Aqueous Accessibility to the Transmembrane Regions of Subunit c of the Escherichia coli F$_1$F$_0$ ATP Synthase*

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Rotary catalysis in F$_1$F$_0$ ATP synthase is powered by proton translocation through the membrane-embedded F$_0$ sector. Proton binding and release occur in the middle of the membrane at Asp-61 on transmembrane helix (TMH) 2 of subunit c. Previously the reactivity of Cys substituted into TMH2 revealed extensive aqueous access at the cytoplasmic side as probed with Ag$^+$ and other thiolate-directed reagents. The analysis of aqueous accessibility of membrane-embedded regions in subunit c was extended here to TMH1 and the periplasmic side of TMH2. The Ag$^+$ sensitivity of Cys substitutions was more limited on the periplasmic versus cytoplasmic side of TMH2. In TMH1, Ag$^+$ sensitivity was restricted to a pocket of four residues lying directly behind Asp-61. Aqueous accessibility was also probed using Cd$^{2+}$, a membrane-impermeant soft metal ion with properties similar to Ag$^+$. Cd$^{2+}$ inhibition was restricted to the I28C substitution in TMH1 and residues surrounding Asp-61 in TMH2. The overall pattern of inhibition, by all of the reagents tested, indicates highest accessibility on the cytoplasmic side of TMH2 and in a pocket of residues around Asp-61, including proximal residues in TMH1. Additionally subunit a was shown to mediate access to this region by the membrane-impermeant probe 2-(trimethylammonium)methyl methanethiosulfonate. Based upon these results and other information, a pocket of aqueous accessible residues, bordered by the peripheral surface of TMH4 of subunit a, is proposed to extend from the cytoplasmic side of cTMH2 to Asp-61 in the center of the membrane.

F$_1$F$_0$ ATP synthase utilizes the energy stored in an H$^+$ or Na$^+$ electrochemical gradient to synthesize ATP in bacteria, mitochondria, and chloroplasts (1–4). The ATP synthase complex is composed of two sectors, i.e. a water-soluble F$_1$ sector that is bound to a membrane-embedded F$_0$ sector. In bacteria, F$_1$ is composed of five subunits in an $\alpha_3$,$\beta_3$,$\gamma$,$\delta$,$\epsilon$ ratio and contains three catalytic sites for ATP synthesis and/or hydrolysis centered at the $\alpha$-$\beta$ subunit interfaces and/or hydrolysis. F$_0$ is composed of three subunits in an $\alpha_1$$\beta_2$$\gamma$$_{10.15}$ ratio and functions as the ion-conducting pathway (5–9). Ion translocation through F$_0$ drives rotation of a cylindrical ring of c-subunits that is coupled to rotation of the $\gamma$ subunit with the ($\alpha$$\beta$)$_3$ hexamer of F$_1$ to force conformational changes in the three active sites and in turn drive synthesis of ATP by the binding change mechanism (1–4, 10–13).

Subunit c of F$_0$ folds in the membrane as a hairpin of two extended $\alpha$-helices. In Escherichia coli, 10 copies of subunit c pack together to form a decameric ring with TMH1$^3$ on the inside and TMH2 on the periphery (6, 14). An atomic resolution structure of the Na$^+$-translocating c$_{11}$ ring from Ilyobacter tartaricus was recently published by Meier et al. (8). In the c$_{11}$ structure, the Na$^+$ binding site is formed by two interacting c subunits. The essential Na$^+$-binding Glu residue, which corresponds to Asp-61 in E. coli, is located in TMH2 at the middle of the lipid bilayer. Subunit a consists of five transmembrane helices, four of which likely interact as a four-helix bundle (15–18). Subunit a lies on the periphery of the c-ring with TMHs 4 and 5 from subunit a and TMH2 from subunit c forming the a-c interface (18–21). During ion translocation through F$_0$, the essential Arg-210 on TMH4 of subunit a is postulated to facilitate the protonation/deprotonation cycle at Asp-61 of subunit c and cause the rotation of the c-ring past the stationary subunit a (3, 4, 19).

Chemical modification of cysteine-substituted transmembrane proteins has been widely used as a means of probing the aqueous accessible regions (22–24). The reactivity of a substituted cysteine to thiolate-directed probes provides an indication of aqueous accessibility because the reactive thiolate species is preferentially formed in an aqueous environment. The aqueous accessibility of the five TMHs in subunit a of E. coli F$_0$ has been probed using Ag$^+$ and NEM (19, 25–27). The results suggest the presence of an aqueous accessible channel in subunit a in the center of TMHs 2–5 extending from the periplasm to the center of the membrane. Protons entering through this periplasmic access channel are postulated to bind to the essential Asp-61 residues of the c-ring and exit to the cytoplasm by a still uncertain pathway at the peripheral face of aTMH4 with protonation/deprotonation of Asp-61 driving c-ring rotation.

