +/−0                   |
| G28C         | 0 0                 | 2 +/−0                   | G28C         | 0 0                 | 2 +/−0                   |
| G30C         | 0 +/−0              | 2 +/−0                   | G30C         | 0 +/−0              | 2 +/−0                   |
| L31C         | 2 0                 | 0                        | L31C         | 2 0                 | 0                        |

* Amino acid substitution in subunit c. WT corresponds to wild type uncBEFH genes in plasmid pDF163. Plasmid pNOC harbors the b21S substitution in pDF163.

* Colony size after 72 h of incubation at 37 °C on minimal plates containing 22 mM succinate.

* Relative yield of c-c cross-link formation was as follows: 0, none; −/−, ≤5%; +, 6–25%; +, 26–50%; ++, 51–75%; +++++, >75% (see Figs. 1 and 2).
Arrangement of Subunit c in $F_0$

**Fig. 1. Immunoblot analysis of Cys substitutions in helix 1.** Membrane vesicles were treated with (+) and without (−) CuP, and 20 μg of protein was separated on a 15% polyacrylamide gel and electroblotted to polyvinylidene difluoride membrane. The blot was probed with antiserum specific to subunit c. Cys substitutions are indicated according to their position within subunit c. CuP-treated membranes from the L8C mutant were incubated with sample buffer containing 5% β-mercaptoethanol for 1 h prior to loading (+/R). Membranes from the uncBEFH-deleted strain JWP109 (c DEL), plasmid pDF163 in strain JWP109 (WT), and plasmid pNOC (bC21S) in strain JWP109 were also analyzed. The positions of the monomer (c) and homodimer (c₂) of subunit c are shown by arrows.

**Fig. 2. Immunoblot analysis of Cys substitutions in helix 2.** Membrane vesicles were treated with (+) and without (−) CuP and (12.5 μg of protein) separated on a 15% polyacrylamide gel, and electroblotted. The blot was probed with antiserum specific to subunit c. Cys substitutions are indicated according to their position within subunit c.

Formation of cross-linked homodimers within the transmembrane segment of helix 2 occurred more extensively but in generally lower yield. The region around Asp⁶¹ was particularly prone to cross-linking and may reflect structural flexibility that relates to proton translocating function. These cross-links are considered further under “Discussion.”

**Cross-link Formation between Helix 1 Double Cys Substitutions**—Double Cys substitutions were introduced into helix 1 to see if cross-linked multimers of subunit c could form (Table II). As discussed above, single Cys substitutions at positions 15, 26, and 30 result in formation of cross-linked homodimers in high yield, and these residues fall on the same face of helix 1 in the NMR structure. We reasoned that Cys substitution at two of these positions should provide information on (i) whether homodimer formation in the single Cys substitutions was due to two helices 1 coming together face to face along this surface (Fig. 3B) or (ii) whether these faces of the two helices neighbor each other as would be the case in a ring type arrangement (Fig. 3C). If the helices are arranged face-to-face (Fig. 3B), then only homodimer formation would occur, whereas in a ring type arrangement cross-linked multimers, up to the number of subunits c in the $F_0$, should be detected. All combinations of double Cys substitutions at positions 15, 26, and 30 led to an extensive ladder of subunit c cross-linked multimers (Fig. 4; Table II).

The G18S mutant was unable to grow on succinate and was present at relatively low levels. Gly is also found at the position corresponding to position 18 in a number of other bacteria (32). In a ring type arrangement of helices, Gly⁶⁸ falls next to Leu⁶⁹ of a neighboring helix 1 (Fig. 3C). Leu at position 19 is also conserved in these bacteria. One exception is in Streptococcus faecalis, where position 18 is a Met and position 19 a Gly. We reasoned that if helix 1 is arranged as depicted in Fig. 3B or Fig. 3C, then changing Leu⁶⁹ to an amino acid with a smaller side chain might revert the phenotype of the mutant G18S.
Further, the G18C/L19C mutant might form cross-linked multimers upon oxidation if the arrangement shown in Fig. 3C is correct, whereas it should only form dimers if the arrangement is like that shown in Fig. 3B. Leu19 in the G18C mutant was changed to Ala and to Cys. The Leu 19 substitutions in the G18C/L19A and G18C/L19C pairs both worked as second site suppressors to allow growth on succinate (Table II). The G18C/L19C mutant membranes also formed subunit c multimers in the presence of oxidant (Table II; Fig. 4).

