Chemical Reactivities of Cysteine Substitutions in Subunit \(a\) of ATP Synthase Define Residues Gating \(H^+\) Transport from Each Side of the Membrane*

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Subunit \(a\) plays a key role in coupling \(H^+\) transport to rotations of the subunit c-ring in \(F_1F_0\) ATP synthase. In \textit{Escherichia coli}, \(H^+\) binding and release occur at Asp-61 in the middle of the second transmembrane helix (TMH) of \(F_0\) subunit \(c\). Based upon the \(A^+\) sensitivity of Cys substituted into subunit \(a\), \(H^+\) are thought to reach Asp-61 via aqueous pathways mapping to surfaces of TMH 2–5. In this study we have extended characterization of the most \(A^+\)-sensitive residues in subunit \(a\) with cysteine reactive methanethiosulfonate (MTS) reagents and Cd\(^{2+}\). The effect of these reagents on ATPase-coupled \(H^+\) transport was measured using inside-out membrane vesicles. Cd\(^{2+}\) inhibited the activity of all \(A^+\)-sensitive Cys on the cytoplasmic side of the TMHs, and three of these substitutions were also sensitive to inhibition by MTS reagents. On the other hand, Cd\(^{2+}\) did not inhibit the activities of substitutions at residues 119 and 120 on the periplasmic side of TMH2, and residues 214 and 215 in TMH4 and 252 in TMH5 at the center of the membrane. When inside-out membrane vesicles from each of these substitutions were sonicated during Cd\(^{2+}\) treatment to expose the periplasmic surface, the ATPase-coupled \(H^+\) transport activity was strongly inhibited. The periplasmic access to N214C and Q252C, and their positioning in the protein at the a-c interface, is consistent with previous proposals that these residues may be involved in gating \(H^+\) access from the periplasmic half-channel to Asp-61 during the protonation step.

The \(H^+\)-transporting \(F_1F_0\) ATP synthases of oxidative phosphorylation utilize the energy of a transmembrane electrochemical gradient of \(H^+\) or \(Na^+\) to mechanically drive the synthesis of ATP via two coupled rotary motors in the \(F_1\) and \(F_0\) sectors of the enzyme (1–4). In the intact enzyme, \(H^+\) transport through the transmembrane \(F_0\) sector is coupled to ATP synthesis or hydrolysis in the \(F_1\) sector at the surface of the membrane. Homologous enzymes are found in mitochondria, chloroplasts, and many bacteria (5). In \textit{Escherichia coli} and other eubacteria, \(F_1\) consists of five subunits in an \(\alpha_3\beta_3\gamma\delta\epsilon\) stoichiometry. \(F_1\) is composed of three subunits in a likely ratio of \(a,b,c_{10}\) in \textit{E. coli} and \textit{Bacillus subtilis} PS3 (6, 7), \(a,b,c_{11}\) in the \(Na^+\) translocating \textit{Ilyobacter tartaricus} ATP synthase (4, 8), and as many as 15 \(c\) subunits in other bacterial species (9). Subunit \(c\) spans the membrane as a helical hairpin with the first transmembrane helix (TMH)\(^c\) on the inside and the second TMH on the outside of the c-ring (8–10). The high resolution x-ray structure of the \textit{I. tartaricus} \(c_{11}\)-ring revealed the sodium binding site at the periphery of the ring with chelating groups to the \(Na^+\) extending from two interacting subunits in an extensive hydrogen bonding network (8, 11). The essential \textit{I. tartaricus} Glu-65 in the \(Na^+\)-chelating site corresponds to \textit{E. coli} Asp-61. Subsequently published structures of \(H^+\) binding c-rings from chloroplasts (12), \textit{Spirulina platensis} (9), and \textit{Bacillus pseudofirmus} (13) have revealed at least two modes of liganding the proton at the essential side chain carboxyl using alternative hydrogen-bonding networks (13).