During H$^+$-driven ATP synthesis, two models for the pathway by which H$^+$ or Na$^+$ exit to the cytoplasm have been proposed. The first model proposes that the ions bound at Asp-61 exit to the cytoplasm via a half-channel composed at least partially by residues in TMH4 of subunit a (25–27). Chemical modification studies of Cys-substituted subunit a of E. coli revealed an aqueous accessible surface of TMH4 that includes

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2 The abbreviations used are: TMH, transmembrane helix; ACMA, 9-amino-6-chloro-2-methoxycacidine; $\beta$-MSH, $\beta$-mercaptoethanol; DMSO, dimethylsulfoxide; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl-MTS; MTSET, 2-(trimethylammonium)ethyl-MTS; NEM, N-ethylmaleimide.
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the essential Arg-210 residue, which extended from the center of the membrane to the cytoplasm, suggesting that the ion exit channel may lie at the a-c interface (19, 25). Alternatively studies of the c-ring from the \textit{L. tartaricus} enzyme indicate that Na\(^+\) can access Glu-65 in the absence of other F\(_0\) subunits, suggesting an intrinsic channel in subunit c (28, 29). However, no such channel was apparent in the crystal structure of the c\(_{11}\)-ring (8).

In a previous study (30), we probed the thiolate reactivity of Cys substitutions in the cytoplasmic half of TMH2 in subunit c. These experiments revealed extensive reactivity to sulfhydryl-directed reagents on the peripheral face of cTMH2, supporting the presence of the cytoplasmic exit channel at the a-c interface. In this study, we extended the survey of aqueous accessibility in transmembrane regions by probing thiolate reactivity of Cys substitutions in TMH1 and in the periplasmic half of TMH2. The reactivity of Cys substituted into these regions proved to be more limited. Only a small region of TMH1, lying directly behind Asp-61, was reactive with Ag\(^+\). In addition to Ag\(^+\), we used Cd\(^{2+}\) as a complementary, membrane-impermeant probe for aqueous accessibility. The survey of Cd\(^{2+}\) sensitivity confirmed that aqueous accessibility from the cytoplasm is much greater for residues packing at the periphery of the c-ring. The experiments reported here distinguish the aqueous accessible and inaccessible regions of the c-ring and strengthen evidence that the cytoplasmic H\(^+\) exit channel is situated at the a-c interface.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—A two-step PCR method that utilizes a mutagenic primer and two wild type primers (31) was used to generate the cysteine substitutions in subunit c. The template plasmid, pCMA113, codes the eight structural genes of F\(_1\)F\(_0\) wherein all endogenous cysteines are replaced with alanine or serine and subunit a is modified with a C-terminal His tag (25). Mutant PCR fragments were transferred into pCMA113 between the BsrGI and BssHII (or PpuMI) restriction sites. The presence of each substitution was confirmed by DNA sequencing through the ligation sites. All mutant plasmids were transferred into the chromosomal unc (\textit{atp}) operon deletion strain JW292 (6). Growth yields of the mutant strains on glucose minimal medium and succinate minimal medium agar plates were assayed as described previously (25).

**Membrane Preparation**—Inside-out membrane vesicles were prepared from JW292 transformant strains as described previously, and protein concentrations were determined using a modified Lowry assay (30).

**ATP-driven ACMA Fluorescence Quenching**—Aliquots of membranes prepared from JW292 transformant strains (1.6 mg at 10 mg/ml) were suspended in 3.2 ml of HMK-NO\(_3\) buffer (10 mM HEPES-KOH, 1 mM Mg(NO\(_3\))\(_2\), 10 mM KNO\(_3\), pH 7.5) and incubated at room temperature for 10 min. ACMA was added to a concentration of 25 \(\mu\)M, and fluorescence quenching was initiated by the addition of 30 \(\mu\)M ATP, pH 7. Each experiment was terminated by adding nigericin to 0.5 \(\mu\)g/ml. The fluorescence measured after the addition of nigericin was used as the base line from which the relative quenching of the mutant membranes was calculated. The 10-min incubation of untreated control samples resulted in no significant inhibition relative to samples assayed immediately after dilution. Treatments with Ag\(^+\) and NEM were carried out using HMK-NO\(_3\) buffer as described previously (30). For treatment with MTS reagents, the MTS derivative was added to a 1.6- mg aliquot of membranes in 160 \(\mu\)l of TMG (50 mM Tris-OAc, pH 7.5, 5 mM MgOAc, 10% glycerol)-acetate buffer at a final concentration of 1 mM. After a 10-min incubation at room temperature, treated membranes were diluted into HMK-NO\(_3\) buffer, and the quenching reaction was carried out as described above. Solutions of MTS reagents were dissolved immediately before use in DMSO solvent. For treatment with Cd\(^{2+}\), a 1.6-mg aliquot of membranes was suspended in 3.2 ml of HMK-Cl buffer (10 mM HEPES-KOH, 5 mM MgCl\(_2\), 300 mM KCl, pH 7.5), and CdCl\(_2\) was added from an aqueous stock solution to the desired concentration. Following an incubation of 10 min at room temperature the fluorescence quenching reaction was carried out as described above. ATP-driven ACMA quenching is more robust in HMK-Cl buffer than in HMK-NO\(_3\) buffer, but HMK-Cl buffer cannot be used to measure the effects of Ag\(^+\) treatment because of precipitation of AgCl.

