Close proximity of a cytoplasmic loop of subunit \textit{a} with \textit{c} subunits of the ATP synthase from \textit{Escherichia coli} *

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SUMMARY

Interactions between subunit $a$ and the $c$ subunits of the *Escherichia coli* ATP synthase are thought to control proton translocation through the $F_o$ sector. In this study cysteine substitution mutagenesis was used to define the cytoplasmic ends of the first 3 transmembrane spans of subunit $a$, as judged by accessibility to 3-N-maleimidyl-propionyl biocytin. The cytoplasmic end of the fourth transmembrane span could not be defined in this way because of the limited extent of labeling of all residues between 186 and 206. In contrast, most of the preceding residues in that region, closer to transmembrane span 3, were labeled readily. The proximity of this region to other subunits in $F_o$ was tested by reacting mono-cysteine mutants with a photo-activated cross-linker. Residues 165, 169, 173, 174, 177, 178, and 182-184 could all be cross-linked to subunit $c$, but no sites were cross-linked to $b$ subunits. Attempts using double mutants of subunit $a$ to generate simultaneous cross-links to 2 different $c$ subunits were unsuccessful. These results indicate that the cytoplasmic loop between transmembrane spans 3 and 4 of subunit $a$ is in close proximity to at least one $c$ subunit. It is likely that the more highly conserved, carboxyl-terminal region of this loop has limited surface accessibility due to protein-protein interactions. A model is presented for the interaction of subunit $a$ with subunit $c$, and its implications for the mechanism of proton translocation are discussed.
INTRODUCTION

The ATP synthase from Escherichia coli is typical of the enzymes found in mitochondria, chloroplasts, and many other bacteria that synthesize ATP (For a recent review see (1)). It is composed of two sub-complexes: an F₁ sector with subunits that contain the catalytic sites, and a membrane-bound Fₒ sector, with subunits that conduct protons across the membrane. In the E. coli enzyme five different subunits are found in F₁: α, β, γ, δ, ε, with a stoichiometry of 3:3:1:1:1. In the Fₒ sector, three different subunits are found: a, b, and c, with a stoichiometry of 1:2:9-12 (2).

Evidence for rotation of subunits through 360 degrees in response to ATP hydrolysis has been provided by direct observation of fluorescently-labeled actin filaments attached to γ or ε (3-6). Therefore, it is thought that the enzyme functions as a rotary motor. Other evidence has been provided that a ring of c subunits is also part of the rotor (7-12). Subunits that make up the stator include δ, a and b, in addition to α and β, which house the catalytic sites. The mechanism by which the proton motive force across the membrane drives rotation of the c oligomer of Fₒ, along with subunits γ and ε, is not known. High resolution structures of F₁, primarily from bovine mitochondria, have provided details about the catalytic sites and their conformational changes (13-15) but less is known about the structure of the Fₒ subunits.

Subunit b is thought to be embedded in the membrane via a hydrophobic region near its N-terminus. Studies of an N-terminal fragment of subunit b, using NMR and detection of disulfide formation, have shown it to be α-helical and dimeric (16). Other studies have shown that a
truncated, soluble form of \( b \) is extended and dimeric (17-21). The N-terminus of subunit \( b \) is thought to interact with subunit \( a \), and its C-terminus is thought to interact with \( \delta \) (22).

Subunit \( c \) is a hydrophobic protein with a conserved aspartic or glutamic acid that is thought to participate in proton translocation steps. NMR studies of the monomeric \( c \) subunit have shown it to be an \( \alpha \)-helical hairpin with two transmembrane spans connected by a short polar loop (23-25), with the conserved D61 residue near the center of the second helix. The conformation of subunit \( c \) appears to be pH dependent, as indicated by NMR studies (26). The number of \( c \) subunits that make up the oligomer in \( F_\circ \) from \( E. \ coli \) is still uncertain, but is likely to be 10 (27).

Subunit \( a \) has been analyzed by surface labeling of unique, engineered cysteine residues. Such studies have established the number of transmembrane spans (28-30) and have characterized the first cytoplasmic loop (31) and the first periplasmic loop (32). These results are summarized in Fig. 1, in which five transmembrane spans are shown. The first cytoplasmic loop is drawn to reflect the limited accessibility of its central region to the reagent MPB¹. Residues important for function (33-36) have been shown to reside in transmembrane spans 4 (R210 and E219) and 5 (H245). In addition, E196, appears to reside in the cytoplasmic loop preceding transmembrane span 4. It is likely that R210 of subunit \( a \) interacts with the essential residue D61 of subunit \( c \) during coupled proton translocation, since disulfide cross-linking studies have shown that transmembrane span 4 of subunit \( a \), between residues 207 and 225, appears to be in contact with subunit \( c \) (37).