In the \(H^+\)-transporting \textit{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 \(a\) (1–4, 14). In the complete membraneous enzyme, the rotation of the c-ring is proposed to be driven by \(H^+\) transport at the subunit a/c interface, with ring movement then driving rotation of subunit \(\gamma\) within the \(\alpha_3\beta_3\) hexamer of \(F_1\) to cause conformational changes in the catalytic sites leading to synthesis and release of ATP (1–4). Subunit \(a\) folds in the membrane with 5 TMHs and is thought to provide access channels to the proton-binding Asp-61 residue in the c-ring (14–17). Interaction of the conserved Arg-210 residue in \(a\) TMH4 with cTMH2 is thought to be critical during the deprotonation-protonation cycle of \(c\)-Asp (18–20), and \(a\) TMH4 and cTMH2 are known to pack in parallel to each other based upon cross-linking (21).

Previously, we probed Cys residues introduced into the 5 TMHs of subunit \(a\) for aqueous accessibility based upon their reactivity with NEM and Ag\(^{+}\) (22–25). Both reagents react preferentially with the thiolate form of the Cys side chain that is expected in a polar, aqueous environment. Two regions of aqueous access were found with distinctly different properties. One region extending from Asn-214 and Arg-210 near the center of the membrane to the cytoplasmic surface of

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2 The abbreviations used are: TMH, transmembrane helix; ACMA, 9-amino-6-chloro-2-methoxycacididine; \(\beta\)MSH, \(\beta\)-mercaptoethanol; MTS, methanethiosulfonate; MTSN, MTS-methyl; MTSEA, MTS-ethylamino; MTSET, MTS-ethyl(trimethylammonium); MTSC, MTS-carboxylethyl; MTSES, MTS-ethylsulfonate; NEM, \(N\)-ethylmaleimide.
TMH4 contains Cys residues that are sensitive to inhibition by both NEM and Ag⁺ (Fig. 1). A second set of Ag⁺-sensitive but NEM-inaccessible residues mapped to the opposite face and periplasmic side of TMH4. Ag⁺-sensitive and NEM-insensitive Cys residues were also found in TMHs 2, 3, and 5, extending from the center of the membrane to the periplasm. The Ag⁺-/NEM-insensitive residues in TMHs 2, 3, 4, and 5 cluster at the interior of the four-helix bundle predicted by cross-linking (Ref. 26 and Fig. 2), 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 probed the reactivity of the most Ag⁺-sensitive Cys residues in subunit a with Cd²⁺ and methanethiosulfonate (MTS) reagents of varying polarity (see Fig. 1 for location). MTS reagent reactivity was confined to 3 Cys residues in TMH4 that are thought to line the aqueous access pathway to the cytoplasm, i.e. N214C, S206C, and S202C. The Cd²⁺ reactivity of inverted membrane vesicles was confined to Cys localized to the cytoplasmic side of subunit a. Cd²⁺-insensitive but Ag⁺-sensitive residues were found to cluster at the center of the proposed four-helix bundle extending to the periplasm (Fig. 2). However, on sonication of the inverted membrane vesicles, these residues became Cd²⁺-sensitive, which supports the proposed aqueous accessibility to this region of the protein from the periplasmic face of the membrane. The residues made accessible by sonication include N214C and Q252C, which pack at the interfaces of TMH4 and TMH5 and the surface of the c-ring. Swiveling of helices at this interface was proposed to gate H⁺ access to cAsp-61 during H⁺-transport-driven ATP synthesis (27). We suggest that these two Cd²⁺-sensitive residues may define the gate of H⁺ access from the periplasmic half-channel to cAsp-61 and then to the second half-channel to the cytoplasm.

EXPERIMENTAL PROCEDURES

Cys-substituted Mutants—The cysteine substitutions used here were constructed in plasmid pCMA113 (22), which encodes a subunit a with a hexahistidine tag on the C terminus and genes encoding the other subunits of F1Fo in which all endogenous Cys had been substituted by Ala or Ser (28). Properties of the substitutions were described previously (22–25). Three new substitutions in the Ser-202 codon were generated here using a two-step PCR protocol with a synthetic oligonucleotide encoding the codon change and two wild-type primers (29). PCR products were transferred into pCMA113 directly using unique HindIII (870) and BsrGI (1913) sites (see Ref. 30) for nucleotide numbering). The new 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 (6).