**2-(Trimethylammonium)ethyl-MTS (MTSET) Modification**—Membranes prepared from JW292 transformant strains were diluted to 20 mg/ml with TMG-Cl buffer (50 mM Tris-HCl, 5 mM MgCl\(_2\), 10% glycerol, pH 7.5) to a volume of 5 ml. Modification was initiated with the addition of 25 \(\mu\)l of 100 mM MTSET in H\(_2\)O prepared immediately before use. After a 10-min incubation at room temperature, free MTSET was removed by passing treated membranes through a G-25 Sephadex centrifuged gel filtration column according to Penefsky (32). The filtrate was solubilized by the addition of 1/10 volume of 50 mM Tris-Cl, pH 7.5, 20% SDS. NEM was added from EtOH solution to 1 mM. After a 15-min incubation at room temperature, \(\beta\)-MSH was added to 4% (v/v) and incubated at 37 \(^\circ\)C for 10 min to reverse MTSET modification and prevent other oxidations during purification. Subunit c was purified from the reaction mixture as described previously (30).

**Electrospray Ionization Quadrupole Ion Trap Mass Spectrometry**—Purified subunit c in CHCl\(_3\):MeOH:H\(_2\)O (5:5:1) was directly injected into an Applied Biosystems 3200 Q TRAP\textsuperscript{®} liquid chromatography-tandem mass spectrometry system using acetonitrile:H\(_2\)O (1:1) as the running solvent. A 5-\(\mu\)l aliquot of 3% trifluoroacetic acid was injected prior to injecting subunit c to increase the abundance of ions within the detectable range for this instrument.

**RESULTS**

**Characterization of Cysteine Substitutions**—Residues in the transmembrane regions of subunit c were mutated to cysteine by oligonucleotide-directed mutagenesis using a plasmid wherein all endogenous cysteines in F\(_1\) were substituted with alanine and wherein the single cysteine in subunit b of F\(_0\) was substituted with serine. Wild type subunits a and c lack endogenous cysteine. The mutant plasmid was transferred into the \textit{E. coli} strain JW292 in which the unc (\textit{atp}) operon encoding F\(_1\)F\(_0\) was deleted from the chromosome. Most of the cysteine substitution mutants grew nearly as well as wild type on both glucose and succinate minimal media (Table 1). Also inverted membrane vesicles from most of the cysteine substitution
TABLE 1
Characterization of cysteine substitution mutants

| Substitution | Colony size on succinatea | Growth yield on glucoseb | Percent quenching with ATPc |
|--------------|--------------------------|--------------------------|-----------------------------|
| TMH1         |                          |                          |                             |
| V15C         | 2.0                      | 104                      | 78                          |
| M16C         | 2.2                      | 105                      | 76                          |
| M17C         | 2.0                      | 108                      | 77                          |
| G18C         | 0.0                      | 62                       | 2±3                         |
| L19C         | 2.3                      | 109                      | 76                          |
| A20C         | 2.0                      | 96                       | 75                          |
| A21C         | 2.1                      | 99                       | 78                          |
| I22C         | 2.5                      | 97                       | 76                          |
| G23C         | 0.1                      | 57                       | 2±3                         |
| A24C         | 2.2                      | 98                       | 75                          |
| A25C         | 1.5                      | 80                       | 46                          |
| I26C         | 2.1                      | 97                       | 76                          |
| G27C         | 0.1                      | 56                       | 2±3                         |
| I28C         | 2.3                      | 108                      | 72                          |
| G29C         | 0.5                      | 59                       | 2±3                         |
| I30C         | 2.0                      | 100                      | 76                          |
| L31C         | 2.0                      | 99                       | 70                          |
| G32C         | 0.1                      | 58                       | 2±3                         |
| TMH2         |                          |                          |                             |
| L59C         | 2.1                      | 90                       | 48                          |
| I66C         | 2.4                      | 105                      | 73                          |
| A67C         | 2.4                      | 100                      | 67                          |
| A68C         | 2.0                      | 108                      | 82                          |
| A69C         | 2.0                      | 85                       | 71                          |
| A70C         | 2.2                      | 103                      | 79                          |
| A71C         | 2.1                      | 106                      | 77                          |
| G58C         | 0.3                      | 57                       | 2±3                         |
| L59C         | 2.1                      | 90                       | 48                          |
| I66C         | 2.4                      | 105                      | 73                          |
| A67C         | 2.4                      | 100                      | 67                          |
| A68C         | 2.0                      | 108                      | 82                          |
| A69C         | 2.0                      | 85                       | 71                          |
| A70C         | 2.2                      | 103                      | 79                          |
| A71C         | 2.1                      | 106                      | 77                          |

