The role of subunit α in proton translocation by the
Escherichia coli F1F0, ATP synthase is poorly under-
stood. In the membrane-bound F0 sector of the enzyme, the H+ binding and release occurs at Asp61 in the middle of the second transmembrane helix (TMH) of subunit c. Protons are thought to reach Asp61 via an aqueous access pathway formed at least in part by one or more of the five TMHs of subunit α. In this report, we have substituted Cys into a 19-residue span of the fourth TMH of subunit α and used chemical modification to obtain information about the aqueous accessibility of residues along this helix. Residues 206, 210, and 214 are N-ethylmaleimide-accessible from the cytoplasmic side of the membrane and may lie on the H+ transport route. Residues 215 and 218 on TMH4, as well as residue 245 on TMH5, are Ag+-accessible but N-ethylmaleimide-inac-
cessible and may form part of an aqueous pocket extending from Asp61 of subunit c to the periplasmic surface.

H+ -transporting F1F0, ATP synthases consist of two structurally and functionally distinct sectors termed F1 and F0 (1). In the intact enzyme, ATP synthesis or hydrolysis takes place in the F1 sector and is coupled to active H+ transport through the F0 sector. Structurally similar F1F0 ATP synthases are present in mitochondria, chloroplasts, and most eubacteria (1). The F1 sector lies at the surface of the membrane and in Escherichia coli consists of five subunits in an α3β3γδε;f1 stoichiometry. The F0 sector spans the membrane and in E. coli consists of three subunits in an α,b,c;ε,τ,θ;ε,τ,ε,τ,θ;ε,τ,ε,τ,θ stoichiometry (2). The structures of several types of F1 have been solved by x-ray crystallography (3–8). In the case of the bovine mitochondrial enzyme crystallized in the presence of Mg2+ and adenosine phosphates, the three α and three β subunits alternate around the central γ subunit, with subunit γ interacting asymmetrically with the three catalytic sites formed at the αβ interface (3–5). In the widely accepted binding change mechanism for ATP synthesis, the alternate tight binding of ADP and P to subsequent release of product ATP are mediated by γ subunit rotation between the alternating catalytic sites (9–11). Rotation of the γ subunit during ATP hydrolysis was demonstrated by attaching an actin filament to an immobilized αβγ complex (12, 13). In the complete membranous enzyme, the rotation of subunit γ is proposed to be driven by H+ transport-coupled rotation of a connected ring of c subunits in the Fc sector of the enzyme, which extend through the lipid bilayer and maintain a fixed linkage with the γ subunit. Rotation of the c ring was also demonstrated using the filament rotation assay (14, 15). The structure of monomeric subunit c has been solved by NMR in a membrane mimetic solvent mixture (16), and the structure of the oligomeric c10 ring was predicted from this structure and cross-linking constraints (17, 18). The proposed subunit packing is now supported by a 3.9 Å x-ray diffraction map of an Fcτ10 subcomplex from yeast mitochondria (19). The c subunit spans the membrane as a hairpin of two α-helices and in the case of E. coli contains the essential aspartyl 61 residue at the center of the second TMH. Asp61 is thought to undergo protonation and deprotonation as each subunit of the oligomeric ring moves past a stationary subunit α. Subunit α is believed to provide access channels to the proton-binding Asp61 residue, but the actual proton translocation pathway remains to be defined (20–23).

The structure of subunit α and its role in H+ translocation are poorly defined. Subunit α is known to fold with five TMHs (24–26) with αTMH4 packing in parallel to cTMH2, i.e. the helix to which Asp61 is anchored (27). The interaction of the conserved Arg210 residue in αTMH4 with cTMH2 is thought to be critical during the deprotonation-protonation cycle of c2TMH6 (29, 28–32). The predicted αTMH4/cTMH2 interactions are in accord with second site revertant analysis (33), and cross-link analysis has confirmed closest neighbor proximity of cTMH2 with αTMH4 over a span of 19 amino acid residues (27). Both modeling and cross-linking experiments indicate that helix 2 of subunit c should be packed on the outside of the ring (17, 34). Electron microscopic studies support the positioning of subunit α and the two b subunits at the periphery of the c ring (35–37).

The chemical labeling of cysteine side chains introduced by site-directed mutagenesis has been used as a means of mapping aqueous accessible regions on several membrane proteins (38–51). Several reagents have been used to modify the genetically introduced Cys to determine accessibility, including NEM (38, 41–44), MTS reagents (40, 48–50), and Ag+ (45–48). Modification of Cys by these reagents depends upon ionization of the Cys sulfhydryl to its thiolate form (52–55), and this is expected to occur preferentially in an aqueous environment (38, 39, 41). In this report, a span of 19 residues in αTMH4 were replaced with Cys and tested for accessibility to water-soluble reagents NEM and Ag+. We found that residues 206, 210, and 214 are NEM-accessible from the cytoplasmic side of the membrane. In contrast, residues 215 and 218 on helix 4 and residue 245 on helix 5 form an Ag+-accessible but NEM-inaccessible pocket bridging αTMH4 and αTMH5. This pocket may form part of the aqueous access pathway extending from Asp61 to the periplasmic surface.

