AE1 protein transports Cl\(^-\) and HCO\(_3\)\(^-\) across the erythrocyte membrane by an electroneutral exchange mechanism. Glu\(^{681}\) of human AE1 may form part of the anion translocation apparatus and the permeability barrier. We have therefore studied the structure of the sequence surrounding Glu\(^{681}\), using scanning cysteine mutagenesis. Residues of the Ser\(^{643}\) (adjacent to the glycosylation site) to Ser\(^{690}\) region of cysteineless mutant (AE1C\(^-\)) were replaced individually with cysteine. The ability of mutants to mediate Cl\(^-\)/HCO\(_3\)\(^-\) exchange in transfected HEK293 cells revealed that extracellular mutants, W648C, I650C, P652C, L655C, and P659C have an important role in transport. By contrast, only transmembrane mutation E681C fully blocked anion exchange activity. The topology of the region was investigated by comparing cysteine labeling with the membrane-permeant cysteine-directed reagent 3-(N-maleimidylpropionyl)biocytin, with or without prior labeling with membrane-impermeant lucifer yellow iodoacetamide (LYIA). Two regions readily label with 3-(N-maleimidylpropionyl)biocytin (Ser\(^{643}\)–Met\(^{663}\) and Ile\(^{684}\)–Ser\(^{690}\)). We propose that poorly labeled Met\(^{664}\)–Glu\(^{683}\) corresponds to transmembrane segment 8 of AE1. Regions Ser\(^{643}\)–Met\(^{663}\) and Ile\(^{684}\)–Ser\(^{690}\) localize, respectively, to extracellular and intracellular sides on the basis of accessibility to LYIA. On the basis of LYIA accessibility, we propose that the Arg\(^{656}\)–Met\(^{663}\) region forms a “vestibule” that leads anions to the transport channel. Glu\(^{681}\) is located 3 amino acids from the C terminus of transmembrane segment 8, which places the membrane permeability barrier within 5 Å of the intracellular surface of the membrane.

AE1 (band 3) belongs to a family of anion exchange proteins that facilitate the movement of Cl\(^-\) and HCO\(_3\)\(^-\) across the plasma membrane. Plasma membrane anion exchange proteins are widely expressed among mammalian tissues, where they participate in the regulation of intracellular pH and volume. Three anion exchanger isoforms have been identified in intact erythrocytes and kidney; AE2, found in kidney, stomach, and lymphocytes; and AE3, found in the brain, retina, and heart (1). All of these anion exchange proteins contain two domains. The highly conserved (70% identity) membrane domain of approximately 55 kDa exchange activity. The cytoplasmic domain of 45–110 kDa is more divergent. In erythrocyte AE1, the cytoplasmic domain anchors the cytoskeleton to the plasma membrane through interactions with ankyrin (2).

AE1 has served as a model for our understanding of membrane protein structure and function because of its high expression in erythrocytes, where it constitutes 50% of the integral membrane protein (3). AE1 was among the first membrane transport proteins to have its cDNA cloned and sequenced (4). Despite the wealth of information regarding anion exchangers, we still do not know which residues of the protein are involved in the transport process. Jennings and co-workers implicated Glu\(^{681}\) in the transport process, since labeling this residue with Woodward’s reagent K (WRK)\(^1\) and reduction with sodium borohydride resulted in altered anion exchange kinetics (5–7). The functional role of Glu\(^{681}\) in AE1 was confirmed in mutagenesis experiments of mouse AE1 (8) and extended to the homologous position of mouse AE2, suggesting that the mechanistic role of Glu\(^{681}\) is conserved among anion exchange proteins (9). WRK chemical modification of AE1 abolishes chloride transport, yet relieves the requirement for proton cotransport during sulfate transport. During sulfate transport in unmodified AE1, a proton, supplied by Glu\(^{681}\), is cotransported. Sulfate/proton cotransport takes place in both inward and outward directions, which implies that Glu\(^{681}\) has access to both the intracellular and extracellular sides of the membrane. Taken together, Glu\(^{681}\) is functionally involved in anion exchange events and may reside at the permeability barrier of AE1. Determination of the location of this residue in the transmembrane region will localize both a part of the transport site and the permeability barrier.