Membrane Preparation—JWP292 transformant strains were grown in M63 minimal medium containing 0.6% glu-
cose, 2 mg/liter thiamine, 0.2 mM uracil, 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 (6). Cells were suspended in TMG-acetate buffer (50 mM Tris acetate, 5 mM magnesium acetate, 10% glycerol, pH 7.5) containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml of DNase I and disrupted by passage through a French press at 1.38 \times 10^8 \text{ nt/m}^2 and membranes prepared as described (31). Cell debris was cleared by low-speed centrifugation of the lysate at 7,700 \times g_{\text{max}} and membranes were collected by high-speed centrifugation of the cleared lysate at 193,000 \times g_{\text{max}}. Membranes were washed once with and then resuspended in TMG-acetate and stored at −80 °C. Protein concentrations were determined using a modified Lowry assay (32).

**ATP-driven Quenching of ACMA Fluorescence**—Aliquots of membranes prepared from JWP292 transformant strains (1.6 mg at 10 mg/ml) were suspended in 3.2 ml of HMK-nitrate buffer (10 mM HEPES-KOH, 1 mM Mg(NO₃)₂, 10 mM KNO₃, pH 7.5) or HMK-chloride buffer (10 mM HEPES-KOH, 5 mM MgCl₂, 300 mM KCl, pH 7.5) and incubated at room temperature for 10 min. ACMA was added to 0.3 μg/ml and fluorescence quenching was initiated by the addition of 30 μl 25 mM ATP, pH 7. Each experiment was terminated by adding nigericin to 0.5 μg/ml. The level of fluorescence obtained after addition of nigericin was used as a normalized 100% baseline in calculating the percent quenching due to ATP-driven proton pumping. For NEM treatment, 160 μl aliquots of 10 mg/ml membrane in TMG-acetate buffer were treated with 5 mM NEM for 10 min at room temperature and then diluted into HMK-nitrate buffer before carrying out the quenching assay. The 10-min incubation of untreated control samples resulted in no significant inhibition relative to samples that were not pre-incubated. The HMK-nitrate buffer is that used in previous experiments with Ag⁺. Treatments with MTS derivatives were performed as for NEM. The MTS compounds were dissolved immediately before use in DMSO solvent at 100 mM and 3.2 μl of reagent mixed with 160 μl of 10 mg/ml membrane in TMG-acetate buffer. After a 10-min incubation at room temperature, the treated membranes were diluted into 3.2 ml HMK-nitrate buffer, and the quenching reaction was carried out as described above. For treatment with Cd²⁺, 160 μl of 10 mg/ml membrane was suspended in 3.2 ml of HMK-chloride buffer. Following an incubation of 10 min at room temperature, the fluorescence quenching reaction was carried out as described above. HMK-chloride buffer was used in these experiments since the ATP-driven ACMA quenching is more robust in this buffer than in HMK-nitrate buffer.

**Sonication of Cd²⁺-treated Membrane Vesicles**—An aliquot of 160 μl of inverted membrane vesicles at 10 mg/ml was diluted into 3.2 ml of HMK-chloride buffer and 3.3 μl of 1 M CdCl₂ added to a final concentration of 1 mM. The sample was then transferred to an ice-water bath and sonicated for 1 min at 80% of maximum power using an Ultrasonics Sonicator Cell Disruptor with a 1.3-cm diameter tip. Following sonication, the membrane vesicles were incubated for 10 min at 20 °C before initiating the quenching assay. The same sonication procedure carried out with control membrane vesicles lacking Cd²⁺ led to negligible effects on their quenching activity, i.e. 90 ± 10% of quenching response of vesicles not subjected to sonication.
that NEM and MTSM are not inhibitory whereas the poten-
disruptive. The inhibition pattern seen with S202C differs in
a protonatable and potentially positively charged group at
not significantly affected by these reagents. The inhibition
NEM reagents, whereas the S202C quenching response was
ions were inhibited significantly by the neutral MTSM and
MTSEA reagent, which, when protonated, would be cationic.
in Fig. 3 was nearly abolished by the membrane-permeant
ACMA quenching response of all three substitutions shown
tested,
most significantly inhibited by two of the MTS reagents
Table 1
Gating Residues in the H⁺ Half-channels of Subunit α

