Aqueous Access Pathways in ATP Synthase Subunit α

REACTIVITY OF CYSTEINE SUBSTITUTED INTO TRANSMEMBRANE HELICES 1, 3, AND 5*²

Received for publication, November 24, 2006, and in revised form, January 10, 2007 Published, JBC Papers in Press, January 18, 2007, DOI 10.1074/jbc.M610848200

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Subunit α is thought to play a key role in H⁺ transport-driven rotation of the subunit c ring in Escherichia coli F₁,F₀ ATP synthase. In the membrane-traversing F₀ sector of the enzyme, H⁺ binding and release occurs at Asp-61 in the middle of the second transmembrane helix (TMH) of subunit c. Protons are thought to reach Asp-61 via aqueous channels formed at least in part by one or more of the five TMHs of subunit α. Aqueous access to surfaces of TMHs 2, 4, and 5 was previously suggested based upon the chemical reactivity of cysteine residues substituted into these helices. Here we have substituted Cys into TMH1 and TMH3 and extended the substitutions in TMH5 to the cytoplasmic surface. One region of TMH3 proved to be moderately Ag⁺-sensitive and may connect with the Ag⁺-sensitive region found previously on the periplasmic side of TMH2. A single Cys substitution in TMH1 proved to be both N-ethylmaleimide (NEM)-sensitive and Ag⁺-sensitive and suggests a possible packing interaction of TMH1 with TMH2 and TMH3. New Ag⁺- and NEM-sensitive residues were found at the cytoplasmic end of TMH5 and suggest a possible connection of this region to the NEM- and Ag⁺-sensitive region of TMH4 described previously. From the now complete pattern of TMH residue reactivity, we conclude that aqueous access from the periplasmic side of F₀ to cAsp-61 at the center of the membrane is likely to be mediated by residues of TMHs 2, 3, 4, and 5 at the center of a four-helix bundle. Further, aqueous access between cAsp-61 and the cytoplasmic surface is likely to be mediated by residues in TMH4 and TMH5 at the exterior of the four-helix bundle that are in contact with the c-ring.

The H⁺-transporting F₁,F₀ ATP synthases of oxidative phosphorylation utilize the energy of a membrane-electrochemical gradient of protons or Na⁺ to mechanically drive the synthesis of ATP via two coupled rotary motors in the F₁ and F₀ sectors of the enzyme (1–3). In the intact enzyme, ATP synthesis or hydrolysis takes place in the F₁ sector at the surface of the membrane, synthesis being coupled to H⁺ transport through the transmembrane F₀ sector. Homologous enzymes are found in mitochondria, chloroplasts, and many bacteria (4). In Escherichia coli and other eubacteria, F₁ consists of five subunits in an α₅β₅γ₁δ₁ε₁ stoichiometry (4). F₀ is composed of three subunits in a likely ratio of α₁β₁δ₁ε₁ in E. coli and Bacillus subtilis PS3 or α₁β₁δ₁ε₁ in the Na⁺-translocating Ilyobacter tartaricus ATP synthase (3, 5–7). Subunit c spans the membrane as a hairpin of two α-helices with the first TMH2 on the inside and the second TMH on the outside of the c-ring (7–9). A high resolution x-ray structure of the I. tartaricus c₁₁₁-ring has revealed the sodium-binding site at the periphery of the ring with chelating groups to the Na⁺ extending from two interacting subunits (7). The essential I. tartaricus Glu-65 in the Na⁺ chelating site corresponds to E. coli Asp-61. In the H⁺-transporting E. coli enzyme, Asp-61 at the center of the second TMH is thought to undergo protonation and deprotonation as each subunit of the c ring moves past a stationary subunit α. In the complete membra- nous enzyme, the rotation of subunit c is proposed to be driven by H⁺ transport at the subunit α/c interface, and this rotation then drives rotation of subunit γ within the α₁β₁δ₁ hexamer of F₁ to cause conformational changes in the catalytic sites leading to synthesis and release of ATP (1–3). The linkage between subunit γ and the c-ring appears to be permanent as these subunits can be cross-linked to each other without loss of function (2).