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§ To whom correspondence should be addressed: Dept. of Biomolecular Chemistry, University of Wisconsin Medical School, Madison, Wisconsin 53706. Tel.: 608-262-1439; Fax: 608-262-5253; E-mail: rhfillin@facstaff.wisc.edu.
Aqueous Accessibility of TMH4 in Subunit a of ATP Synthase

EXPERIMENTAL PROCEDURES

Construction of Cys-substituted Mutants—Cysteine substitutions were introduced by a two-step PCR method using a synthetic oligonucleotide that contained the codon change and two wild type oligonucleotides [56]. Most substitutions had already been generated in this lab (27) and were transferred to a plasmid containing the entire unc (atp) operon, in which all endogenous Cys had been substituted by Ala [57], and a hexahistidine tag on the C terminus of subunit a [24], using two unique BsmBI sites that lie within the subunit a gene (nucleotide positions 1110 and 1727) [58]. The presence of the mutation was confirmed by sequencing the cloned fragment through the ligation junctions. For these studies, the plasmids were transformed into JW2922 (2), a strain with a chromosomal deletion of the entire unc operon. The ωH245C/D119H mutant described in Fig. 12 was constructed in a pDF163-like plasmid [59]. Plasmid pDF163 contains the HindIII (870 to 3218) fragment of unc DNA cloned between these sites in plasmid pBR322 and encodes subunits a, b, c, and γ. For this experiment, the plasmid was transformed into strain JWP109 (27), a strain with a chromosomal deletion of unc genes coding subunits a, b, c, and γ.

Comparative Growth Studies—The Cys-carrying strains were plated on glucose-containing minimal medium with 0.1 mg/ml ampicillin. Single colonies were then tested for growth on sucrose-containing minimal plates with scoring for growth at 72 h, as well as growth yield in liquid minimal medium containing 0.04% glucose.

Membrane Preparation—Plasmid transformants of strain JW2922 were grown in M63 minimal medium containing 0.6% glucose, 2 mg/liter thiamine, 0.2 mg/ml uracil, 0.2 mg/liter arginine, 0.02 mg/ml dihydroxybenzoic acid, and 0.1 mg/ml ampicillin, supplemented with 10% LB broth, and harvested in the late exponential phase of growth (2). The cells were suspended in TMG-acetate buffer (50 mM Tris acetate, 5 mM magnesium acetate, 10 mM glycerol, pH 7.5) containing 1 mM DTT, 1 mM nigericin (final concentration, 0.5 μM) and incubated at room temperature with mixing for 30 min. The solution was then passed through a French press at 124 MPa and membranes were collected by centrifugation at 10,000 × g for 15 min at 4 °C. The membranes were also pretreated at 10 mg/ml in TMG-acetate buffer

ATP-driven Quenching of ACMA Fluorescence—The membranes were suspended in 3.2 ml of the HMK buffers described below, containing either chloride or nitrate as the counter ion. ACMA was added to 0.3 μM/ml final concentration, and 30 μl of 0.1 x ATP, pH 7.0 was added to initiate quenching of fluorescence. The reaction was terminated by addition of 8 μl of 288 μM nigericin (final concentration, 0.5 μg/ml). The level of fluorescence obtained after the addition of nigericin was normalized to 100% in calculating the percentage of quenching caused by ATP-driven proton pumping. For NEM treatment, 80 μl of membranes at 15 mg/ml in TMG-acetate buffer were incubated at room temperature for 15 min prior to dilution into HMK-chloride buffer (10 mM Hepes-KOH, 5 mM MgCl₂, and 300 mM KCl, pH 7.5). For AgNO₃ treatment, 160 μl of membranes at 10 mg/ml were suspended in HMK-nitrate buffer (10 mM Hepes-KOH, 1 mM Mg(NO₃)₂, and 10 mM KNO₃, pH 7.5) containing 40 μM AgNO₃ and incubated at room temperature for 15 min before carrying out the quenching assay. Identical results were observed using silver acetate instead of AgNO₃. As a control, the membranes were also pretreated at 10 mg/ml in TMG-acetate buffer with 5 mM AgNO₃ and then diluted into HMK-nitrate buffer before carrying out the same quenching assay.

NADH-driven Quenching of Quinacrine Fluorescence in Stripped Membrane Vesicles—Membranes in TMG-acetate were centrifuged and resuspended in TEG buffer (1 mM Tris acetate, 0.5 mM EDTA, 10% glycerol, pH 8.0) and incubated for 30 min at 30 °C. The membranes were centrifuged again, washed once with TEG buffer, and then resuspended in TMG-acetate buffer at 10 mg/ml. The stripped membrane vesicle suspension (480 μg) was added to 3.2 ml of HMK-chloride buffer, and following addition of quinacrine to 1.657 μg/ml (final concentration), 16 μl of 10 mM NADH was added to initiate quenching of fluorescence. The reaction was terminated by addition of 8 μl of 288 μM nigericin (final concentration, 0.5 μg/ml). For NEM treatment, 480 μg of stripped membranes at 10 mg/ml were incubated at room temperature with 5 mM NEM for 15 min prior to dilution in HMK-chloride buffer. For diclofenecarboxylamide treatment, diluted stripped membranes at 150 μg/ml in HMK-chloride buffer were treated with 18.75 μM diclofenecarboxylamide at room temperature for 15 min prior to the quenching measurement.