| Mutation and location | +Ag/
|-----------------------|-------|
|                       | Ag⁹ |  | +MTSM/
|                       |  |  | MTSM⁹ | +MTSEA/
|                       |  |  | MTSEA⁹ | +MTSET/
|                       |  |  | MTSET⁹ | +MTSES/
|                       |  |  | MTSES⁹ | +MTSCE/
|                       |  |  | MTSCE⁹ |
| WT                    | 0.90 ± 0.05  | 0.94 ± 0.04  | 0.88 ± 0.06  | 0.95 ± 0.07  | 0.94 ± 0.05  | 0.95 ± 0.07 |
| 1-2 loop              | M93C | 0.12 ± 0.00  | 0.98 ± 0.04  | 0.91 ± 0.05  | 0.97 ± 0.04  | 0.96 ± 0.01  | 0.96 ± 0.01 |
| TMH 2                 | D119C | 0.07 ± 0.01  | 1.06 ± 0.10  | 0.97 ± 0.05  | 1.00 ± 0.06  | 0.99 ± 0.03  | 0.98 ± 0.03 |
|                       | L120C | 0.08 ± 0.01  | 1.00 ± 0.04  | 0.98 ± 0.03  | 0.94 ± 0.08  | 1.01 ± 0.03  | 1.00 ± 0.03 |
| 3-4 loop & TMH4       | S199C | 0.13 ± 0.01  | 0.99 ± 0.12  | 0.97 ± 0.13  | 0.96 ± 0.06  | 0.98 ± 0.04  | 0.98 ± 0.04 |
|                       | S202C | 0.06 ± 0.00  | 0.96 ± 0.05  | 0.20 ± 0.05  | 0.99 ± 0.01  | 0.99 ± 0.01  | 0.99 ± 0.04 |
|                       | K203C | 0.03 ± 0.03  | 0.96 ± 0.05  | 0.99 ± 0.01  | 1.00 ± 0.00  | 0.98 ± 0.03  | 1.00 ± 0.01 |
|                       | S206C | 0.05 ± 0.03  | 0.60 ± 0.04  | 0.27 ± 0.10  | 0.98 ± 0.03  | 0.98 ± 0.03  | 0.95 ± 0.07 |
|                       | N214C | 0.09 ± 0.04  | 0.48 ± 0.11  | 0.36 ± 0.16  | 0.93 ± 0.08  | 0.94 ± 0.09  | 0.90 ± 0.14 |
|                       | M215C | 0.05 ± 0.01  | 0.90 ± 0.06  | 0.91 ± 0.11  | 0.97 ± 0.06  | 0.96 ± 0.08  | 0.95 ± 0.08 |
| TMH 5                 | I249C | 0.07 ± 0.03  | 0.87 ± 0.13  | 0.88 ± 0.14  | 1.00 ± 0.03  | 0.93 ± 0.08  | 0.98 ± 0.03 |
|                       | Q252C | 0.09 ± 0.01  | 0.96 ± 0.04  | 0.91 ± 0.03  | 0.97 ± 0.05  | 1.00 ± 0.00  | 0.94 ± 0.08 |
| C terminus            | Y263C | 0.11 ± 0.00  | 0.82 ± 0.05  | 0.80 ± 0.00  | 0.74 ± 0.01  | 0.73 ± 0.11  | 0.81 ± 0.08 |

* The results presented are the average of two or more determinations ± S.D.
* The +Ag/Ag ratios are taken from Ref. 25.
* MTS reagents were added at a concentration of 2 mM.

RESULTS

Testing of Ag⁺-sensitive Residues for Inhibition by Other Cysteine Reactive Reagents—We initially did a survey of the most Ag⁺-sensitive residues in subunit α to determine their susceptibility to inhibition of the ATP-driven ACMA quenching response by five MTS reagents of varying size and charge (Fig. 1). Of the residues tested, three Cys substitutions were most significantly inhibited by two of the MTS reagents tested, i.e. S202C, S206C and N214C with MTSM and MTSEA (Table 1). The three MTSM or MTSEA sensitive substitutions were insensitive to inhibition by the anionic MTSES and MTSCE reagents or the membrane impermeant and cationic MTSET reagent as were the other nine Cys substitutions tested (Table 1). A comparison of the inhibition seen with select reagents and the S202C, S206C, and S214C substitutions is shown in Fig. 3. Two of these substitutions corre-
respond to the two NEM-sensitive residues in subunit α, and
NEM was included in these experiments for comparison. The ACMA quenching response of all three substitutions shown in Fig. 3 was nearly abolished by the membrane-permeant MTSEA reagent, which, when protonated, would be cationic. The quenching response of the S206C and N214C substitutions were inhibited significantly by the neutral MTSM and NEM reagents, whereas the S202C quenching response was not significantly affected by these reagents. The inhibition seen with the S206C and N214C substitutions could be attributed to modifications that increase the bulk of the amino acid side chain or reduce its polarity. However, the introduction of a protonatable and potentially positively charged group at these positions by modification with MTSEA is clearly more disruptive. The inhibition pattern seen with S202C differs in that NEM and MTSM are not inhibitory whereas the potentially cationic MTSEA is very inhibitory. A polar side chain at