The mechanism by which subunit \( a \) contributes to the proton conducting path of the ATP synthase is not clear, but it is likely to play such a role. Models have been presented in which subunit \( a \) contributes amino acids that make up two half-channels, one opening to the periplasm and one to the cytoplasm, that allow access to the proton binding site on subunit \( c \) (38,39).
Previously identified residues in subunit \( a \) (33-36), thought to be important in proton access to subunit \( c \), are found within transmembrane spans, near the periplasmic surface (32). So far, it is not clear how proton access might be controlled at the cytoplasmic surface. These studies were undertaken to examine the structure of the cytoplasmic loop between transmembrane spans 3 and 4, and to determine which other \( F_o \) subunits those residues were near.
EXPERIMENTAL PROCEDURES


*Materials.* Restriction enzymes were obtained from New England Biolabs. Synthetic oligonucleotides were obtained from Operon Technologies. DNA sequencing was done by Lone Star Labs. MPB and TFPAM-3 were obtained from Molecular Probes. Nickel-nitrilotriacetic acid (Ni-NTA) resin and DNA miniprep kits were obtained from Qiagen. Mouse anti-HA antibody was obtained from Roche. Goat anti-mouse IgG-alkaline phosphatase conjugate, goat anti-rabbit IgG-alkaline phosphatase conjugate, avidin-conjugated alkaline phosphatase, 5-bromo-4-chloro-3-indoylphosphate p-toluidine salt (BCIP), p-nitro blue tetrazolium chloride (NBT), SDS-polyacrylamide gels, PVDF membranes, and low and broad range protein molecular weight standards were obtained from Bio-Rad. N-Octyl-β-D-glucoside was purchased from Anatrace. All other chemicals were purchased from Sigma or Fisher. The UV lamp was purchased from UVP, model UVL-56, with wavelength 365nm. Anti-β antibodies and anti-c antibodies were gifts of Dr. R. Capaldi, University of Oregon and Dr. K. Altendorf, University of Osnabrück, Osnabrück, Germany, respectively.

*Plasmids, mutagenesis, growth and expression.* The plasmids, pLN6HisHA (28), pLN7HisHA (31), pLN46HisHA (32), pTW1HisHA (28), pARP2HisHA (39), and pDP1018HisHA were used for the construction of mutants. Plasmid pDP1018HisHA was constructed by ligation of the 770 base pair BsaH I-Afl III fragment from pBJA1018 (39) and the 2592 base pair BsaH I-Afl III fragment from pLN7HisHA. These plasmids produce subunit a that includes an HA epitope (YPYDVPDYA), derived from the hemagglutinin protein of human influenza virus, and a His$_6$ tag at the C-terminus of the protein. These tags have no effect on function. These plasmids differ primarily in the placement of unique restriction sites that are
necessary for cassette mutagenesis. Mutagenesis and growth of cultures were carried out as described previously (32).

Preparation of inside-out membrane vesicles. Inside-out membrane vesicles were made from a 250 ml culture (per experimental sample) in LB medium grown to $A_{600} = 1.0$. Cells were resuspended in 10 ml of 50 mM Tris-HCl, 10 mM MgSO$_4$, pH 7.5 and passed through a French press at 14,000 p.s.i. Cell debris and unbroken cells were removed by a low speed spin at 8000 rpm for 15 min. The supernatant was then centrifuged at 50,000 rpm for 1 h at 4°C in a Beckman Ti-70 rotor. The pellet was resuspended and used in the experiments or stored at -80°C.

Labeling of membrane vesicles. The inside-out membrane vesicles were labeled in 200 mM Tris-HCl (pH 8.0), with 120 μM MPB at room temperature for 15 min. The reaction was stopped by adding β-mercaptoethanol to a final concentration of 20 mM. The vesicles were then centrifuged at 50,000 rpm for 45 min and subunit $a$ was purified by Ni-NTA resin as described below.

Cross-linking of subunit $a$ by TFPAM-3. This cross-linker is expected to span 10-15 Å. The cross-linking was carried out by the methods described previously (31). The membrane vesicles were suspended in 50 mM MOPS (pH 7.0), 5 mM EDTA and 10% of glycerol. It was incubated with 200 μM TFPAM-3 for 60 min at room temperature, and the reaction was terminated by addition of 15 mM cysteine. After addition of 5 mM ATP, cross-linking was activated by UV light. The reactions were terminated after 2 h at room temperature.