**RESULTS**

Cys Substitutions in TMH4 of Subunit a—In this study, Cys substitutions in the fourth TMH of subunit a were transferred to a His-tagged version of subunit a in a plasmid that coded for the pure unc (atp) operon in which all the endogenous Cys in Fₒ and F₁ were substituted by Ala (57). These plasmids were transformed into JW2922, a strain with a chromosomal deletion of the entire unc operon. The growth of transformant strains was tested on glucose and sucrose-containing minimal medium. The Cys substitutions in subunit a reported here have little effect on growth, with the exception of R210C, G213C, and E219C, which grew poorly or not at all on succinate and exhibited low growth yields with glucose as a carbon source (Table I).

Sulfhydryl-specific Reagents Inhibit Function of the aN214C Mutant—We wished to examine the aqueous accessibility of residue 214. Previously, Jiang and Fillingame (27) demonstrated that aN214C forms a cross-link with cM65C and aA62C, indicating that this residue may be in close proximity to the proton-binding Asp₁₅₉ residue of subunit c. The capacity of several sulfhydryl-specific reagents to inhibit ATP-driven quenching of ACMA fluorescence by aN214C inverted membrane vesicles was tested. NEM and nonpolar MTS reagents, i.e. ethylamino- and carbamoylmethyl- MTS, inhibited quenching in aN214C membranes (Fig. 1) but not in control membranes lacking the aN214C (data not shown). The larger, charged MTS reagents, i.e. trimethylammonium- and sulfonylcarboxyl MTS, were not inhibitory. NEM was found to inhibit quenching in aN214C membranes maximally at 5 mM but also quite significantly at 1 mM (Fig. 2). The [14C]NEM labeling studies described below were carried out with 1 mM NEM. The inhibition of ATP-driven quenching observed here may be due to a direct block in proton transport through Fₒ, because NEM also blocked passive, Fₒ-mediated proton translocation by stripped membrane vesicles (Fig. 3). This is indicated by the increase in NADH-driven quenching of quinacrine fluorescence after NEM treatment, which can be attributed to a decrease in the proton leakiness of the stripped membrane vesicles.

The program was developed at the National Institutes of Health and is available on the World Wide Web at rsb.info.nih.gov/nih-image.
An 80-nm N214C membranes is inhibited by a variety of sulfhydryl-specific reagents, and the uncoupler nigericin was added to 0.5 mM ATP-driven quenching of ACMA fluorescence by stripped membranes containing 22 mM succinate.

**TABLE I**

| Mutation | Growth on glucose | Growth on succinate |
|----------|-------------------|---------------------|
| Wild type | 100              | 2.1–2.8             |
| S206C    | 103              | 2.5                 |
| L207C    | 83               | 1.5                 |
| G208C    | 107              | 1.5                 |
| L209C    | 99               | 3.1                 |
| R210C    | 50               | 0                   |
| L211C    | 107              | 2.8                 |
| F212C    | 99               | 2.5                 |
| G213C    | 67               | 1.2                 |
| N214C    | 99               | 2.1                 |
| M215C    | 102              | 1.8                 |
| Y216C    | 102              | 2.0                 |
| A217C    | 104              | 2.6                 |
| G218C    | 93               | 2.6                 |
| E219C    | 63               | 0.2                 |
| L220C    | 93               | 2.1                 |
| E221C    | 102              | 2.8                 |
| F222C    | 102              | 2.5                 |
| I223C    | 104              | 2.9                 |
| L224C    | 102              | 2.7                 |
| Δunc     | 65               | 0                   |

* Yield in liquid minimal medium containing 0.04% glucose, expressed as a percentage of wild type.

* Colony size after incubation for 72 h on minimal medium plates containing 22 mM succinate.

* Wild type is the Δunc strain JWP292 transformed with a plasmid containing the entire unc operon with all endogenous cysteines changed to alanine.

**Fig. 1.** ATP-driven quenching of ACMA fluorescence by aN214C membranes is inhibited by a variety of sulfhydryl-specific reagents. An 80-μl aliquot of membranes at 10 mg/ml in TMG-acetate were treated with sulfhydryl-specific reagents for 15 min at room temperature before being diluted into 3.2 ml of HMK-chloride buffer containing 0.3 μg/ml ACMA. ATP was added to 0.94 mM (first arrow), and the uncoupler nigericin was added to 0.5 mM (second arrow) at the times indicated. The traces indicate no treatment (trace 1), 0.5 mM NEM (trace 2), 1 mM NEM (trace 3), and 5 mM NEM (trace 4).

**Fig. 2.** Concentration dependence of NEM inhibition of ATP-dependent quenching by aN214C membranes. Membranes at 10 mg/ml in TMG-acetate were treated for 15 min at room temperature with the indicated amounts of NEM, added from a 0.5 mM stock in ethanol, prior to the quenching measurement as described in the legend to Fig. 1. The traces indicate no treatment (trace 1), 0.5 mM NEM (trace 2), 1 mM NEM (trace 3), and 5 mM NEM (trace 4).