Subunit α is thought to provide access channels to the proton-binding Asp-61 residue in the c-ring, and candidate residues lining a possible aqueous access pathway were tentatively defined (10–13). Subunit α is known to fold with five TMHs (14–16) with TMH4 packing in parallel to TMH2 of subunit α (17), i.e. the helix to which Asp-61 is anchored. Interaction of the conserved Arg-210 residue in aTMH4 with cTMH2 is thought to be critical during the deprotonation-protonation cycle of cAsp-61 (10, 18, 19). Cross-linking of Cys residues introduced into subunits α and c, or b and c, supports the po- sitioning of subunit α and the two b subunits at the periphery of the c ring (17, 20, 21). Little is known about the structure or three-dimensional arrangement of the TMHs in subunit α. Several sets of second site suppressor mutations in one TMH, to the primary mutation in a second TMH, had suggested possible helical-helical interactions (13, 14, 18, 22). More recently, we have introduced pairs of Cys residues into putatively apposing TMHs and tested for zero-length, cross-linking of the Cys thiol groups to disulfide bonds. Cross-links were found with 8 different Cys pairs and define a juxtaposition of TMHs 2–3, 2–4,

* This work was supported by United States Public Health Grant Gm23105 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

² The abbreviations used are: TMH, transmembrane helix; ACMA, 9-amino-6-chloro-2-methoxyacridine; NEM, N-ethylmaleimide.

The on-line version of this article (available at http://www.jbc.org) contains a supplemental table summarizing the properties of all Cys substitutions made in subunit α.

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| Location and mutation | Growth on glucose | Growth on succinate | Quenching with ATP |
|-----------------------|-------------------|---------------------|--------------------|
|                       | %                 |                     |                    |
| TMH1                  |                   |                     |                    |
| W39C                  | 102               | 2.3                 | 78 ± 1             |
| T40C                  | 102               | 2.6                 | 78 ± 0             |
| I41C                  | 103               | 2.3                 | 76 ± 1             |
| N42C                  | 93                | 2.2                 | 68 ± 3             |
| I43C                  | 93                | 2.4                 | 65 ± 5             |
| D44C                  | 93                | 2.0                 | 45 ± 3             |
| S45C                  | 100               | 2.2                 | 68 ± 9             |
| M46C                  | 101               | 2.4                 | 70 ± 6             |
| F47C                  | 101               | 2.2                 | 68 ± 2             |
| F48C                  | 100               | 2.2                 | 71 ± 3             |
| S49C                  | 100               | 2.4                 | 71 ± 1             |
| V50C                  | 99                | 2.2                 | 69 ± 9             |
| V51C                  | 100               | 2.0                 | 79 ± 2             |
| L52C                  | 98                | 2.3                 | 70 ± 6             |
| G53C                  | 100               | 2.7                 | 69 ± 12            |
| L54C                  | 99                | 2.0                 | 55 ± 0             |
| L55C                  | 99                | 2.0                 | 67 ± 8             |
| F56C                  | 100               | 2.1                 | 58 ± 2             |
| L57C                  | 100               | 2.2                 | 68 ± 10            |
| V58C                  | 97                | 2.2                 | 58 ± 2             |
| L59C                  | 98                | 2.2                 | 65 ± 12            |
| F60C                  | 97                | 2.2                 | 62 ± 8             |
| R61C                  | 99                | 2.2                 | 66 ± 1             |
| S62C                  | 102               | 2.5                 | 78 ± 1             |

1–2 cytoplasmic loop

| G96C                  | 112               | 2.6                 | 77 ± 1             |
| K97C                  | 100               | 2.1                 | 78 ± 3             |
| S98C                  | 100               | 2.2                 | 76 ± 2             |
| K99C                  | 111               | 2.4                 | 76 ± 2             |
| L100C                 | 100               | 2.2                 | 73 ± 3             |
| I101C                 | 100               | 2.4                 | 76 ± 1             |
| A102C                 | 105               | 2.4                 | 74 ± 2             |
| P103C                 | 99                | 2.4                 | 74 ± 2             |
| L104C                 | 100               | 2.4                 | 74 ± 2             |
| A105C                 | 100               | 2.4                 | 72 ± 1             |