Purification and detection of subunits. After reaction, membrane vesicles were resuspended in extraction buffer (200 mM Tris-HCl (pH 8.0), 1.5% octyl glucoside, 0.1% deoxycholate, 0.5% cholate, 10 mM β-mercaptoethanol, 10 mM imidazole, and 1% Tween 20). Subunit $a$ was
purified using Ni-NTA as described previously (32). Samples of purified subunit \( a \) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane as described previously (31). For detection of subunit \( a \), previously published procedures were followed (29). For detection of subunit \( b \), the blocked membrane was incubated at room temperature for 2 h with \( b \)-antibody at a dilution of 1:1000. After washing three times with TBS/Tween 20, it was incubated with goat anti-mouse IgG-alkaline phosphatase conjugate at a dilution of 1:1000 for 1 h. After another three washings with TBS/Tween 20, color was developed as described above. For detection of subunit \( c \), the blocked membrane was incubated at room temperature for 2 h with \( c \)-antibody at a dilution of 1:5000. After washing three times with TBS/Tween 20, it was incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate at a dilution of 1:1000 for 1 h. After another three washings with TBS/Tween 20, color was developed.

\textit{Molecular models.} The molecular models shown were created from the Protein Data Bank file 1c99, model 1, using RasMol (40).
RESULTS

A series of 46 mono-substituted cysteine mutants of subunit $a$ that are thought to be near the cytoplasmic surface were constructed. All mutants grew in succinate minimal medium, indicating the ability to carry out oxidative phosphorylation. Inverted inner membrane vesicles were prepared from these mutants, and were then labeled with MPB to test the surface accessibility of each residue. The results for mutations between residues 167 and 174 are shown in Fig. 2, and the results for mutations between residues 177 and 184 are shown in Fig. 3. The results of all mutants are summarized in Table I. For comparative purposes, each labeling experiment contained G70C, a residue shown previously (29) to label strongly in membrane vesicles, i.e., from the cytoplasmic surface, and E131C, a residue that was shown to label poorly in membrane vesicles (28), but strongly from the periplasmic surface (29). In panel A, the results of MPB labeling in membrane vesicles, followed by Ni-NTA purification of subunit $a$ are shown. In panel B an immunoblot of the corresponding samples is shown, to confirm that the level of protein is similar.

The proposed cytoplasmic loop between transmembrane spans 3 and 4—In Fig. 2, the results of labeling of residues K167C, M168C, K169C, G170C, I171C, G173C, and F174C are shown. Only K167C, lane 4, shows no sign of labeling, while F174C, lane 10, labels as well as G70C, lane 2. Other residues show intermediate levels of labeling. In Fig. 3 the results of labeling of residues E177C, L178C, T179C, P182C, F183C, and N184C are shown. All of the residues label to an intermediate degree except T179C, which is near the background level. The results of H185C, W186C, A187C, F188C, I189C, V191C, and N192C are shown in Table I. None of them shows significant labeling, except for H185C and W186C. Other residues between 193 and 206 show only a trace of labeling, at most.

The cytoplasmic ends of transmembrane spans 1, 2, and 3—Residues F60, R61, V63 and A64 near the end of transmembrane span 1 were analyzed and the results are presented in Table I.
In this group, only A64C, lane 7, is labeled significantly when compared to the controls, G70C and E131C. The results of labeling I101C, A102C, and P103C, three residues near the cytoplasmic end of transmembrane span 2, are shown in Table I. Only I101C, is labeled above a background level. The results of labeling residues near the cytoplasmic surface of transmembrane span 3, L160C, I161C, L162C, F163C, Y164C, and S165C, are also presented in Table I. None of these residues were labeled to a significant extent.

Cross-linking analysis—A series of cross-linking experiments was conducted to determine if any of the residues in the cytoplasmic loop between transmembrane spans 3 and 4 are near subunits \( b \) or \( c \). Mono-substituted cysteine mutants at the following positions were reacted with the photoactivated cross-linker TFPAM-3: 165, 167-171, 173-175, 177-179, and 182-184. After UV activation, nine residues showed evidence of cross-linking to subunit \( c \), and the results are presented in Fig. 4. In panel 4A, samples S165C, K169C, G173C, and F174C are probed with anti-HA for detection of subunit \( a \), on the left side, and the same samples are probed with anti-\( c \) antibodies on the right side. The \( a-c \) cross-link can be seen in both blots, and is dependent upon UV activation, designated by the “+” sign. Similarly, as shown in panel 4B, samples E177C, L178C, P182C, and F183C are probed with anti-HA for detection of subunit \( a \), on the left, and with anti-\( c \) antibodies on the right side. Residue N184C was also found to cross-link to subunit \( c \) in a similar manner (results not shown).