**Fig. 3.** NEM inhibits passive proton flux through stripped Cys214 membrane vesicles. Stripped membranes at 10 mg/ml in TMG-acetate were treated for 15 min with 5 mM NEM or 18.75 μM dicyclohexylcarbodiimide at room temperature before being diluted into 3.2 ml of HMK-chloride buffer containing 1.875 μg/ml quinacrine. NADH was added to 50 μM, and nigericin was added to 0.5 μg/ml at times indicated. The traces indicate no treatment (trace 1), dicyclohexylcarbodiimide treatment (trace 2), and NEM treatment (trace 3).

**NEM Inhibition of Quenching with Other aTMH4 Cys Substitutions**—The Δunc strain was transformed with the set of plasmids containing single Cys substitutions in aTMH4. Inside-out membrane vesicles were prepared from these strains. The membranes were treated with 5 mM NEM, and ATP-dependent quenching of ACMA fluorescence was tested for each substitution. Representative quenching traces for several mutants are presented in Fig. 4. NEM inhibited quenching most strikingly with two Cys substitutions, aS206C (Fig. 4) and aN214C (Fig. 1), whereas proton translocation activity remained largely unchanged in the other 17 mutants tested (Table II).

Characterization of NEM Reaction with Residues S206C and N214C—In the models of subunit α discussed elsewhere (24–27, 32), residue 206 is located on the cytoplasmic face of the membrane, whereas residue 214 is located near the center of the lipid bilayer. Because NEM reacts preferentially with the ionized form of Cys (52, 53), the pH dependence of NEM inhibition of the two residues was examined. When S206C membranes were treated with NEM at pH 7.5, inhibition of quenching was observed. However, NEM treatment at pH 7.0 resulted in no inhibition of quenching and indicated a higher level of protonation of the sulfhydryl side chain at this pH (Fig. 5A). In contrast, quenching with N214C membranes was inhibited by NEM treatment at either pH, indicating that this Cys residue is subject to ionization at pH 7.0 (Fig. 5B). The Cys214 residue must therefore have an unusually low pKₐ and be in an unexpectedly hydrophilic environment for a residue centered in the middle of the membrane. The Cys214 side chain may be close to the essential αR210 residue, which could provide charge neutralization, leading to the low pKₐ.

**[14C]NEM Labeling of aTMH4 Cys Substitutions**—To check whether NEM reacted with other residues without causing inhibition of quenching, inside-out membrane vesicles from the
aTMH4 mutants were treated with 1 mM [14C]NEM for 1 h to determine the aqueous accessibility of each residue. The hexa-
histidine-tagged subunit α was then purified by Ni²⁺/H⁺ affinity chromatography, and the amount of label incorporated into subunit α was quantified by scintillation counting and PhosphoImager analysis (Fig. 6). Cys substitutions at positions 206, 210, and 214 were strongly labeled with NEM, indicating that this hydrophilic reagent has access to these residues, possibly via the same aqueous channel by which protons move through the enzyme.

Silver as a Probe of Aqueous Accessibility to aTMH4—Silver has been used to map aqueous pores of several membrane proteins (45–48), and it was shown to inhibit the Na⁺/K⁺-ATPase (63). Silver ion has an ionic radius of 1.26 Å, which is close to that of Na⁺ (0.97 Å) and H₂O⁺ (1.54 Å) (64). It forms a covalent bond with sulphydryl groups of Cys (45, 55, 65). In our initial experiments with αN214C membranes, Ag⁺ treatment caused an inhibition of ATP-driven quenching of ACMA fluorescence, the extent of inhibition varying with the amount of Ag⁺ used (Fig. 7), whereas membranes lacking the Cys substitution were unaffected by Ag⁺ treatment. The extent of inhibition also proved to depend upon the amount of membrane added to the cuvette (Fig. 8). The amount of AgNO₃ needed for a given extent of inhibition increased proportionally with the amount of membrane present and suggested that the silver

**Table II**

| Mutation | Quenching with ATP* | +NEM/NEM² |
|----------|---------------------|-----------|
| WT       | 76 ± 6              | 1.01 ± 0.16 |
| S206C    | 73 ± 3              | 0.13 ± 0.02 |
| L207C    | 35 ± 9              | 0.67 ± 0.09 |
| G208C    | 81 ± 4              | 0.96 ± 0.02 |
| L209C    | 41 ± 8              | 1.02 ± 0.05 |
| R210C    | 3 ± 2               |           |
| L211C    | 69 ± 10             |           |
| F212C    | 74 ± 14             | 0.99 ± 0.27 |
| G213C    | 9 ± 2               | 1.02 ± 0.26 |
| N214C    | 52 ± 11             | 0.18 ± 0.05 |
| M215C    | 73 ± 2              | 0.95 ± 0.16 |
| Y216C    | 39 ± 2              | 1.33 ± 0.29 |
| A217C    | 71 ± 22             | 1.18 ± 0.23 |
| G218C    | 42 ± 0.2            | 1.17 ± 0.23 |
| E219C    | 26 ± 3              | 1.02 ± 0.14 |
| L220C    | 42 ± 17             | 1.31 ± 0.20 |
| I221C    | 75 ± 7              | 1.04 ± 0.03 |
| F222C    | 71 ± 0.2            |           |
| I223C    | 67 ± 5              | 1.02 ± 0.11 |
| L224C    | 58 ± 14             | 0.97 ± 0.27 |

*Measured in a buffer containing 10 mM Hepes-KOH, pH 7.5, 5 mM MgCl₂, 300 mM KCl, with and without 5 mM NEM.