2–3 periplasmic loop

| Y128C                 | 99                | 2.3                 | 73 ± 5             |
| I129C                 | 100               | 2.4                 | 73 ± 2             |
| A130C                 | 100               | 2.4                 | 71 ± 2             |
| E131C                 | 100               | 2.5                 | 69 ± 1             |
| H132C                 | 99                | 2.1                 | 74 ± 6             |
| V133C                 | 99                | 2.3                 | 71 ± 4             |
| L134C                 | 100               | 2.1                 | 70 ± 3             |
| G135C                 | 100               | 2.2                 | 59 ± 3             |
| L136C                 | 113               | 2.4                 | 54 ± 4             |
| P137C                 | 100               | 2.1                 | 64 ± 4             |

TMH3

| A138C                 | 101               | 2.0                 | 74 ± 1             |
| L139C                 | 97                | 2.5                 | 65 ± 1             |
| R140C                 | 99                | 2.0                 | 69 ± 6             |
| V141C                 | 100               | 1.5                 | 77 ± 9             |
| V142C                 | 100               | 2.5                 | 74 ± 3             |
| P143C                 | 101               | 2.5                 | 69 ± 3             |
| S144C                 | 101               | 2.4                 | 72 ± 5             |
| A145C                 | 101               | 2.3                 | 70 ± 3             |
| D146C                 | 96                | 2.0                 | 54 ± 5             |
| V147C                 | 90                | 2.0                 | 70 ± 2             |
| N148C                 | 99                | 2.0                 | 70 ± 2             |
| V149C                 | 99                | 2.3                 | 73 ± 1             |
| T150C                 | 102               | 2.5                 | 68 ± 4             |
| L151C                 | 101               | 2.5                 | 71 ± 0             |
| S152C                 | 101               | 2.3                 | 71 ± 1             |
| M153C                 | 101               | 2.4                 | 68 ± 4             |
| A154C                 | 100               | 2.5                 | 69 ± 1             |
| L155C                 | 100               | 2.3                 | 60 ± 4             |
| G156C                 | 100               | 2.3                 | 67 ± 6             |
| V157C                 | 101               | 2.4                 | 72 ± 1             |
| F158C                 | 101               | 2.2                 | 69 ± 2             |
| I159C                 | 101               | 2.2                 | 69 ± 4             |

3–4 cytoplasmic loop

| L160C                 | 100               | 2.3                 | 64 ± 2             |
| I161C                 | 99                | 2.3                 | 70 ± 4             |
| L162C                 | 99                | 2.2                 | 67 ± 2             |
| F163C                 | 99                | 2.2                 | 69 ± 8             |
| Y164C                 | 88                | 2.0                 | 74 ± 4             |

2–5, 3–4, 3–5, and 4–5 in a four-helix bundle (23). The aqueous-accessible residues defined in this study and in previous studies (11, 12) will be discussed in the context of the new cross-linking based model.

The differential reactivity of cysteines introduced by site-directed mutagenesis has been used as a means of probing aqueous-accessible regions of several membrane proteins. The reagents used, including methanethiosulfonate derivatives (24, 25), NEM (26, 27), and Ag⁺ (28, 29), react preferentially with the ionized, thiolate form of the Cys side chain and thus can serve as an indicator of the polarity of the environment. Previously, we probed Cys residues introduced into TMHs 2, 4, and 5 for aqueous accessibility based upon their reactivity with NEM and Ag⁺ (11, 12). We found two regions of aqueous accessibility in the transmembrane segments of subunit α with differing properties. One region consists of Ag⁺- and NEM-sensitive residues on one side of TMH4 extending from Asn-214 and Arg-210 near the center of the membrane to the cytoplasmic surface. A second set of Ag⁺ -sensitive but NEM-inaccessible residues maps to the opposite face and periplasmic side of TMH4 and from the center of the membrane to the periplasmic surfaces of TMH2 and TMH5. The Ag⁺-sensitive residues in TMHs 2, 4, and 5 cluster at the interior of the four-helix bundle predicted by cross-linking (23) and could interact to form a continuous aqueous pathway extending from the periplasmic surface to the center of the membrane. In the experiments reported here, we have focused on TMH1, TMH3, and the cytoplasmic end of TMH5.