a Colony size in mm. Average colony size of wild type is 2.2 mm.
b Expressed as percentage of wild type growth yield in 0.04% glucose liquid medium. Average growth yield of the unc background is 60 ± 5%.
c As observed in HMK-NO₃ buffer. Average quenching of wild type is 75 ± 5%.

strains were functional in ATP-driven proton pumping as assayed by quenching of ACMA fluorescence (Table 1). Replacement of any of the glycine residues in TMH1 abolished growth on succinate medium and ATP-driven proton pumping. Glycine residues at positions 23, 25, 27, and 29 are highly conserved, and Gly at positions 18 and 32 are moderately conserved (33). Replacement of these small side chains with a larger one likely disrupts the tight helical packing between subunits in the c-ring. The properties of substitutions on the periplasmic side of TMH2 are reported here as well as the growth properties of the L59C mutant that was erroneously reported previously as being inactive (30). The properties of this substitution as well as substitutions on the periplasmic side of TMH2 are reported here.

Sensitivity of TMH1 to Thiolate-reactive Probes—We tested the aqueous accessibility of Cys substitutions in cTMH1 in inverted membrane vesicles by examining the sensitivity of ATP-driven proton pumping to inhibition by cysteine-modifying reagents. These reagents react preferentially with the thiolate form of cysteine, which should predominate in an aqueous environment. Inhibition of ATP-driven proton pumping by the thiolate-directed reagents is thought to reflect an obstruction of the aqueous, proton-translocating pathway. Disulfide formation has not been observed between Cys residues substituted into adjacent c subunits in the absence of an oxidizing agent and cannot account for the loss of activity (14). Inverted membrane vesicles carrying substitutions in cTMH1 were treated with 5 mM N-ethylmaleimide or 1 mM methyl-MTS, 2-carboxymethyl-MTS, 2-aminoethyl-MTS (MTSEA), sulfonatoethyl-MTS, or MTSET. None of the substitutions in TMH1 were sensitive to inhibition by NEM or to the MTS reagents (data not shown). In contrast, four of the Cys substitutions tested (at positions 20, 24, 25, and 28) were strongly inhibited by Ag⁺. Typical experiments contrasting Ag⁺-sensitive and -insensitive substitutions are shown in Fig. 1.

Sensitivity of TMH2 to Thiolate-reactive Probes—Our previous experiments revealed widespread sensitivity of Cys substitutions in the cytoplasmic half of TMH2 to inhibition by Ag⁺ and MTS reagents (30). In this study we expanded the testing of aqueous accessibility to the periplasmic half of this TMH including positions 66–71. As in TMH1, none of these periplasmic substitutions in TMH2 were sensitive to NEM or to MTS reagents (data not shown). However, Cys substituted at position 66 was very sensitive to inhibition by Ag⁺ versus the moderate sensitivity of Cys substituted at positions 68 and 69. The Ag⁺ sensitivity of Cys substitutions in both transmembrane regions of subunit c is summarized in Fig. 2.