*The results presented are the averages of three or more determinations ± S.D.
Aqueous Accessibility of TMH4 in Subunit a of ATP Synthase

Louis N. Oosawa, Peter L. Swenson, Blake E. Roche, Yoko H. Harada, and Susan M. Gottschalk

CONCENTRATION-DEPENDENT INHIBITION OF ATP-DRIVEN ACMA QUENCHING IN aN214C MEMBRANES BY AG⁺—The ability of DTT to reverse the inhibition of quenching caused by treatment of membranes with AgNO₃ was also explored (Fig. 11). We found that the addition of DTT rapidly reversed AgNO₃ inhibition with aN214C membranes. In contrast, inhibition was very slowly reversed with aN214C membranes, indicating that the hydrophilic DTT may have more direct access to the cytoplasmically located S206C than the membrane-buried Cys214 residue.

INVOLVEMENT OF aTMH5 IN PROTON CONDUCTANCE—In the models of subunit a suggested previously (20, 22, 24–26, 32), Gly218 in TMH4 is thought to be adjacent to His245 in TMH5. This model is supported by several second site suppressor pairs between TMHs, including the aG218D mutation, which was corrected by a second mutation, aH245G (66). We wondered whether the Ag⁺-accessible region of aTMH4 was also bounded by aTMH5. The aH245C substitution, located in aTMH5, has been characterized as a nonfunctional mutant. However, partial function can be restored by the introduction of a second mutation, aD119H (24). Membranes carrying the aH245C/D119H double mutant were treated with silver, and ATP-driven quenching of ACMA fluorescence was measured (Fig. 12). Ag⁺ inhibits proton pumping in this mutant, suggesting that several helices of subunit a may be involved in the formation of an Ag⁺-accessible cavity extending into the interior of subunit a. The boundaries of this cavity will be investigated further in the future.

DISCUSSION

In this paper, we report on the aqueous accessibility of cysteine residues substituted into the fourth transmembrane helix in subunit a of the rotary ATP synthase. aTMH4 is thought to interact with Asp61 of subunit c during proton translocation and may play a part in forming an aqueous access channel to cAsp61 from one or both sides of the membrane. The proximity...
of αTMH4 to cTMH2 was initially postulated on the basis of second site suppressor analysis (33) and is now supported by intermolecular Cys-Cys cross-links between cTMH2 and αTMH4 (27). Aqueous access to Asp61 of subunit c had been postulated previously based upon the pH-sensitive function of the cA24D mutant (67) and is further supported by the discovery that simultaneous mutation of three residues surrounding Asp61 makes the enzyme sensitive to inhibition by Li⁺ (68).

**Fig. 9.** ATP-driven ACMA quenching by Cys215 and Cys218 membranes is inhibited by Ag⁺ but insensitive to inhibition by NEM. M215C (A) and G218C (B) membranes at 10 mg/ml in TMG-acetate were diluted into 3.2 ml of HMK-nitrate buffer, and AgNO₃ was added to 40 μM, or NEM was added at 5 mM to membranes in TMG-acetate buffer prior to dilution into HMK-nitrate buffer. Following incubation for 15 min at room temperature, the quenching measurement was made as described in the legend to Fig. 1. The traces indicate no treatment (trace 1), 5 mM NEM treatment (trace 2), and 40 μM AgNO₃ treatment (trace 3).

**Fig. 10.** Cys215 and Cys218 are Ag⁺-sensitive and NEM-insensitive, whereas Cys206 and Cys214 are both Ag⁺- and NEM-sensitive. A 160-μl aliquot of membranes at 10 mg/ml in TMG-acetate was diluted into 3.2 ml of HMK-nitrate buffer, and AgNO₃ was added to 40 μM for 15 min, or NEM was added to 5 mM for 15 min prior to dilution into HMK-nitrate, as described in the legend to Fig. 9. The results are presented as the ratios of quenching in the presence of Ag⁺ or NEM to the quenching in the absence of a reagent. The gray bars represent the quenching ratio ± Ag⁺ treatment, whereas the black bars represent the quenching ratio ± NEM treatment. Each bar represents the average ratio from n = 2 determinations ± S.D.

**Fig. 11.** Cys206 is more accessible to DTT than Cys214. An 80-μl aliquot of membranes at 10 mg/ml in TMG-acetate were diluted into 3.2 ml of HMK-nitrate buffer, AgNO₃ was added to 20 μM, and the sample incubated for 15 min at room temperature. DTT was then added to 1 mM from a 1 M stock, and the reaction was incubated for the indicated length of time prior to the quenching measurement. A depicts quenching traces from S206C membranes; B depicts traces from N214C membranes. The traces indicate control not treated with AgNO₃ (trace 1), DTT treatment 1 h (trace 2), DTT treatment 15 min (trace 3), DTT treatment 1 min (trace 4), and no DTT treatment (trace 5).
Aqueous Accessibility of TMH4 in Subunit a of ATP Synthase

FIG. 12. ATP-driven ACMA quenching in αH245C/D119H membranes is inhibited by Ag⁺. A 160-μl aliquot of membranes at 10 mg/ml in TMG-acetate was diluted into 5.2 ml of HMK-nitrate buffer, and AgNO₃ was added to 40 or 80 μM. Samples were incubated for 15 min at room temperature prior to the quenching measurement. The traces indicate control not treated with AgNO₃ (trace 1), 40 μM AgNO₃ (trace 2), and 80 μM AgNO₃ (trace 3).