Our current knowledge of AE1 topology comes from both experimental data and hydrophathy plots. Hydrophathy plots show regions of strong hydrophobicity in the N-terminal half of the membrane domain corresponding to the first seven transmembrane segments of AE1. However, our limited knowledge of the topology of the membrane domain of AE1 has recently been reviewed (10). The site of N-linked glycosylation, Asn\(^{642}\) (11), roughly marks the boundary between the first half of the membrane domain, with well defined transmembrane segments, and the second half, with ill defined topology (Fig. 1) (12). A chymotryptic cleavage site in intact erythrocytes has been identified at Tyr\(^{553}\) (13), localizing this residue to the external face. Antibody accessibility studies have mapped the extreme C terminus and two other loops to the cytoplasm (14, 15).

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\(^{\ddagger}\) The abbreviations used are: WRK, Woodward’s reagent K; AE1C\(^-\), cysteineless AE1; BCEE-A-M, 2,7-bis(2-carboxyethyl)-5 and 6-carboxyfluorescein, acetoxymethyl ester; biotin maleimide, 3-(N-maleimidylpropionyl)biocytin; LYIA, lucifer yellow iodoacetamide, dipotassium salt; stilbene maleimide, 4-acetamido-4-maleimidystilbene-2,2’-disulfonic acid, disodium salt; TLCK, N-p-tosyl-l-lysine chloromethyl ketone; TPCK, N-tosyl-l-phenylalanine chloromethyl ketone; PBS, phosphate-buffered saline.
We have chosen to study the topology of the initial portion of the second half of AE1 membrane domain, beginning at the well defined external site of N-glycosylation (from Ser643 to Ser690), using introduced cysteine scanning mutagenesis. Mutation of individual amino acids to cysteine represents a minor structural modification, relative to other methods in use to define topology and therefore has some potential advantages. Our goals were to define the membrane topology of an functionally important region of human AE1 anion exchange protein and to validate the use of cysteine scanning mutagenesis to the study of protein topology of mammalian membrane proteins. We have previously constructed a version of human AE1, lacking all cysteines and characterized it as functional (16). This mutant forms the basis for the introduction of cysteine residues at defined sites.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were from New England Biolabs. ECL chemiluminescent reagent, horseradish peroxidase conjugated to sheep anti-mouse IgG, and Hyperfilm and Immobilon membranes were from Amersham Pharmacia Biotech. Biocytin hydrazide, BCECF-AM, LYIA, biotin maleimide, and stilbene maleimide were from Molecular Probes, Inc. (Eugene, OR). Poly-L-lysine and nigericin were from Sigma. Coverslips were from Fisher.

Construction of Mutant Anion Exchangers—A human AE1 cDNA construct, called AE1C, in which all five cysteine codons were mutated to serine was previously constructed (16) in the expression vector pRBG4 (18). Individual introduced cysteine codons were cloned into AE1C to yield mutants, each with a unique cysteine codon. Introduced cysteine mutants at amino acids 645–647 were not constructed, because their codons overlap with the Smal site (nucleotides 2048–2053), used to clone introduced cysteine mutants into AE1C. Mutagenesis was performed using a polymerase chain reaction megaprimer mutagenesis strategy (19, 20). Polymerase chain reaction primers were designed using graphics program (Whitehead Institute for Medical Research). Polymerase chain reaction was performed using an ERI-COMP thermal cycler and either Vent DNA polymerase (Boehringer Mannheim). Mutants were verified by DNA sequencing.

Protein Expression—Anion exchangers were expressed by transient transfection of human embryonic kidney 293 (HEK293) cells (21), as described previously (22), except that calcium phosphate transfection was performed with 1 mg of anion exchanger plasmid added at 2.8 μg of anion exchanger plasmid with 4.2 μg of pRBG4 carrier/100-mm tissue culture dish. Cells were grown at 37 °C in a 5% CO2 environment in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 5% (v/v) fetal calf serum (Life Technologies) and to 37 °C. Cells were then washed with PBSCM. Sedimented cells were then lysed with 250 μl of IPB containing 2% (v/v) bovine serum albumin, 200 μM TPCK, 200 μM TLCK, and 2 mM phenethylsulfonfluoride on ice for 15 min. In experiments to measure activity to label with biotin maleimide, samples were prepared as described above for samples. After immunoprecipitation, samples were electrophoresed on 8% acrylamide gels (23) and transferred to Immobilon membranes (24). Biotinylated proteins were detected by incubation of the blot with 10 ml of 1:2500 diluted streptavidin-biotinylated horseradish peroxidase (Amersham Pharmacia Biotech) in TBST buffer (TBST buffer (0.1% (v/v), Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.5), containing 0.5% (w/v) bovine serum albumin). After a 1.5-h incubation, blots were washed with TBST. Blots were visualized using ECL reagent and Hyperfilm (Amersham Pharmacia Biotech).