Subunit a Required for Access of Membrane-impermeant MTSET to G58C—Previously we showed that modification of the G58C substitution by NEM does not take place in the absence of subunit a (30). However, the route of access by NEM to this membrane-embedded position was uncertain because NEM can dissolve in and permeate membranes (34). To determine whether subunit a was required for aqueous access by thiolate-directed reagents from the cytoplasm, we probed this position with MTSET, which was shown previously to be membrane-impermeant (34). Membrane vesicles were treated with 0.5 mM MTSET and the non-reacting MTSET was then removed by gel filtration. Subunit c was purified from treated membranes and subjected to mass spectrometry to detect the MTSET modification. In spectra not shown here, unmodified subunit c appeared as a set of peaks identified as unmodified subunit c G58C (8330 Da) and multiple oxidation products, which are easily formed with the eight Met residues in the pro-
tein during purification in the absence of β-MSH. MTSET-modified subunit c appeared as a set of peaks shifted by 117 Da relative to unmodified subunit c, i.e. a shift consistent with the addition of S-(CH2)2-N(CH3)3. In similar experiments using membrane vesicles from a subunit a deletion strain, the intensity of the MTSET-modified subunit c peak was dramatically reduced by more than 6-fold. Surprisingly the intensity of the MTSET-modified peaks suggested that 30% of c subunits were being modified by MTSET. However, the constitutive positive charge of the MTSET adduct is likely to have enhanced the ionization of the modified subunit c during electrospray ionization, necessitating additional experiments.

Because of extensive oxidations during purification in the absence of β-MSH and the possibly artificial inflation of the peak intensity of the MTSET-modified subunit c, we pursued an alternative method to detect MTSET modification. The extent of MTSET modification in these experiments was assessed by measuring the extent of protection from subsequent reaction of the G58C Cys with NEM following solubilization of the membranes with SDS. Membrane vesicles were treated with 0.5 mM MTSET, and free MTSET was removed by gel filtration. The filtrate was then solubilized in SDS to expose the G58C Cys to reaction with NEM. Finally β-MSH was added to prevent further reaction with NEM and reverse the MTSET modification. The β-MSH also helped to prevent methionine oxidation during the purification of subunit c. Subunit c was purified and subjected to mass spectrometry (Fig. 3). The primary peak in these spectra was the G58C subunit c modified by NEM (8456 Da). The secondary peak at 8473 Da is most likely an oxidation product of the protein. The extent of protection by MTSET was determined by measuring the intensity of the peak corresponding to unmodified subunit c (8330 Da). In the presence of subunit a this unmodified peak accounted for 12% of the total subunit c in the experiment shown in Fig. 3A and an average of 10 ± 2% in three identical experiments. In similar experiments using membrane vesicles lacking subunit a, this unmodified peak disappeared suggesting that no c subunits were protected from NEM modification (Fig. 3B).

**FIGURE 2.** Sensitivity of Cys substitutions to inhibition by Ag+. Inhibition after treatment with 40 μM Ag+ was calculated from relative quenching values. Each determination is the average of ≥2 trials, and the error bars represent the S.D. Inhibition values for residues 52–65 were taken from Ref. 30. Cys substitutions at positions 18, 23, 29, 54, 55, and 61 abolished quenching activity. The two helices are aligned vertically based upon the WHATIF model from the crystal structure (8).

**FIGURE 3.** Modification of G58C with MTSET in the presence and absence of subunit a. Membrane vesicles containing subunit a (A) or lacking subunit a (B) were treated with 0.5 mM MTSET and, following solubilization with SDS, were then treated with 1 mM NEM as described under “Experimental Procedures.” The MTSET derivatization was then reversed by treatment with β-MSH to generate unmodified subunit c. Subunit c was purified from the SDS-solubilized membrane fraction as described previously (30) and then subjected to electrospray ionization quadrupole ion trap mass spectrometry. Peaks corresponding to unmodified subunit c at 8330 Da and NEM-modified subunit c at 8456 Da are labeled. In the experiment shown in A, unmodified subunit c accounts for 12% of the total subunit c in the experiment shown.

**Cd2+ Sensitivity of Cys Substitutions in Subunit c**—Cadmium has been used previously to probe channel gating in Shaker K+ channels.
channels and in the cystic fibrosis transmembrane conductance regulator (35, 36). Cd\(^{2+}\) has an ionic radius similar to that of Ag\(^{+}\) (0.95 and 1.15 Å, respectively), and Cd\(^{2+}\), like Ag\(^{+}\), is a soft Lewis acid that forms a covalent bond with the thiolate form of cysteine (37). We tested the sensitivity of the previously described Cys substitutions to inhibition by Cd\(^{2+}\) as an alternative probe of aqueous accessibility. The concentration dependence for Cd\(^{2+}\) inhibition for several substitutions is shown in Fig. 4. A concentration of 300 μM was selected as optimal in distinguishing degrees of sensitivity. Representative experiments for inhibition by 300 μM Cd\(^{2+}\) are shown in Fig. 5. In TMH1, only I28C was sensitive to treatment with Cd\(^{2+}\) as an alternative probe of aqueous accessibility. The concentration dependence for Cd\(^{2+}\) inhibition for several substitutions is shown in Fig. 4. A concentration of 300 μM was selected as optimal in distinguishing degrees of sensitivity. Representative experiments for inhibition by 300 μM Cd\(^{2+}\) are shown in Fig. 5. In TMH1, only I28C was sensitive to treatment with Cd\(^{2+}\). In TMH2, Q52C, M57C, G58C, L59C, and I63C were highly sensitive to the treatment, and A62C and P64C/A20P were moderately sensitive (Fig. 6). Wild type ATP-driven proton pumping was inhibited ~10% after treatment with 300 μM Cd\(^{2+}\) (Fig. 5).