The following double cysteine mutants were constructed and tested for cross-linking using TFPAM-3: S165C/P182C, K169C/P182C, G173C/P182C, F174C/P182C, E177C/P182C, L178C/P182C, S165C/F183C, K169C/F183C, G173C/F183C, F174C/F183C, E177C/F183C, L178C/F183C, S165C/N184C, K169C/N184C, G173C/N184C, F174C/N184C, E177C/N184C, and L178C/N184C. In each case only a single cross-linked product was seen, with no indication of an \( a-c_2 \) product (results not shown).
DISCUSSION

The labeling results presented here provide further information about the junctions of the first three transmembrane spans of subunit \( a \), and this information is summarized in Fig. 5. This model differs from that shown in Fig. 1 in two respects. The junctions of the transmembrane spans at the cytoplasmic surfaces have been adjusted to reflect the results of labeling, and the periplasmic loop between spans 4 and 5 has been adjusted, as discussed below. Previous work (29) had identified residue T37C, at the amino-terminal end of transmembrane span 1, as accessible by MPB labeling, but residues W39C and D44C were not. At the carboxyl-terminal end of transmembrane span 1, residues 67-70 labeled strongly, while residue 64 labeled weakly (29). In this work, residues F60C, R61C, and V63C were shown not to label, relative to the weak labeling of residue A64C. The results indicate a core transmembrane segment of approximately 26 residues from 38 to 63. This matches well the calculated hydropathy peak centered near residues 50-53 (41,42). The carboxyl-terminal end of transmembrane span 2 was analyzed in previous work (32). Residues D119C, L120C, and P122C were inaccessible to MPB labeling, while residues D124C and P127C were labeled. Near the amino-terminal end of transmembrane span 2, residues 92-98 were all labeled by MPB (31). Here it was shown that residue I101C was labeled to a small degree relative to residues A102C and P103C. These results indicate a core transmembrane segment of approximately 22 residues from 102 to 123, which again matches closely the calculated hydropathy peak centered near residues 109-111 (41,42). The amino-terminal end of transmembrane span 3 was analyzed in previous work (32), and residues V142C, S144C, and D146C were all shown to be labeled with MPB. At the carboxyl-terminal end of transmembrane span 3 residue K169C was shown to be labeled with fluorescein-maleimide (30) and G172C was shown to be labeled with MPB (28). Here, residues 160-165, and K167C were shown to be inaccessible to MPB, while M168C was labeled. Therefore, a core transmembrane
segment of approximately 21 residues exists from 147 to 167, which again matches well the calculated hydropathy peak centered near residues 156-158 (41,42).

In contrast, the results presented here give no indication of the amino-terminal end of transmembrane span 4. Previous studies had indicated that residue E196C could be labeled from the cytoplasmic surface, but those results occurred under slightly more strenuous labeling conditions (30), or the labeling was rather weak (28). In this study the labeling of all residues tested between 185 and 206 was seen to be very weak, and only a few showed a level of labeling that could be considered above background. While it is likely that absolute levels of labeling would differ under different conditions and with different maleimido-reagents, the lack of relative differences seen here makes it impossible to recognize the junction of transmembrane span 4 with the cytoplasmic surface. This also applies to the periplasmic ends of both transmembrane spans 4 and 5, where only three residues were found to be labeled by MPB (29). In studies of \textit{lac} permease, single amino acid insertions (43) or deletions (44,45) have been used as indicators of the ends of transmembrane spans. In previous work reported by this lab (39,41), insertions of alanine after residues 202 and 225 did not seriously impair function of the ATP synthase, while those after positions 212, 217, and 222 did. These results are consistent with a hydrophobic core segment in transmembrane span 4 approximately between residues 202-225. Likewise, consideration of insertions of alanine (39) and MPB labeling (29) identifies a core segment in transmembrane span 5 between residues 238 and 259. The loop now identified between residues 226 and 236 is consistent with the marginal effects of alanine insertions after residues 225, 229, and 233. This is in contrast to the loss of function seen with insertions after residues 217, 222, 238 and 243 (37,39).