After analyzing each sample for incorporation of biotin, samples were normalized for variations in recovery of AE1 protein as follows. The blots from above were stripped by incubation in 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, at 5 °C for 15 min. Blots were then processed as immunoblots (above), using 10 ml of TBSTM (TBST, containing 5% (w/v) nonfat dry milk powder (Carnation)) and 3 μl of monoclonal anti-AE1 antibody, 1F12 (25). After washing (as above), blots were probed with 10 ml of 1:3000 diluted horseradish peroxidase conjugated to sheep anti-mouse IgG and subsequently processed with ECL reagent (see above).

Cell Surface Processing Assay—The fraction of anion exchanger protein cell processed to the cell surface was assessed by labeling intact, whole cells with the radioiodinated antibody described above. A second half of AE1 membrane domain, beginning at the C-terminal 13 amino acids of human AE1 was synthesized, yielding peptide: NH2-CEGRDEYDEVAMPY-COOH. The peptide was coupled to keyhole limpet hemocyanin and subsequently injected into two rabbits numbered 1657 and 1658. Serum from each rabbit was sacrificed and exsanguinated, and sera designated 1657 and 1658 were isolated. Both sera have high anti-AE1 titer. Peptide synthesis and antisera production were completed by SynPop (Dublin, CA).

Immunoprecipitation—Lysates of whole tissue culture cells were prepared by incubation of cells from one-half of a 100-mm dish, with 250 μl of IPB buffer (1% (v/v) Nonidet P-40, 5 mM EDTA, 0.15 M NaCl, 0.5% (w/v) sodium deoxycholate, 10 mM Tris-HCl, pH 7.5), containing 2 mg/ml bovine serum albumin, 200 μM TPCK, 200 μM TLCK, and 2 mM phenethylsulfonfluoride, on ice for 15–30 min. Insoluble material was removed by centrifugation for 15 min at 16,000 × g in an IEC Micromax microcentrifuge. The sample was precleared with 2 μl of preimmune rabbit serum and protein A-Sepharose. Each immunoprecipitation used 2 μl of anti-AE1 antibody 1658 and was incubated at 4 °C for 1 h. The immunoprecipitates were washed two times with IPB containing 0.1 mg/ml A-Sepharose (Pharmacia, Piscataway, NJ) and resuspended in 10 mM Tris-HCl, pH 7.5, 5 mM, and 250 mM NaCl, pH 7.5. Samples were eluted from the resin by incubation for 5 min at 65 °C with SDS-polyacrylamide gel electrophoresis sample buffer containing 2% (v/v) 2-mercaptoethanol (23).

Chemical Accessibility Assays—HEK293 cells grown in 100-mm tissue dishes were transfected with wild type or mutant AE1 cDNA, as described above. Forty-eight hours post-transfection, cells were labeled by incubation with 1 mg/ml trypsin in PBS (140 mM NaCl, 3 mM KCl, 6.5 mM NaH2PO4, 1.5 mM KH2PO4, pH 7.4, for 20 min at room temperature with gentle, occasional mixing. Then 10 μl of 2 mM biotin maleimide (in dimethyl sulfoxide) was added to the + and − samples, and the samples were incubated with occasional mixing for 15 min at room temperature. Reactions were stopped by the addition of 0.5 ml of 2% (v/v) 2-mercaptoethanol in Dulbecco’s modified Eagle’s medium and incubated at room temperature for 10 min. Cells were sedimented as above and washed with PBSCM. Sedimented cells were then lysed with 250 μl of IPB containing 2% (v/v) bovine serum albumin, 200 μM TPCK, 200 μM TLCK, and 2 mM phenethylsulfonfluoride on ice for 15 min. In experiments to measure activity to label with biotin maleimide, samples were prepared as described above for −samples. After immunoprecipitation, samples were electrophoresed on 8% acrylamide gels (23) and transferred to Immobilon membranes (24). Biotinylated proteins were detected by incubation of the blot with 10 ml of 1:2500 diluted streptavidin-biotinylated horseradish peroxidase (Amersham Pharmacia Biotech) in TBST buffer (TBST buffer (0.1% (v/v), Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.5), containing 0.5% (w/v) bovine serum albumin). After a 1.5-h incubation, blots were washed with TBST. Blots were visualized using ECL reagent and Hyperfilm (Amersham Pharmacia Biotech).