* We were not able to confirm the high Ag⁺ sensitivity of the L195C and V198C substitutions reported previously (25) and did not include these substitutions in this study.

4 The reaction of [³⁵C]NEM with many of the residues listed in Table 1 has been reported previously and normalized to the most NEM-reactive resi-
due S206C (22, 23). The reactivity of additional Cys substitutions with
³⁵C]NEM is reported in Angervin, C.A., Ph.D Thesis, University of Wiscon-
sin, Madison, 2004. These residues include: V198C (10%), S199C (100%), L200C (29%), L201C (14%), S202C (61%), K203C (72%), P204C (5%), and V205C (9%), where the relative reactivity is normalized to that of S206C at 100%.
N214C mutant. The Cd\(^{2+}\) sensitivity of the TMH Cys substitutions surveyed in this study is compared in Fig. 7. About half of the mutants were inhibited significantly by Cd\(^{2+}\) whereas the other half were as resistant or only slightly more sensitive to inhibition than wild type. The M93C substitution proved to be Cd\(^{2+}\)-insensitive (data not shown).

Ag\(^{+}\)-sensitive but Cd\(^{2+}\)-insensitive Residues Locate to the Periplasmic Side of the Membrane—In the current topographical models of subunit \(a\), the Ag\(^{+}\)-sensitive but Cd\(^{2+}\)-insensitive D119C and L120C substitutions are located on the periplasmic side of TMH2 and would be most easily accessed from the aqueous compartment at the interior of the inverted membrane vesicles used here (Fig. 1). To test whether the Cd\(^{2+}\) insensitivity of these residues might be due to Cd\(^{2+}\) impermeability to the interior aqueous compartment, we disrupted L120C membrane vesicles by sonication during the course of Cd\(^{2+}\) treatment. The sonication treatment led to a marked increase in the Cd\(^{2+}\) inhibition of ATP-driven ACMA quenching as shown in Fig. 8. Similar increases in Cd\(^{2+}\) sensitivity were seen on sonication of M215C and Q252C membrane vesicles that have substitutions in TMH4 and TMH5 respectively (Fig. 9). The Cd\(^{2+}\) sensitivity of D119C and N214C membrane vesicles was also markedly increased by

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**FIGURE 3.** Inhibition of ATP-driven proton pumping by NEM, MTSM, and MTSET with membrane vesicles from the S202C, S206C, and N214C substitutions. ATP-driven quenching of ACMA fluorescence by inverted membrane vesicles was assayed as described under “Experimental Procedures” using HMK-nitrate buffer. Fluorescence quenching was initiated by the addition of ATP at 20 s and terminated by the addition of nigericin at 100 s. The return to the maximum fluorescence reached after the addition of nigericin was used to calculate the relative inhibition of quenching. Fluorescence quenching by mutant vesicles treated with DMSO (control), 5 mM NEM, 2 mM MTSM, and 2 mM MTSEA.

**FIGURE 4.** ATP-driven proton pumping activity with different substitutions in Ser-202 of subunit \(a\). The activity of the S202A, S202I, S202M, and S202K substitutions are compared with wild type.