Fig. 13. Location of NEM- and Ag⁺-sensitive residues in a hypothetical model of subunit α. Subunit α modeled by Rastogi and Girvin (Ref. 22; Protein Data Bank code 1c17). Positions of NEM-reactive Cys residues are shown in red; the Arg²¹⁰ side chain is also indicated. Ag⁺-sensitive, NEM-insensitive residues are shown in yellow. The model was constructed by standard NMR structure calculation methods using α-helical backbone constraints for TMHs 2–5 and five helix-helix contacts suggested from second site suppressor analysis. In topological models (24, 32) the loops at the top of the structure are thought to extend into the cytoplasm, and loop 4,5 is thought to extend into the periplasm, placing residue 214 in αTMH4 at the center of the membrane. The figure is drawn from the program MOLMOL (77).

Additionally, the analogous enzyme from Propionigenium modestum, with a very homologous subunit c, alternatively transports Na⁺, Li⁺, or H⁺ (69, 70). Thus, it seems likely that these various ions gain access to the membrane-embedded carboxyl of subunit c via a water-filled channel. We have substituted cysteine over the length of αTMH4 and tested the susceptibility of each substitution to modification with water-soluble, thiol-modifying reagents. The approach has been used previously to define surfaces of membrane proteins with aqueous accessibility (38–51).

Reactivity of Cys Substitutions with NEM, Ag⁺, and MTS Reagents—The ionized sulphydryl group of cysteine is the form that preferentially reacts with the thiol-specific reagents used here. For example, the reactivity of MTS reagents with the thiolate is preferred by a factor of 10⁹ over reaction with a nonionized thiol group (54). Similarly, NEM (52, 53) and Ag⁺ (45, 55) react preferentially with ionized thiylates rather than with neutral thiols. The differential reactivity of substituted cysteines can thus provide information about the ionization state of different residues, which in most cases should be related to aqueous accessibility, i.e. the interpretation given in similar studies of other membrane proteins (38, 39, 41). The means by which NEM penetrates the membrane to react with Cys residues is not certain. It may be sufficiently lipid-soluble to gain access to transmembrane Cys via the hydrophobic phase of the membrane. However, modification should only be observed with those residues subject to ionization. It is also conceivable that uncharged MTS reagents could access reactive Cys residues via the lipid phase, although reactivity would again depend upon the ionization state of the sulphydryl group. It is of interest that Ag⁺ reacts with transmembrane residues that are NEM-insensitive. This may indicate that NEM-sensitive residues need to be bounded by larger aqueous cavities, sufficient in size to accommodate the bulkier NEM molecule.

Table III

| Position | Substitution | Growth on Succinate | Reference |
|----------|--------------|---------------------|-----------|
| Ser²⁰⁶   | Ala          | + + +               | Ref. 73   |
| Leu      | Slow         |                     |           |
| Arg²¹⁰  | Ala          | 0                   | Ref. 74   |
| Val      | 0            | Ref. 75             |
| Ile      | 0            | Ref. 75             |
| Gln      | 0            | Ref. 75             |
| Glu      | 0            | Ref. 75             |
| Lys      | 0            | Ref. 75             |
| Gly²¹³  | Asp          | 0                   | Unpublished data |
| Asn²¹⁴  | Val          | + + +               | Ref. 75   |
| Leu      | +            | Ref. 75             |
| Glu      | + + +        | Ref. 75             |
| Gln      | + + +        | Ref. 75             |
| His      | 0            | Ref. 75             |
| Gly²¹⁸  | Ala          | + + +               | Ref. 75   |
| Val      | +            | Ref. 75             |
| Asp      | +            | Refs. 66 and 75     |
| Lys      | –            | Ref. 75             |

* In most cases ATP-driven proton pumping was also measured, and the decrease in activity correlates with the growth on succinate parameter.

Properties of αTMH4 mutations constructed previously in NEM or Ag⁺-sensitive residues

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modification results in a block to proton transport through this region of the protein. Inhibition of proton translocation by attachment of the ethylmaleimide group is consistent with evidence from mutagenesis studies that bulky substitutions are not as well tolerated as smaller ones at these positions (Table III). The fact that the three residues found to label with NEM line one face of αTMH4 suggests that NEM could be gaining access to these residues via an aqueous pathway formed at this face of an α-helix.

The Cys206 residue appears to titrate as the pH of the medium is lowered from 7.5 to 7.0, as indicated by the dramatic decrease in NEM reactivity, suggesting that this residue is accessible to the bulk solvent. The solvent accessibility of this residue is also supported by the rapid reversal of Ag⁺ inhibition by diithiothreitol. On the other hand, the reactivity of Cys214 is unaffected by lowering the pH of the medium to 7.0, suggesting that this residue remains ionized at a lower pH than Cys206, even though topological analysis would place residue 214 in the middle of the membrane. Despite its low apparent pH of 5.5–6.0, Cys214 does not appear to be generally accessible to the aqueous medium based upon the slow reversal of Ag⁺ inhibition by DTT. We suggest that the low pKᵣ of Cys214 at the center of the membrane may be due to salt bridge formation with the proximal Arg210 residue.