**EXPERIMENTAL PROCEDURES**

**Construction of Cys-substituted Mutants**—The cysteine substitutions generated here were transferred into plasmid pCMAl13 (11), which contains a hexahistidine tag on the C terminus of subunit α (14) and genes encoding the entire unc (atp) operon from which all endogenous Cys had been substituted by Ala or Ser (30). Cys substitutions were introduced by a two-step PCR method using a synthetic oligonucleotide, which contained the codon change, and two wild type primers (31). PCR products were transferred into pCMAl13 directly using...
unique HindIII (870) or PflMI (1136) and BsrGI (1913) sites (see Ref. 32 for nucleotide numbering). TMH3 mutations were transferred into pVF196 (14) using unique PflMI (1136) and PshAI (1805) sites and then transferred to pCMA113 using HindIII and BsrGI sites as described above. All mutations were confirmed by sequencing the cloned fragment through the ligation junctions. All experiments were performed with the mutant whole operon plasmid derivative of pCMA113 in the unc operon deletion host strain JWP292 (5). All plasmid transformant strains were tested for growth on succinate and glucose as described (11).

**Membrane Preparation**—Plasmid transformants of strain JWP292 were grown in M63 minimal medium containing 0.6% glucose, 2 mg/liter thiamine, 0.2 mM uracil, 1 mM L-arginine, 0.02 mM dihydroxybenzoic acid, and 0.1 mg/ml ampicillin, supplemented with 10% LB medium, and harvested in the late exponential phase of growth (5). Cells were suspended in TMG-acetate buffer (50 mM Tris acetate, 5 mM magnesium acetate, 10% glycerol, pH 7.5) containing 1 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml DNase I and disrupted by passage through a French press at 1.38 × 10⁸ nt/m², and membranes were prepared as described (33). The final membrane preparation was suspended in TMG-acetate buffer and stored at −80 °C. Protein concentrations were determined using a modified Lowry assay (34).

**ATP-driven Quenching of ACMA Fluorescence**—Membranes were suspended in 3.2 ml of HMK buffer (10 mM Hepes-KOH, 1 mM Mg(NO₃)₂, and 10 mM KNO₃, pH 7.5). ACMA was added to 0.3 μg/ml final concentration, and 30 μl of 0.1 mM ATP, pH 7.0, was added to initiate quenching of fluorescence. The reaction was terminated by the addition of 8 μl of 288 μM nigericin (0.5 μg/ml final concentration). The level of fluorescence obtained after the addition of nigericin was normalized to 100% in calculating the percentage of quenching due to ATP-driven proton pumping. For AgNO₃ treatment, 160 μl of membranes at 10 mg/ml was suspended in HMK buffer containing 40 μM AgNO₃ and incubated at room temperature for 15 min before carrying out the quenching assay. For NEM treatment, membranes at 10 mg/ml in TMG-acetate buffer were treated with 5 mM NEM for 15 min at room temperature and then diluted into HMK-nitrate buffer before carrying out the same quenching assay.

**RESULTS**

**Properties of Cys Substitutions in TMH 1, 3, and 5 of Subunit a**—In this study, Cys substitutions in TMH1, TMH3, and TMH5 and adjacent loop regions were generated in a His-tagged version of subunit a. The substitutions were expressed from a plasmid encoding subunits of F₁ and F₅ in which all endogenous Cys were substituted by Ala or Ser (11). These plasmids were transformed into strain JWP292, which carries a chromosomal deletion of the entire unc (atp) operon. The growth of these strains on minimal medium was compared using glucose or succinate as carbon sources (Table 1). The Cys substitutions reported here had minimal effects on growth with either carbon source. Additionally, ATP-driven quenching of ACMA fluorescence was performed on inside-out membrane vesicles of the substituted strains to evaluate ATPase-coupled H⁺ pumping function (Table 1). The quenching response for most mutants was close to that observed with the control, Cys-free wild type membranes, which typically showed quenching responses of 76 ± 6%. Of the 72 Cys-substituted mutants tested, the D44C mutant showed the most severely reduced quenching response at...
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45%, with seven other mutants (at residues 54, 56, 58, 135, 136, 146, and 155) showing more modest reductions in quenching to the range of 50–60%.