The above results show that despite being of a size similar to Ag\(^{+}\), Cd\(^{2+}\) is a more selective probe with a more restricted pattern of sensitivity than that of Ag\(^{+}\). Angevine and Fillingame (25) reported that ~80% of Ag\(^{+}\) added in an inhibition assay associates with the membrane under typical experimental conditions. Because of its higher positive charge density, Cd\(^{2+}\) might not dissolve in the membrane as easily as Ag\(^{+}\) and thus might be more selective than Ag\(^{+}\). To measure the extent to which Cd\(^{2+}\) ions associate with the membrane, control membrane vesicles without Cys substitutions were treated with 300 μM Cd\(^{2+}\) under the same conditions as in an ATP-driven H\(^{+}\)-pumping assay. Membrane vesicles were separated from the buffer by centrifugation, washed, and resuspended in 2% SDS solution. Samples of the supernatant and solubilized membrane vesicles were subjected to inductively coupled plasma optical emission spectroscopy analysis. Under these conditions, 91% of the Cd\(^{2+}\) remained in the supernatant fraction with only 9% dissolving in the membrane.

![FIGURE 4. Concentration dependence of Cd\(^{2+}\) sensitivity. ATP-driven quenching of ACMA fluorescence by inverted membrane vesicles was assayed as described in Fig. 1. Inhibition by Cd\(^{2+}\) present at a range of concentrations was calculated from relative quenching values. The Cys substitutions shown are G58C (○), I28C (▲), M57C (●), A62C (●), A24C (□), and no Cys (◆).](image)

![FIGURE 5. Inhibition of ATP-driven proton pumping by Cd\(^{2+}\). ATP-driven quenching of ACMA fluorescence by inverted membrane vesicles was assayed as described in Fig. 1. The return to maximum fluorescence after the addition of nigericin (N) was used to calculate the inhibition values given in Fig. 6. The traces are representative of the effect of 300 μM Cd\(^{2+}\) on vesicles containing no Cys substitutions (A), the insensitive I26C substitution (B), the highly sensitive I28C substitution (C), and the moderately sensitive A20P/P64C substitution (D).](image)

![FIGURE 6. Sensitivity of Cys substitutions to inhibition by Cd\(^{2+}\). Inhibition after treatment with 300 μM Cd\(^{2+}\) was calculated from the relative quenching values. Each determination is the average of ≥2 trials, and the error bars represent the S.D.](image)

The experiments reported in Figs. 5 and 6 were done in HMK-Cl buffer, which is optimal for the ATPase-coupled ACMA quenching assay. Cd\(^{2+}\) does form soluble complexes with chloride ions in aqueous solutions such as the HMK buffer...
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used here. The soluble complexes reported include CdCl\(^+\), CdCl\(_2\), and CdCl\(_2^-\) (37), so we are uncertain that free Cd\(^{2+}\) is the sole inhibitory species in chloride-containing buffers. To test the possible role of chloride in Cd\(^{2+}\) inhibition, assays were also carried out in chloride-free HMK-NO\(_3\) buffer with a series of mutants (Table 2). The nonspecific inhibition of H\(^+\) pumping by wild type vesicles increased from 5% in HMK-Cl to 15% in HMK-NO\(_3\) using 300 \(\mu\)M Cd\(^{2+}\). The relative sensitivity of most Cys substitutions was not affected by the buffer system used (Table 2). However, the sensitivity of four substitutions to inhibition by 300 \(\mu\)M Cd\(^{2+}\) was markedly increased in the HMK-NO\(_3\) buffer versus the HMK-Cl buffer (i.e. A24C, A62C, A20P/P64C, and M65C). The depth of these four substitutions in the membrane is such that they lie at the border demarcating the Cd\(^{2+}\)-sensitive and -insensitive regions. The absence of chloride complexes might allow free Cd\(^{2+}\) to penetrate to these residues more efficiently. Using the formation constants for Cd\(^{2+}\)-chloride complexes reported in Ref. 37 we estimated that 92% of Cd\(^{2+}\) in HMK-Cl buffer is complexed with chloride with the majority as CdCl\(^+\) (47%) and CdCl\(_2\) (37%) with free Cd\(^{2+}\) representing only 8% of the total Cd\(^{2+}\) present.