Silver Used to Probe αTMH4 Cys Substitutions—Silver has been used previously as an irreversible covalent modifier of Cys introduced into several membrane proteins (45–48). The irreversibility of the Ag⁺ inhibition under the conditions used here is indicated by an experiment where Ag⁺ pretreatment of membrane vesicles protected Cys215 from becoming labeled with [¹⁴C]NEM after solubilization of the membrane with SDS (experiment not shown). Further, if membranes are treated with silver at this face of an α-helix/H9251 via Cys215 mutant enzymes still exhibited inhibition by Ag⁺, and Cys215 mutant enzymes still exhibited inhibition by Ag⁺, as we have discussed elsewhere (76), such swiveling may be coupled mechanically to other helical movements that drive stepwise rotation of the c ring.

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REFERENCES

1. Senior, A. E. (1988) Physiol. Rev. 68, 177–231
2. Jiang, W., Hermolin, J., and Fillingame, R. H. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 4966–4971
3. Abramians, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628
4. Gibbons, C., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2000) Nat. Struct. Biol. 7, 1055–1061
5. Menz, R. I., Walker, J. E., and Leslie, A. G. W. (2001) Cell 106, 331–341
6. Shirakihara, Y., Leslie, A. G. W., Abramians, J. P., Walker, J. E., Ueda, T., Sekimoto, Y., Kambara, M., Saika, K., Kagawa, Y., and Yoshida, M. (1997) Structure 5, 825–836
7. Bianchet, M. A., Hullihen, J., Pedersen, P. L., and Amzel, L. M. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 11065–11070
8. Grath, G. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 3446–3448
9. Boyer, P. D. (1989) FASEB J. 3, 2164–2178
10. Leslie, A. G., and Walker, J. E. (2000) Philos. Trans. R. Soc. Lond-Biol. Sci. 365, 465–471
11. Senior, A. E., Nadanaciva, S., and Weber, J. (2002) Biochim. Biophys. Acta 1553, 188–211
12. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997) Nature 386, 259–262
13. Kinosita, K., Jr., Yasuda, R., Noji, H., and Adachi, K. (2000) Philos. Trans. R. Soc. Lond-Biol. Sci. 355, 473–488
14. Sambongi, Y., Ito, Y., Tanabe, M., Ono, H., Iwamoto-Kihara, A., Ueda, I., Yanagida, T., Wada, Y., and Futai, M. (1999) Science 286, 1722–1724
15. Panke, O., Gumbiowski, K., Junge, W., and Engelbrecht, S. (2000) FEBS Lett. 524, 34–38
16. Girvin, M. E., Rastogi, V. K., Abildgaard, F., Markley, J. L., and Fillingame, R. H. (1998) Biochemistry 37, 8817–8824
17. Dmitriev, O. Y., Jones, P. C., and Fillingame, R. H. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 7785–7790
18. Fillingame, R. H., Jiang, W. P., and Dmitriev, O. Y. (2000) J. Bioenerg. Biomembr. 32, 433–439
19. Stock, D., Leslie, A. G. W., and Walker, J. E. (1999) Science 286, 1700–1705
20. Fillingame, R. H., Jiang, W. P., Dmitriev, O. Y., and Jones, P. C. (2000) Biochim. Biophys. Acta 1458, 387–403
21. Fillingame, R. H., Jiang, W. P., Dmitriev, O. Y. (2000) J. Exp. Biol. 203, 9–17
22. Rastogi, V. K., and Girvin, M. E. (1999) Nature 402, 263–268
23. Cain, B. D. (2000) J. Bioenerg. Biomembr. 32, 365–371
24. Valiyaveetil, F. I., and Fillingame, R. H. (1998) J. Biol. Chem. 273, 16241–16247
25. Long, J. C., Wang, S., and Vik, S. B. (1998) J. Biol. Chem. 273, 16235–16240
26. Wada, T., Long, J. C., Zhang, D., and Vik, S. B. (1999) J. Biol. Chem. 274, 17353–17357
27. Jiang, W., and Fillingame, R. H. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 6607–6612
28. Fillingame, R. H. (1998) in The Bacteria (Kruilich, T. A., ed) Volume XII, pp. 345–391, Academic Press, New York
29. Hatch, L. F., Cox, G. B., and Howitt, S. M. (1995) J. Biol. Chem. 270, 29407–29412
30. Valiyaveetil, F. I., and Fillingame, R. H. (1997) J. Biol. Chem. 272, 32635–32641
31. Wang, H. Y., and Oster, G. (1998) Nature 396, 279–282
32. Vik, S. B., Long, J. C., Wada, T., and Zhang, D. (2000) Biochim. Biophys. Acta 1458, 457–466
33. Fillingame, R. H., and Fillingame, R. H. (1994) J. Biol. Chem. 269, 2562–2567
34. Jones, P. C., Jiang, W. P., and Fillingame, R. H. (1998) J. Biol. Chem. 273, 17178–17185
35. Birkenhager, R., Hoppert, M., Deckers-Hebestreit, G., Mayer, F., and Altendorf, K. (1995) Eur. J. Biochem. 229, 58–67
36. Takeyasu, K., Omote, H., Nettikadan, S., Tokumasu, F., Iwamoto-Kihara, A., and Futai, M. (1996) FEBS Lett. 392, 110–113
37. Singh, S., Turina, P., Bustamante, C. J., Keller, D. J., and Capaldi, R. (1996) FEBS Lett. 397, 30–34
38. Yamamura, N., Konishi, S., Iwaki, S., Kimura-Someya, T., Nada, S., and Yamaguchi, A. (2001) J. Biol. Chem. 276, 20330–20339
39. Li, J., Xu, Q., Cortes, D. M., Perez-O., Laskay, A., and Karlin, A. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 11605–11610
40. Wilson, G., and Karlin, A. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 1241–1248
41. Mordoch, S. S., Granot, D., Lebendiker, M., and Shuldiner, S. (1999) J. Biol. Chem. 274, 19480–19486
42. Frillinges, S., Sahin-Tath, M., Wu, J., and Kaback, H. R. (1998) FASEB J. 12, 1281–1289
43. Kawai, I., Zen, K. C., Hu, Y., and Kaback, H. R. (2001) Biochemistry 40.
Aqueous Accessibility of TMH4 in Subunit α of ATP Synthase