Inhibition of ATPase-coupled H⁺ Transport by Ag⁺ or NEM in TMH1 and TMH3—Inside-out membrane vesicles with Cys substitutions in residues 39–62 encompassing αTMH1 or residues 128–164 encompassing TMH3 were treated with 40 μM AgNO₃ or 5 mM NEM in chloride-free assay buffer, and the effect on ATPase-coupled H⁺ transport was measured by the quenching of ACMA fluorescence. Examples for the five moderately Ag⁺-sensitive mutants are shown in Fig. 1. In each of the five mutants, Ag⁺ reduced the quenching response by greater than 50%. NEM had negligible inhibitory effects on the quenching response for three of the substitutions shown but significantly reduced the quenching response for the D44C and D146C mutants (Fig. 1). As discussed in greater detail below, the NEM sensitivities of residues D44C and D146C are unusual in that only a few other transmembrane Cys in subunit a react with NEM. The complete surveys of the Ag⁺ sensitivity and NEM sensitivity of residues in TMH1 and TMH3, including periplasmic loop 2–3 and portions of loop 3–4, are shown in Fig. 2 and Fig. 3, respectively. The activity of all transmembrane Cys substitutions in TMH1 was relatively insensitive to Ag⁺ or NEM treatment with the dramatic exception of D44C (Fig. 2). The R140C, S144C, and D146C substitutions within the predicted membrane spanning sequence of TMH3 (residues 138–159) were inhibited by Ag⁺ with S144C showing the greatest sensitivity (>70% inhibition) (Fig. 3). As mentioned previously, D146C was also sensitive to inhibition by NEM. Two Cys substitutions at positions 130 and 136, which are predicted to reside in the periplasmic loop between TMHs 2 and 3, were also inhibited by ≥50% upon Ag⁺ treatment. Residues 161 and 162 at the beginning of the 3–4 cytoplasmic loop also showed modest sensitivity to Ag⁺ treatment, i.e. 40–50% inhibition.

Inhibition of ATPase-coupled H⁺ Transport by Ag⁺ or NEM in TMH5—In a previous report (12), we demonstrated that Ag⁺-sensitive residues in TMH4 extended to the cytoplasmic surface but had not completed a survey of residues near the cytoplasmic surface in TMH5. The Ag⁺ and NEM sensitivities of 8 such residues are shown in Fig. 4. Cys substitutions at positions 262 and 263 resulted in significant sensitivity to Ag⁺ inhibition, activity being inhibited by 72 and 89%, respectively. However, these two substitutions show striking differences in NEM sensitivity with Y262C showing 75% inhibition versus a more modest 24% inhibition for Y263C. The L264C substitution was also moderately sensitive to inhibition by Ag⁺, whereas other residues were resistant to inhibition by Ag⁺ or NEM.

DISCUSSION

Prior to this study, we found that Ag⁺-sensitive Cys residues in TMHs 2, 4, and 5 localized to distinct helical surfaces that we concluded were likely to be aqueous-accessible (11, 12). The ionic radius of Ag⁺ is close to that of Na⁺ or H₃O⁺ (28), and the ionized Cys thiolate that is thought to be the reactive species would be formed preferentially in an aqueous environment. It is unclear exactly why Ag⁺ modification of these thiolates results in inhibition of ATP-driven H⁺ translocation, but minimally, the Ag⁺ sensitivity indicates that these residues are likely to be aqueous-accessible. The purpose of this study was to test whether these putative aqueous access pathways extended to residues lining TMH1 and TMH3 and to the cytoplasmic end of TMH5. The Cys residues to which the Ag⁺ and NEM sensitivity have now been mapped are shown in a topological model of subunit a in Fig. 5. We will discuss the results within the context of a model for interaction of these helices, the model being based upon zero-length cross-linking between Cys residues introduced into adjacent TMHs (23). The cross-sectional packing of TMHs 2, 3, 4, and 5 predicted by this model is shown in Fig. 6. The Cys-Cys cross-links were also used to estimate the relative depth of placement of adjacent TMHs in the lipid bilayer, as indicated in Fig. 5, by minimizing vertical distances between cross-linkable Cys pairs. The depth of placement of

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3 The 8 cross-linkable Cys-Cys pairs used to construct the model are: 120/144 (TMHs 2–3), 119/218 and 20/218 (TMHs 2–4), 120/245 and 120/246 (TMHs 2–5), 148/219 (TMHs 3–4), 148/245 (TMHs 3–5), and 218/248 (TMHs 4–5).
TMH4 in subunit a was based upon its cross-linking to TMH2 of subunit c (17), and the positioning of the L. tartaricus c-ring in a lipid bilayer was determined by cryo-electron crystallography (35). The cross-linkable Cys pairs form a near horizontal band on the periplasmic side of TMHs 2–5, extending from residues 119 and 120 in TMH2 to residues 245, 246, and 248 in TMH5 (23). The cross-linking results indicate that these helices could cross each other and pack as a four-helix bundle.