**Fig. 3. Schematic representations of helices 1 and 2 and a model for their arrangement in F0.** The relative position of α-carbons of selected residues, as determined by the NMR model,1 is depicted using modified α-helical wheel projections of the helices. A, the positions of α-carbons of selected residues in helix 1; B and C, two potential arrangements of helix 1 from analysis of single Cys substitutions; D, positions of α-carbons of selected residues in helix 2; E, a section of the ringlike arrangement of subunit c in F0 with subunits packed in a front-to-back manner with α-carbon positions as indicated by the NMR model.

**Table II**

| Helix 1 | Helix 2 |
|---------|---------|
| Substitution | Growth on succinate | Cross-link | Substitution | Growth on succinate | Cross-link |
| L8C/I30C | 2.5 | M | M65C/A67C | 1.5 | 0 |
| M11C/V15C | 2 | M | I66C/G68C | 0.5 | D |
| M11C/M16C | 2 | D | A67C/G68C | 2.5 | d |
| M11C/M17C | 2 | D | A67C/V69C | 0.1 | 0 |
| A14C/M16C | 2.5 | M | A67C/L70C | 2 | d |
| V15C/I26C | 2 | M | V68C/G71C | 2.5 | D |
| V15C/I30C | 1.2 | M | G69C/L70C | 0.2 | 0 |
| M16C/G18C | 0.1 | M | L70C/L72C | 1.5 | M |
| M16C/G18C/L19A | 1.5 | M |
| G18C/L19A | 2 | 0 |
| G18C/L19C | 1 | M |
| A21C/G23C | 0 | m |
| I26C/I30C | 1.5 | M |

a Amino acid substitution in subunit c.
b Colony size after 72 h of incubation at 37 °C on minimal plates containing 22 mM succinate.
c Extent of cross-link formation was as follows: M, high yield multimers (a ladder of subunit c cross-links seen on the immunoblot); m, relatively low yield multimers; D, dimer; d, low yield dimer; 0, none. See Figs. 4–6.

Further, the G18C/L19C mutant might form cross-linked multimers upon oxidation if the arrangement shown in Fig. 3C is correct, whereas it should only form dimers if the arrangement is like that shown in Fig. 3B. Leu19 in the G18C mutant was changed to Ala and to Cys. The Leu19 substitutions in the G18C/L19A and G18C/L19C pairs both worked as second site suppressors to allow growth on succinate (Table II). The G18C/L19C mutant membranes also formed subunit c multimers in the presence of oxidant (Table II; Fig. 4).
Fig. 5. Immunoblot analysis of double Cys substitutions in helix 1. Membrane vesicles were treated with (+) and without (−) CuP, and 25 μg of protein was separated on a 10–15% polyacrylamide gradient gel and electroblotted. The blot was probed with antiserum specific to subunit c. The blots are presented and marked as described for Fig. 2.

To further investigate this arrangement, double Cys substitutions were generated at positions 14/16, 16/18, 16/18 with the L19A suppressor substitution, and 21/23. All of the mutants formed subunit c multimers upon oxidation (Fig. 5A). The M16C/G18C mutant grew poorly on succinate, whereas the M16C/G18C/L19A grew well. Cys substitutions at positions 16 and 18 gave the most defined ladder of subunit c multimers, as shown in Fig. 5, A and B. The ladder clearly extends to distinct multimers at the position of c10 and lighter multimers at the position of c12.