10491–10499
44. Yan, R. T., and Maloney, P. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5973–5976
45. Lu, Q., and Miller, C. (1995) Science 268, 304–307
46. Kriegler, S., Sudweeks, S., and Yakel, J. L. (1999) J. Biol. Chem. 274, 3934–3936
47. del Camino, D., and Yellen, G. (2001) Neuron 32, 649–656
48. Egan, T. M., Haines, W. R., and Vogt, M. M. (1998) J. Neurosci. 18, 2350–2359
49. Rassendren, F., Buell, G., Newbolt, A., North, R. A., and Surprenant, A. (1997) EMBO J. 16, 3446–3454
50. Karlin, A., and Akabas, M. H. (1998) Methods Enzymol. 293, 123–145
51. Bass, R. B., Coleman, M. D., and Falke, J. J. (1999) Biochemistry 38, 9317–9327
52. Gorin, G. Martie, P. A., and Doughty, G. (1966) Arch. Biochem. Biophys. 115, 593–597
53. Friedman, M. (1973) The Chemistry and Biochemistry of the Sulphhydryl Group in Amino Acids, Peptides and Proteins, 1st Ed., Pergamon Press, Oxford
54. Roberts, D. D., Lewis, S. D., Ballou, D. P., Olson, S. T., and Shafer, J. A. (1986) Biochemistry. 25, 5595–5601
55. Danee, I. G. (1986) Polyhedron 5, 1037
56. Barrick, S. (1996) Methods Mol. Biol. 37, 203–215
57. Kuo, P. H., Ketchum, C. J., and Nakamoto, R. K. (1998) FEBS Lett. 426, 217–220
58. Gay, N. J., and Walker, J. E. (1981) Nucleic Acids Res. 9, 2187–2194
59. Fraga, D., and Fillingame, R. H. (1989) J. Biol. Chem. 264, 6797–6803
60. Mosher, M. E., White, L. K., Hermolin, J., and Fillingame, R. H. (1985) J. Biol. Chem. 260, 4897–4914
61. Fillingame, R. H. (1973) J. Bacteriol. 124, 879–883
62. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
63. Hussain, S., Meneghini, E., Moosmayer, M., Lacotte, D., and Anner, B. M. (1994) Biochim. Biophys. Acta 1190, 402–408
64. Weast, R. C., and Astle, M. J. (eds) (1982) CRC Handbook of Chemistry and Physics, 63 Ed., F179, CRC Press, Inc., Boca Raton, FL
65. Bell, R. A., and Kramer, J. R. (1999) Environ. Toxicol. Chem. 18, 9–22
66. Hartzog, P. E., and Cain, B. D. (1994) J. Biol. Chem. 269, 32313–32317
67. Zhang, Y., and Fillingame, R. H. (1994) J. Biol. Chem. 269, 5473–5479
68. Zhang, Y., and Fillingame, R. H. (1995) J. Biol. Chem. 270, 87–93
69. Dimroth, P. (2000) Biochim. Biophys. Acta 1458, 374–386
70. Kaim, G. (2001) Biochim. Biophys. Acta 1505, 94–107
71. Bennett, M. A. (1962) J. Biol. Chem. 237, 611–622
72. Morris, L. J. (1966) J. Lipid Res. 7, 717–732
73. Howitt, S. M., Gibson, F., and Cox, G. B. (1988) Biochim. Biophys. Acta 936, 74–80
74. Cain, B. D., and Simoni, R. D. (1986) J. Biol. Chem. 261, 10043–10050
75. Cain, B. D., and Simoni, R. D. (1989) J. Biol. Chem. 264, 3292–3300
76. Fillingame, R. H., Angevine, C. M., and Dmitriev, O. Y. (2002) Biochim. Biophys. Acta 1555, 29–36
77. Koradi, R., Billeter, M., and Wüthrich, K. (1996) J. Mol. Graphics 14, 51–55
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Christine M. Angevine and Robert H. Fillingame

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