A striking feature of the proposed four-helix bundle is the clustering of Ag+/H11001-sensitive residues at its center (Fig. 6); these residues could interact to form an aqueous access pathway from the periplasm. The clustered residues include the very Ag+/H11001-sensitive residues 119 and 120 from TMH2, 215 from TMH4, and 245 and 252 from TMH5. The more moderately sensitive residues 140 and 144 in TMH3 might also line this access pathway. A close proximity of residues 140 and 144 in TMH3 and the Ag+-sensitive face of TMH2 is supported by the observed cross-linking of Cys residues introduced at positions 120 and 144. The silver sensitivity of residues 130 and 136 in the periplasmic loop between these helices could indicate a possible packing interaction of the loop with the periplasmic ends of these helices. Residue 146, which is moderately sensitive to both NEM and Ag+, would lie at the opposite, peripheral face of TMH3 (Fig. 6), and its presence at that position is not easily reconciled with the clustering of other Ag+-sensitive residues at the center of the four-helix bundle.

Residue 44 in TMH1 could also contribute to the aqueous access pathway from the periplasm. The D44C replacement is moderately sensitive to both Ag+ and NEM. The D44C substitution leads to a fairly profound reduction in ATP-driven ACMA quenching and may suggest an important functional role for this residue. The Asp-44 residue may play an important, albeit not totally essential, role in the passage of protons through F0 during ATPase/synthase-coupled H+ transport. Howitt et al. (36) have previously reported that the D44N substitution results in blockage of passive F0-mediated H+ translocation, albeit without significantly affecting in vivo activity and growth on glucose or succinate. D44C membranes, which had been stripped of F1, remained H+ impermeable and are also blocked in passive F0-mediated H+ translocation (experiments not shown). We suggest that TMH1 may pack between

4 Cys residues at positions 142, 144, and 146 were labeled by biotin-maleimide from the periplasmic surface and led Zhang and Vik (37) to conclude that this region extended into the periplasmic loop. Such a placement, outside of the hydrophobic core of the lipid bilayer, conflicts with the cross-linking results (23), but the biotin-maleimide reactivity suggests that this transmembrane region may have unusual aqueous accessibility.

5 Of the 162 Cys substitutions tested to date, only 12 show reductions in the ATP-driven quenching of similar or greater magnitude with the R210C, G213C, and H245C, showing quenching responses in the range of 0–10%, and the D44C, D119C, L207C, L209C, Y216C G218C, E219C, L220C, and Q252C substitutions, showing quenching responses in the range of 25–45% (see Supplemental Table 2 online).

FIGURE 4. Differing sensitivity of Cys substitutions at the C-terminal end of TMH5 to Ag+ and NEM inhibition. Membranes were treated as described in the legend for Fig. 1. The substitution tested is indicated in each panel.
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FIGURE 5. Position of Ag⁺-sensitive residues in a two-dimensional topological model of subunit a. Residues that are most sensitive to inhibition by Ag⁺ are shown in red (>90% inhibition), orange (71–90% inhibition), and brown (51–70% inhibition). The relative depth of placement of the five TMHs of subunit a in the lipid bilayer is based upon cross-linking experiments as described under "Discussion."

FIGURE 6. Cross-sectional view of Ag⁺-sensitive residues in the five TMHs of subunit a. The relative position of Ag⁺-sensitive residues in TMHs 2–5 is depicted by cross-sectional views of α-helical wheels. The juxtaposition of TMHs 2–5 to each other is based upon the cross-linking experiments described in Schwem and Fillingame (23). The most Ag⁺-sensitive residues are shown in red (>90% inhibition) and orange (71–90% inhibition). The position of the moderately Ag⁺-sensitive residues 146 and 146 in TMH3 are shown in brown (51–70% inhibition). The other residues shown in black depict the positions of Cys that form cross-links between different TMHs. The placement of TMH1 between TMHs 2 and 3 is based upon the possible connectivity of Ag⁺-sensitive residues at the interface of these helices. The position of the αArg-210 side chain is also indicated. The green shaded area represents the helical face of TMH4 that cross-links to TMH2 of subunit c.