Cross-link Formation between Helix 2 Double Cys Substitutions—The Cys substitutions in helix 1 are consistent with subunit c being arranged in a ring, implying that a similar arrangement must exist for helix 2. A variety of double Cys substitutions were generated in helix 2, falling at different offsets around the helix (Table II; Fig. 3D). Double Cys substitutions between positions 67–72 were of particular significance, since this region was not subject to high yield dimer homodimer formation in the respective single Cys substitutions. Further, the region starting at Met65 appears to be of a continuous and regular α-helix in the NMR structure (Fig. 3D). Of the eight mutants constructed, L70C/L72C formed multimers upon oxidation (Fig. 6). The propensity of L70C/L72C to form a cross-linked multimer can satisfy a number of arrangements of helix 2 relative to helix 1. However, an oligomeric arrangement of subunit c in a ring with the interacting faces of helix 1 and helix 2 packed as in the NMR model and with helix 1 on the inside and helix 2 on the outside fits well with the cross-linking data (Fig. 3E). This places the critical Asp61 toward the interior, positioned within a four-helix bundle.

Cross-link Formation between Helix 1 and Helix 2 Double Cys Substitutions—Cysteines were introduced into both helix 1 and helix 2 to determine their orientation with respect to each other (Table III). We reasoned that a Cys on helix 1 of one subunit c should be able to form a “diagonal” cross-link with a Cys on helix 2 of a neighboring subunit c in the oligomeric ring structure and thus elicit the formation of cross-linked multimers.

Three of the double Cys mutants, A14C/L72C, A20C/I66C, and A21C/M65C, formed extensive cross-linked multimers upon oxidation (Fig. 7). M17C/V68C and M17C/L72C also formed cross-linked multimers but at a lower yield (data not shown). The diagonal cross-linked products fit a model where each subunit c monomer has a structure similar to that proposed in the NMR structure, with each subunit c arranged in a ring as depicted in Fig. 3E. The A14C/L72C and A21C/M65C results are the most compelling, since the A14C and A21C mutants do not form homodimers, and the L72C mutant forms very low yield homodimers. In these cases, the cross-linked multimers of subunit c must be due to disulfide bond formation between helix 1 and helix 2. In the case of A20C/I66C, the single Cys substitution to either position does give rise to a noticeable c-c homodimer. Hence, in this case, multimer formation could result from cross-linked products of Cys66–Cys68 and Cys66–Cys68, as well as helix 1/helix 2 cross-links. In some mutants, such as A14C/V68C and M16C/G69C, a cross-linked product corresponding to a subunit c trimer was identified that satisfied the arrangement. The ability of A20C/V68C to form a cross-linked band up to c5 (results not shown), can be rationalized if the larger side chain of the A20C substitution is forced to occupy a position on the opposing side to where it normally packs. This must also be the case for c-c dimer formation to occur in the A20C and G27C mutants as well as several of the helix 2 single Cys mutants in this vicinity.

DISCUSSION

Singly and doubly Cys-substituted mutants of subunit c were generated here to determine by disulfide cross-link formation the arrangement of subunits in F0. The results confirm that subunit c is folded in a hairpin-like structure with two transmembrane helices. Double Cys substitutions that formed cross-linked multimers on oxidation but showed little or no homodimer formation as single Cys substitutions provide the most compelling evidence for a ringlike arrangement of subunits, as shown in Fig. 3E. The defining double Cys substitutions include the A14C/M16C, M16C/G18C, and G18C/L19C...
pairs in helix 1 and the L70C/L72C pair in helix 2. Multimers formed by “diagonal” cross-linking between helix 1 and helix 2 define the orientation of helices with respect to each other and provide further evidence for a ringlike arrangement. The key double Cys substitutions that fall into this category are A14C/L72C and A21C/M65C. The cross-linking data fit remarkably well with the proposed NMR model of monomeric subunit c (24).1 In fact, the NMR model was used as a basis for predicting residues that were likely to cross-link in the experiments described above.