**FIGURE 5.** Inhibition of ATP-driven proton pumping by Cd\(^{2+}\) with S206C-substituted subunit \(a\). ATP-driven quenching of ACMA fluorescence was measured as described under “Experimental Procedures” using HMK-chloride buffer. Membrane vesicles were treated with varying concentrations of CdCl\(_2\) for 10 min at room temperature prior to initiation of the assay with ATP. The return of the fluorescent signal to the maximum reached after the addition of nigericin was used to calculate the inhibition values given in Fig. 6. The activity and extent of Cd\(^{2+}\) inhibition of wild-type (\(A\)) and S206C (\(B\)) membrane vesicles are compared.
sonication during Cd\textsuperscript{2+} treatment (Fig. 10). Of the Ag\textsuperscript{+}-sensitive but Cd\textsuperscript{2+}-insensitive substitutions identified in Fig. 7, I\textsubscript{249}C membrane vesicles were the only set where simultaneous sonication during Cd\textsuperscript{2+} treatment did not increase the inhibition of the ACMA quenching response (Fig. 10).

Probing the Periplasmic Exposure of His-245 with Cd\textsuperscript{2+}—His-245 is likely to pack at the center of the helical bundle mediating aqueous access from the periplasm and perhaps serve as a buffer to the protons entering the half-channel (33). The H245C substitution results in an inactive enzyme, which led us to probe the Ag\textsuperscript{+} sensitivity of H245C in the active D119H/H245C suppressor strain, where we found the ACMA quenching response to be only modestly sensitive to inhibition by Ag\textsuperscript{+} (23). To test possible access to H245C from the periplasm, we compared the effects of Cd\textsuperscript{2+} in sonicated and unsonicated inverted membrane vesicles. As shown in Fig. 11A, Cd\textsuperscript{2+} treatment of sonicated membrane vesicles resulted in an ~40% inhibition of the ACMA quenching response. In contrast, the initial rate of ATP-driven ACMA quenching by unsonicated membrane vesicles was little affected by Cd\textsuperscript{2+} treatment (Fig. 11B). However, as the ATP-driven ACMA quenching reaction progressed beyond 20 s, gradual inhibition ensued (Fig. 11B). Because these membrane vesicles had been incubated in HMK-chloride buffer with 1 mM Cd\textsuperscript{2+} for 10 min prior to the addition of ATP, we postulate that the gradual access of Cd\textsuperscript{2+} to H245C during enzyme function must result from dynamic movements that enhance aqueous access from the cytoplasmic side of the membrane.

**DISCUSSION**

Only three of twelve of the most Ag\textsuperscript{+}-sensitive Cys substitutions in subunit \textit{a} proved to be sensitive to any of the MTS reagents tested, as assessed by inhibition of ATP-driven ACMA quenching activity. Inhibition was only observed with the smallest of the reagents tested, i.e. MTSM and MTSEA, and then with only the S\textsubscript{202}C, S\textsubscript{206}C, and N\textsubscript{214}C substitutions. The size or possibly the charge of the groups on MTSET, MTSCE, and MTSES may prevent access to any of the twelve Cys substitutions tested in Table 1. It is unclear whether MTSM and MTSET have access to the nine substitutions that are insensitive to inhibition by these reagents. Five of these substitutions, i.e. D\textsubscript{119}C, L\textsubscript{120}C, M\textsubscript{215}C, I\textsubscript{249}C, and Q\textsubscript{252}C, are not accessible to labeling with \textsuperscript{14}C]NEM (22, 23), so there may be a correlation between NEM and MTS-reagent accessibility. On the other hand, the S199C, S202C, and S203C are all labeled by \textsuperscript{14}C]NEM, although NEM is not inhibitory (23, 25). It is thus possible that some of the Cys sub-

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**FIGURE 6.** Concentration dependence of Cd\textsuperscript{2+} inhibition of ATP-driven proton pumping with different Cys substitutions in subunit \textit{a}. The percent inhibition of ATP-driven proton pumping was calculated as described in Fig. 5.

**FIGURE 7.** Relative inhibition of ATP-driven proton pumping by Cd\textsuperscript{2+} with Cys substitutions in TMHs 2, 4, and 5 of subunit \textit{a}. Membrane vesicles were treated with 1 mM Cd\textsuperscript{2+} and the extent of inhibition calculated as described in Fig. 5. The position of the substituted residues in a two dimensional model of subunit \textit{a} is indicated in Fig. 1.