The labeling of the residues in the putative loop between transmembrane spans 3 and 4 showed a strikingly asymmetric pattern. Twelve out of thirteen residues tested between 168 and 184 were found to be labeled by MPB, while none of the twenty residues tested between 185 and
206 showed significant labeling. In consideration of the bulky nature of the reagent MPB, it is likely that the amino-terminal half of this loop is highly exposed, given the extensive labeling pattern. The lack of labeling at the carboxyl-terminal end of the loop is consistent with significant protein-protein interactions. This asymmetry correlates with amino acid sequence conservation, in which conservation for residues 190-225 in subunit $a$ is high (46), while for residues prior to 190 conservation is low. Evidence for a surface of interaction between transmembrane span 4 of subunit $a$ and the carboxyl-terminal transmembrane span of subunit $c$ has been provided by studies of engineered disulfides by Jiang and Fillingame (37). In the structural model of subunit $c$ (26) determined at pH 8, shown in Fig. 6, these residues form a curved surface, suggesting that transmembrane span 4 of subunit $a$ wraps around that surface. If E196 of subunit $a$ were part of an $\alpha$-helical extension of transmembrane span 4 then it would be found near the loop of subunit $c$ (residues 41-43), since L207C of subunit $a$ forms a disulfide with I55C of subunit $c$, and residues 43 to 55 in subunit $c$ are all $\alpha$-helical in the structural model at pH 8. This possibility is illustrated in Fig. 6, in which residues 190-225 of subunit $a$ are modeled as an $\alpha$–helix.

The labeling pattern of residues 168-184 is consistent with a connection from transmembrane span 3 to the top of a ring of $c$ subunits, with high surface accessibility. The accessibility of these residues to MPB resembles that of the residues near the cytoplasmic surface of transmembrane spans 1 and 2 of subunit $a$. The results of cross-linking with TFPAM-3 indicate that this region is near subunit $c$, i.e. at a distance of 10-15 Å. The range of sites that can be cross-linked indicates that both the end of transmembrane span 3 and the center of the second cytoplasmic loop are near subunit $c$. It is likely that the efficiency of cross-linking was too low to detect a doubly cross-linked product. Therefore, it remains uncertain if all positions cross-link to
the same subunit $c$. Although this loop is not predicted to be entirely $\alpha$-helical (42), the pattern of labeling and cross-linking from residues 173-179 (GFTKELT) is consistent with an $\alpha$–helix.

The proposed interactions between subunit $a$ and subunit $c$ discussed above have important implications for the mechanism of proton translocation, of which several proposals have been described recently (26,47). As shown in Fig. 6, $aR210$ (blue) would be near the $cD61$ (red). That would place $aE219$ pointing into the bundle of transmembrane spans of subunit $a$ (30), and $aE196$ at the top near the loop of subunit $c$. The residues in subunit $a$ that cross-link to subunit $c$ are likely to be near the adjacent subunit $c$ in the ring. It has been proposed that the proton motive force causes protonation of a network of residues in subunit $a$ near the periplasmic surface (32), including $aE219$ and $aH245$. This could cause a twisting or bending of transmembrane span 4 of subunit $a$ allowing protonation of $cD61$ from the periplasmic side. This would cause $aR210$ to move away from $cD61$, as subunit $c$ changes to its protonated conformation (26), and the $cD61$ rotates to its new position. The conformational changes in the transmembrane span 4 of subunit $a$ and in the loop region of subunit $c$, would be transmitted to the E196 region of subunit $a$. This could promote the deprotonation of $cD61$ of the adjacent subunit $c$, perhaps by the exposure of $aE196$ and the introduction of water. At this point, the $aR210$ would now be in position to be attracted electrostatically to $cD61$, but since it would be to the adjacent subunit $c$, one step of rotation will have occurred.

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Footnote:

1 Abbreviations: HA epitope, mouse hemagglutinin epitope; MPB, 3-N-maleimidyl-propionyl biocytin; Ni-NTA, Nickel-nitrioltriacetic acid; TBS, 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl; TFPAM-3, N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimido-propionamide
Table 1: Relative Labeling of Mono-Substituted Cysteine Mutants with MPB