The formation of cross-linked homodimers from single Cys substitutions in helix 1 conflict with the model shown in Fig. 3E. The predominance of Gly and Ala in residues involved in protein-protein contact show the greatest sequence conservation (34). Met11, Val15, and Leu19 on the interior helical face are selectively labeled by 3-(trifluoromethyl)-3-(iodophenyl)diazirine, in addition to other selectively labeled residues elsewhere in the protein, which led to the suggestion that these residues were on one face of an α-helix that was exposed to the fatty acyl phase of the lipid bilayer (35). In the ring of subunit c suggested here, the diameter of the space in the central core is ≈25 Å, and it seems likely that lipid molecules will be present and account for the 3-(trifluoromethyl)-3-(iodophenyl)diazirine accessibility. Similar situations with centrally located lipid are seen with the bacteriorhodopsin trimer of the purple membrane (36) and presumed for the homooligomeric ring of the light-harvesting complex LH2 (37).

A variety of experiments support the idea that Asp61 in E. coli and equivalent residues in other species bind and release protons in the transport step coupled to ATP synthesis (15, 40). The essential Asp61 in helix 2 of the E. coli protein can be moved to position 24 in helix 1 with retention of function (41). The NMR model of monomeric subunit c shows Ala24 of helix 1 in close proximity to Asp61 of helix 2, but the side chains point to opposite surfaces of the packed helices. How then is function retained in the Asp interchange mutant? In the model depicted here, the Ala24 and Asp61 side chains are positioned within the center of a four-helix bundle formed by the front and back face of two adjacent monomers (Fig. 3E). The interchange of the essential carboxyl from one position to another can be
rationalized in such an arrangement. The model can also be used to rationalize the position of essential liganding residues in subunit c of the Na\(^+\)-translocating F\(_{1}\)F\(_{0}\) ATPases and a Li\(^+\) binding variant of the E. coli F\(_{1}\)F\(_{0}\) ATPase. Kaim et al. (17) have shown that residues Gln\(^{32}\), Glu\(^{65}\), and Ser\(^{66}\) are essential for Na\(^+\) binding in the P. modestum enzyme, i.e. residues at positions equivalent to residues 28, 61, and 62 in E. coli. The conserved residues in P. modestum (Gln, Glu, and Thr) have been identified at equivalent positions within the transmembrane helices of subunit c in the Na\(^+\)-F\(_{1}\)F\(_{0}\) ATPase of Acetobacterium woodii (42). In both enzymes, Pro is found at a position equivalent to Ala\(^{24}\) in E. coli. In the model depicted here, each of these side chains points toward the center of the four-helix bundle formed by neighboring subunits. The model also explains why V\(^{61}\)Al\(#\rightarrow\)AE\(^{61}\)S(G/A) substitutions in E. coli subunit c enable Li\(^+\) binding (18); i.e. the Ser\(^{62}\) hydroxyl can serve as a liganded group opposite the Glu\(^{61}\) carboxyl.

The F\(_{1}\)F\(_{0}\) ATP synthases share many structural similarities with a family of so called vacuolar or V\(_{1}\)V\(_{0}\) ATPases. The V\(_{1}\)V\(_{0}\) subunit c of the F\(_{1}\)F\(_{0}\) ATPases, respectively. Helices 1 and 3 of V-type subunit c show a striking enrichment and conformational changes would be relayed from the site of ion binding/release to the polar loop of subunit c, and conformational changes in the polar loop then drive movement of the γε complex from loop to loop in a circular fashion. The key question obviously remaining is whether rotations take place within the membrane sector.

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Arrangement of the Multicopy $\text{H}^+\text{-translocating}$ Subunit c in the Membrane Sector of the $\text{Escherichia coli}\text{F}_1\text{F}_0$ ATP Synthase
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