TMH2 and TMH3, as indicated in Fig. 6, and bring the Asp-44 residue close to the cluster of other Ag⁺-sensitive residues in TMH2 (i.e. Met-115, Asp-119, Pro-122, and Asp-126), and Ser-144 in TMH3. In this proximity, the Asp–44 residue could potentially form a salt bridge with Arg-140 in TMH2. Arg-140 could also potentially form a salt bridge with Asp-119 in TMH2. These possible proximities are now being tested by cross-linking and additional mutational analysis.

We also report two new Ag⁺-sensitive Cys substitutions at the cytoplasmic boundary of TMH5 (Fig. 5). The Y263C substitution is one of the most Ag⁺-sensitive residues seen in the protein, showing 89% inhibition upon Ag⁺ treatment.⁶ V262C is more moderately sensitive to Ag⁺ but also very sensitive to NEM, showing >75% inhibition upon treatment with NEM. At the cytoplasmic side of TMH4, the principle residues implicated in H⁺ transport include the very Ag⁺-sensitive residues 202, 203, and 206. Residue 206 is also very sensitive to NEM. A single NEM-accessible pocket could extend from residues 206 in TMH4 and 262 in TMH5 and possibly connect with the NEM-sensitive N214C residue at the center of the membrane. The critical Arg-210 residue would then locate to the center of this pocket. This interface is presumed to pack at the peripheral surface of the c-ring and participate with cAsp-61 in proton-translocation and c-ring rotation. The Ag⁺ sensitivity of residues 262 and 263 may indicate a connectivity of the aqueous-accessible space at the cytoplasmic surface of TMH5. The very Ag⁺-sensitive Y263C substitution would face the interior of the four-helix bundle with the NEM-sensitive V262C facing the periphery Fig. 6).

In summary, from the now complete survey of the Ag⁺ and NEM reactivity of Cys-substituted TMHs in subunit a, we hypothesize that there are two aqueous half-channels in subunit a extending from the center of the lipid bilayer to the cytoplasmic or periplasmic surfaces. When the chemical modification data are correlated with the cross-linking data (23), the Ag⁺-sensitive residues that extend to the periplasmic surface cluster at the center of the proposed four-helix bundle of TMHs 2–4 and may bridge to the interface of TMH1 as it packs between TMH2 and TMH3. The cytoplasmic half-channel begins in the region of Asn-214 and Arg-210 at the center of the membrane and extends along the outer face of TMH4 to Ser-202 at the surface of the lipid bilayer. Cys residues introduced into this region are both NEM- and Ag⁺-sensitive, and this aqueous pocket may extend to the NEM-sensitive V262C substitution at the peripheral face of TMH5. The residues at the peripheral faces of TMHs 4 and 5 are presumed to interact with the c-ring during the protonation and deprotonation of Asp-61 of subunit c. The key question raised by this model relates to the mechanism of gating of the two half-channels. The protonated cAsp-61 is hypothesized to release its proton to the cytoplasmic half-channel due to interaction with αArg-210 with a consequent lowering of its p Kis (13). Reprotonation from the periplasm would seem to require a change in the positioning of these residues to break the salt bridge and raise the pKsα to facilitate protonation. If protons gain access to this site via a periplasmic pathway at the center of the four-helix bundle, how

⁶ The Y263C cysteine was labeled by biotin-maleimide from the cytoplasmic surface (16), and some substitutions at this position (e.g. Lys, Glu, Gly) were shown to significantly disrupt proton translocation through F₀ (38).
are the H\(^+\) transferred to cAsp-61 on the surface of the c-ring? It seems possible that the protons could collect at the junction defined by the very Ag\(^+\)-sensitive Q252C and M215C substitutions and that a swiveling of these helices would enable movement of H\(^+\) to the periphery. Such swiveling could also simultaneously move the aArg-210 away from cAsp-61 to facilitate its reprotonation.

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