**DISCUSSION**

In this study we extended a survey of aqueous accessibility in the transmembrane regions of subunit c by probing Cys substitutions for inhibition by the thiolate-reactive probes Ag\(^+\), Cd\(^{2+}\), NEM, and a series of MTS reagents. We demonstrated that the cytoplasmic half of TMH2 and region around Asp-61 show greatest aqueous accessibility as judged by the inhibition assay. Accessibility on the periplasmic half of TMH2 diminished as the Cys substitutions were moved from the center of the membrane to the periplasmic surface. These results support a model in which the outer helices of the c-ring form at least part of the cytoplasmic proton exit channel.

Of the 13 functional Cys substitutions in TMH1 that were probed with Ag\(^+\), inhibition was restricted to a pocket of four residues lying directly behind Asp-61 (Fig. 7A). Replacement of residue 20, 24, or 25 with Cys conferred sensitivity to inhibition by Ag\(^+\) but not by any of the other reagents we tested. The I28C replacement conferred sensitivity to Cd\(^{2+}\) as well as Ag\(^+\), whereas the other Ag\(^+\)-sensitive residues in TMH1 were unaffected by Cd\(^{2+}\). Ag\(^+\) could be accessing more sites because of its lower charge density or increased membrane permeability. Aqueous accessibility of these four residues is not unexpected because of their proximity to Asp-61. These residues seem to interact in a functional way as they pack in the aqueous pocket surrounding Asp-61. The essential Asp residue can be moved to position 24 with retention of function, suggesting that the side chains at positions 24 and 61 occupy a similar location (38). Ala-20 can be replaced with Pro to compensate for a non-functional P64A substitution in TMH2 with the wild type Pro-64 lying one helical turn toward the periplasm from Asp-61 (39). Mutations of Ile-28 prevent reaction of Asp-61 with dicyclohexylcarbodiimide suggesting that Ile-28 contributes to the dicyclohexylcarbodiimide binding pocket around Asp-61 (40, 41), and an I28E substitution can compensate for the replacement of Asp-61 with Gly (42). In the *I. tartaricus* subunit c, Ile-28 is replaced at an equivalent position by Gln-32, the side chain of which directly chelates with bound Na\(^+\) (8).

Our previous study showed that Ag\(^+\) reactivity with Cys substitutions in TMH2 is widespread, including the face of TMH2 that is

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**TABLE 2**

Comparison of Cd\(^{2+}\) sensitivity in HMK-Cl versus HMK-NO\(_3\) buffers

| Substitution | Inhibition of H\(^+\) pumping |
|--------------|-------------------------------|
|              | HMK-Cl | HMK-NO\(_3\) |
| No Cys       | 5      | 14      |
| A20C         | 6      | 13      |
| I22C         | 6      | 13      |
| A24C         | 13     | 50      |
| Q52C         | 80     | 89      |
| M57C         | 79     | 85      |
| V60C         | 7      | 21      |
| A62C         | 38     | 90      |
| I63C         | 94     | 95      |
| P64C\(^a\)   | 50     | 95      |
| M65C         | 35     | 57      |
| V68C         | 8      | 13      |
| G69C         | 13     | 23      |

\(^a\) Constructed with an A20P suppressor mutation.

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**FIGURE 7. Structural modeling of the Ag\(^+\)- and Cd\(^{2+}\)-sensitive residues in the c-ring.** Sensitive residues are highlighted on a model of the *E. coli* c-ring derived from the x-ray structure of the c-ring from *I. tartaricus* using the program WHATIF (8). A, TMH2 is shown as a ribbon to reveal the surface of TMH1 that interacts with TMH2. Asp-61 in one of the c subunits is colored cyan. The positions of Ag\(^+\)-sensitive residues in TMH1 of the same c subunit are highlighted in red. B, the outer ring of helices is shown in a surface representation. Asp-61 is colored cyan. The positions of Cd\(^{2+}\)-sensitive residues showing >75% inhibition are highlighted in red where the S2', S9', and 63' numbers indicate residues packing next to Asp-61 from an adjacent subunit in the c-ring. The vertical positions of Arg-50, Phe-54, Tyr-73, and Met-75 are indicated.
thought to be less accessible because of its interaction with TMH1. We suggested that this extensive reactivity might indicate that this helix is dynamic at least over the time course of the experiment. We would not expect the same mobility in TMH1 because the inner helices of the c-ring are thought to pack tightly against neighboring subunits. The I30C substitutions at the center of the ring can be cross-linked to each other without loss of function, arguing against any functional necessity for mobility of this helix (6). Moreover the lack of extensive reactivity of Cys substitutions outside the pocket surrounding Asp-61 would suggest that TMH1 does not contribute significantly to an aqueous pathway to the cytoplasmic surface of the c-ring.