| Mutant  | Labeling\(^a\) | Mutant  | Labeling\(^a\) |
|---------|----------------|---------|----------------|
| F60C    | +              | P182C   | +++            |
| R61C    | +              | F183C   | ++             |
| V63C    | +              | N184C   | +++            |
| A64C    | +++            | H185C   | ++             |
| I101C   | ++             | W186C   | ++             |
| A102C   | +              | A187C   | +              |
| P103C   | +              | F188C   | +              |
| L160C   | +              | I189C   | +              |
| I161C   | +              | V191C   | +              |
| L162C   | +              | N192C   | +              |
| F163C   | +              | L193C   | +              |
| Y164C   | +              | I194C   | +              |
| S165C   | +              | L195C   | +              |
| K167C   | +              | E196C   | +              |
| M168C   | ++             | G197C   | +              |
| K169C   | +              | V198C   | +              |
| G170C   | ++             | S200C   | +              |
| I171C   | +++            | L201C   | +              |
| G173C   | ++++           | S202C   | +              |
| F174C   | +++++          | K203C   | +              |
| E177C   | ++             | P204C   | +              |
| L178C   | +++            | V205C   | +              |
| T179C   | +              | S206C   | +              |

\(^a\) Labeling is scored as indicated in Fig. 2 and 3. “+” indicates a level of labeling that is not significantly higher than background. “+++++” indicates the highest level of labeling seen, that of G70C.
FIGURE LEGENDS

FIG. 1. The transmembrane model of subunit α. The cytoplasmic loop between residues 64 and 100 is drawn to indicate that the central region has limited accessibility to the reagent MPB, but the segments nearest the membrane are highly exposed. The periplasmic loop between residues 124-146 has been drawn to reflect its partial exposure throughout this region.

FIG. 2. Labeling of subunit α from the cytoplasmic surface: residues 167, 168, 169, 170, 171, 173 and 174. A. Protein standards in kD are indicated at the left. MPB labeling in membrane vesicles of G70C, E131C, K167C, M168C, K169C, G170C, I171C, G173C, and F174C is shown. The position of subunit α is indicated by the arrowhead. B. Immunoblot of the samples in panel A.

FIG. 3. Labeling of subunit α from the cytoplasmic surface: residues 177, 178, 179, 182, 183, and 184. A. Protein standards in kD are indicated at the left. MPB labeling in membrane vesicles of G70C, E131C, E177C, L178C, T179C, P182C, F183C, and N184C is shown. The position of subunit α is indicated by the arrowhead. B. Immunoblot of the samples in panel A.

FIG. 4. Cross-linking of subunit α to subunit c using TFPAM-3. A. Protein standards, 103 kD, 77 kD, 50 kD, 34.3 kD, 28.8 kD, and 20.7 kD are shown in the left and center lanes. Four cysteine mutants at the indicated positions were analyzed in preparations of membrane vesicles. Samples were reacted with TFPAM-3, and then exposed to uv radiation. After
incubation, subunit \(a\) was extracted with detergent and purified. The resulting blots were probed with anti-\(a\) antibodies, on the left, or with anti-\(c\) antibodies, on the right. The plus (+) sign indicates uv exposure, while the minus (-) sign indicates identical handling without the uv exposure. The expected sizes of the \(a\), \(c\), and \(a\)-\(c\) cross-linked subunits are indicated. B. Four additional cysteine mutants were treated in an identical fashion. Note that uncross-linked subunit \(c\) also co-purified with His-tagged subunit \(a\), and that the \(c\)-antibodies recognized a second band that might be a noncovalent \(c\)-dimer co-migrating with standard 28.8 kD.

**FIG. 5.** Transmembrane spans of subunit \(a\). Only residues analyzed in this study are colored. Residues that were labeled with MPB in membrane vesicles are colored dark blue, while those with little or no labeling are colored light blue. Residues that were cross-linked using TFPAM-3 to subunit \(c\) are identified with a red asterisk. Transmembrane spans were determined as described in the text.

**FIG. 6.** Interacting surfaces of subunit \(a\) and subunit \(c\). The structure of subunit \(c\), with deprotonated D61, as determined by NMR (26), is shown on the right. Residues that formed disulfides with subunit \(a\) residues are colored cyan (37). In subunit \(c\), residue D61 is colored red, and all other residues are colored in the CPK scheme. Transmembrane span 4 of subunit \(a\) and its amino-terminal extension to residue 190 are shown on the left. Residues in subunit \(a\) that form disulfides to residues in subunit \(c\) are colored magenta (37). In subunit \(a\), residue R210 is colored blue, and other residues are colored in the CPK scheme. Expected membrane boundaries are marked by horizontal lines: subunit \(a\) (K203 and I225) and subunit \(c\) (T51 and V78).
Figure 2
Figure 4
Close proximity of a cytoplasmic loop of subunit a with c subunits of the ATP synthase from Escherichia coli

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