**FIGURE 8.** Effect of sonication during Cd\textsuperscript{2+} treatment on inhibition of ATP-driven proton pumping with L120C membrane vesicles. Inverted membrane vesicles were treated or not treated with 1 mM Cd\textsuperscript{2+} and subjected or not subjected to 1 min of sonication as described under “Experimental Procedures.”
Institutions studied here are modified by the MTS reagents and that the modification is not inhibitory. For S202C, S206C, and N214C, the greater sensitivity to inhibition by MTSEA versus MTSM can probably be attributed to the disruptive effects caused by a positively charged amine introduced into these transmembrane positions. For S202C this conclusion is supported by the mutagenesis studies where the S202K substitution was much more disruptive than the S202M or S202I substitutions. In the case of S206C, the disruption of function by MTSM and NEM can be attributed at least partially to introduction of a bulky group at this position. This conclusion is supported by mutagenesis studies where the S206A substitution is non-disruptive (34), whereas the S206L substitution nearly abolishes function (35). The size of the group introduced at N214C also appears to be a critical factor in inhibition of function in that the N214L substitution is much more debilitating than the N214V substitution (36).

The three residues showing MTS reagent sensitivity, i.e. S202C, S206C, and N214C, are thought to pack at the interface of a TMH4 and the peripheral surface of cTMH2 of the c-ring. The rate of reaction of MTS reagents with ionized thiolates is 100 times that of reaction with a neutral thiol (37), so it is very likely that the reactive Cys residues report an aqueous accessible space in this transmembrane region of the protein. S206C and N214C are the only two substitutions in subunit a that are sensitive to major inhibition by NEM and, in models derived from cross-linking evidence and the c-ring x-ray structures, would pack around the MTSEA-sensitive pocket in subunit c that surrounds Asp-61 (38). The only Cys substitution in subunit c that is inhibited by NEM is cG58C, and it also packs in this pocket (38).

The activity of all Ag⁺-sensitive Cys substitutions mapping toward the cytoplasmic side of aTMH4 and aTMH5 was inhibited by treatment with CdCl₂ (Fig. 1). Cd²⁺-sensitive residues 199, 202, 203, and 263 are likely to cluster at the cytoplasmic surface of TMHs 4 and 5 where they could interact with the Cd²⁺-sensitive cQ52C substitution in subunit c (39). In contrast to these residues, the activity of Cys substitutions at positions 119, 120, 214, 215, and 252 was not inhibited by Cd²⁺. Each of these substitutions localize more toward the center or periplasmic side of the membrane. For each of these
substitutions, sonication of inside-out membrane vesicles during Cd\(^{2+}\) treatment to expose the periplasmic surface resulted in strong inhibition of ATP-driven ACMA quenching activity. We conclude that Cd\(^{2+}\) cannot permeate the *E. coli* inner membrane and that the permeability barrier to Cd\(^{2+}\) explains the lack of inhibition seen with the 119, 120, 214, 215, and 252 substitutions in the absence of sonication. Residues 206 and 214 would pack directly above and below the essential Arg-210 residue in an \(\alpha\)-helical model of TMH4 (Fig. 1). Based upon these results we predict that Ser-206 provides aqueous access to the half-channel extending to the cytoplasm and that Asn-214 is accessed from the periplasmic half-channel at the interior of the four-helix bundle formed by TMHs 2–4 (Figs. 1 and 2).

The accessibility of residues 214 and 252 from the periplasm, and their positioning in the protein at the interface of the c-ring (Fig. 2), is consistent with a previous proposal that these residues may be involved in gating H\(^{+}\) access from the periplasmic half-channel to cAsp-61 during the protonation step (27). Based upon previous cross-linking experiments, gating was proposed to occur via helical swiveling of both \(\alpha\)TMH4 and \(\alpha\)TMH5, with simultaneous movement of Asn-214 and Gln-252 toward the c-ring interface, and with the coordinated movement of Arg-210 away from a deprotonated c-subunit to facilitate its reprotonation from periplasmic half-channel. The effects of sonication reported here on Cd\(^{2+}\) inhibition support the idea of aqueous access from the periplasm to not only residues Asn-214 and Gln-252, but also to Asp-119, Leu-120, Met-215, and His-245 in the predicted periplasmic half-channel at the interior of the four-helix bundle formed by TMHs 2–4 (Figs. 1 and 2).

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