Ag⁺ has been used as a probe of the aqueous accessibility of Cys substitutions in a Shaker K⁺ channel (24). The Ag⁺-sensitive residues were more recently mapped onto an x-ray structure of the Kv1.2 K⁺ channel (43).³ With the exception of three bulky residues (Trp-362, Trp-363, and Leu-371) that were partially buried and not fully solvent-accessible, the Ag⁺-sensitive residues at eight other positions were solvent-exposed, and the Ag⁺-insensitive positions were buried within the protein. These results support the use of Ag⁺ as a probe for aqueous accessible regions within transmembrane proteins and reinforce the argument that the Ag⁺-sensitive substitutions in the transmembrane regions of subunit c are likely to lie in aqueous accessible positions.

To confirm and refine the pattern of aqueous accessibility in the c-ring, we surveyed Cys reactivity in transmembrane regions of subunit c using Cd²⁺. Cd²⁺ has an ionic radius close to that of Ag⁺ and H₂O⁺ but proved to be more selective.⁴ This selectivity may be in part due to the decreased association of Cd²⁺ with the membrane versus that previously seen with Ag⁺. That is, under the conditions used here, most of the Cd²⁺ ions remain in aqueous solution and do not partition into the membrane, whereas most of the Ag⁺ ions associate with the membrane under equivalent conditions (25). The presence of chloride in the ATP-driven H⁺ pumping assay may have also contributed to the selectivity of Cd²⁺. Cd²⁺ is known to form soluble complexes with chloride (37) that would effectively increase the size of the ion and prevent access to narrower cavities. Free Cd²⁺ may be able to penetrate farther into the membrane explaining the increased sensitivity of the A24C, A62C, P64C, and M65C substitutions at the border of the Cd²⁺-sensitive pocket when assayed in chloride-free buffer. Some of the chloride complexes might also be less inhibitory because of charge neutralization.

The pattern of cadmium sensitivity in subunit c confirms that the cytoplasmic half of TMH2 is the most accessible membrane-embedded region of the subunit (Fig. 7B). The Cd²⁺ inhibition pattern is similar to that of MTSEA reported previously (30) where MTSEA-sensitive residues formed a pocket around Asp-61. This pocket includes residues 57, 58, 59, 62, 63, 64, and 65. The Cd²⁺ inhibition pattern is more restricted and includes primarily residues 57, 58, and 59 on the cytoplasmic side of Asp-61.

FIGURE 8. Reagent accessibility at the interface of subunits a and c. The interface of subunit a and subunit c is shown where subunit a is represented by four cylinders corresponding to TMHs 2–5 with the subunit c monomer represented by a model derived from the x-ray structure of the c-ring from l. tartaricus using the program WHATIF (8). The vertical placement of the subunits relative to one another is based on cross-linking between cTMH2 and aTMHs 4–5 (20, 21). Overlapping colored regions indicate sensitivity to Ag⁺ (green), Cd²⁺ (yellow), MTSEA (red), and NEM (purple).

We show here that the membrane-impermeant probe MTSET has access from the cytoplasm to G58C in the region of subunit c that is sensitive to Cd²⁺ and MTSEA. MTSET modified one in 10 subunits of the c-ring, a stoichiometry consistent with that of NEM modification measured previously (30). MTSET cannot access this membrane-embedded region of subunit c in the absence of subunit a. The simplest explanation for the limited stoichiometry of modification and the requirement of subunit a for modification is that subunit a mediates aqueous access from the cytoplasm to the Cd²⁺- and MTSEA-sensitive face of cTMH2. This face of cTMH2 most likely packs against the Ag⁺-sensitive residues on the cytoplasmic side of TMH4 of subunit a (Fig. 8). This peripheral surface of aTMH4 was shown to cross-link to the peripheral surface of the c-ring (20). Further the three NEM-sensitive residues in these subunits (cG58C, aS206C, and aN214C) would pack at this interface (25, 30) as would all of the residues in either subunit showing sensitivity to both Cd²⁺ and MTSEA (30). The reactivity pattern for all reagents shows that cTMH2 is more accessible than cTMH1 and provides further evidence for a proton exit pathway to the cytoplasm that is likely formed at the interface of TMH2 in subunit c and the peripheral surface of TMH4 in subunit a.

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³ C. Miller, personal communication.
⁴ Ionic radii are: Ag⁺, 1.15 Å; Cd²⁺, 0.95 Å; and H₂O⁺, 1.41 Å.
⁵ H. Dong and R. H. Fillingame, manuscript in preparation.
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