Anion Exchange Assays—HEK293 cells were grown on top of 7 × 11-mm glass coverslips in 60-mm tissue culture dishes and transfected as described above. After two post-transfection, coverslips were rinsed with serum-free Dulbecco’s modified Eagle’s medium (Life Technologies) and loaded with BCECF-AM dye by incubation in 4 ml of serum-free Dulbecco’s modified Eagle’s medium, containing 2 μM BCECF, for 20–30 min, at 37 °C. Coverslips were mounted in a custom-built quartz cuvette, with perfusion capabilities. Intracellular pH was monitored by measuring fluorescence at excitation wavelengths of 440 and 502 nm measuring fluorescence at excitation wavelengths of 440 and 502 nm.
Ringer’s buffer containing 140 mM sodium gluconate (chloride-free buffer). Both buffers were bubbled continuously with air containing 5% carbon dioxide. Intracellular pH was calibrated by the nigericin-high potassium method (27), using three pH values from pH 6.5 to 7.5. Transport rates were determined by linear regression of the initial linear rate of change of pH, using Kaleidagraph software (Synergy Software).

**RESULTS**

Construction of Introduced Cysteine Mutants—To probe the topology of the region surrounding the functionally essential Glu$^{681}$ residue of human AE1 protein, we constructed an array of introduced cysteine mutants. In this array, each residue from the site of AE1 glycosylation (Asn$^{642}$) to Ser$^{690}$ was individually mutated to cysteine. Each cysteine mutant was cloned into AE1C$^{2}$, an AE1 mutant with all endogenous cysteine codons mutated to serine (16). Fig. 1 shows the sites of cysteine mutants constructed in topologically well defined regions of the protein, to verify the labeling methodology. S$^{555}$C represents a control site for the extracellular surface of the protein, since it is found between two chymotryptic cleavage sites in intact erythrocytes (13); S$^{595}$C lies in a hydrophilic region and is separated from S$^{555}$C by a highly hydrophobic sequence, which defines S$^{595}$ as an intracellular site; K$^{892}$C is adjacent to the C terminus of the protein, previously mapped to the inside of the cell (14), thereby defining K$^{892}$ as an intracellular site; S$^{574}$ is found in the hydrophobic region between Ser$^{555}$ and Ser$^{595}$ and therefore is probably in a transmembrane segment.

**FIG. 1. Topology model for membrane domain of human AE1.** The model has been truncated at the junction between the cytoplasmic domain and membrane domain, as defined by the sites of high protease sensitivity. T, trypsin; C, chymotrypsin. The branched structure at Asn$^{642}$ represents N-linked glycosylation. The model is based on data on the accessibility of residues to LYIA (J. Fujinaga, X.-B. Tang, and J. R. Casey, manuscript in preparation) and on data from glycosylation scanning mutagenesis (36).

**FIG. 2. Labeling human AE1 introduced cysteine mutants with biotin maleimide.** AE1C$^{2}$ is a cDNA construct encoding a form of human AE1 in which all cysteine codons were replaced by serine codons. Into this cysteineless background, individual cysteine mutants were cloned, to generate mutants each with only a single introduced cysteine codon. HEK293 cells were transfected with cDNA for AE1C$^{2}$ and introduced cysteine mutants at sites shown at the top. Cells were harvested and incubated with 2 mM biotin maleimide for 15 min at room temperature. After solubilization, samples were immunoprecipitated with anti-AE1 antibody, subjected to electrophoresis on 8% acrylamide gels, and transferred to PVDF membrane. A, biotin incorporation was detected using horseradish peroxidase-streptavidin and ECL. B, the blot from A was stripped and probed with monoclonal anti-AE1 antibody (IVF12) to detect the level of AE1 expression.
Labeling Introduced Cysteines with Biotin Maleimide—To begin to determine the topology of each residue in the introduced cysteine mutant array, we labeled HEK293 cells expressing a single introduced cysteine mutant with the membrane-permeant, cysteine-directed reagent, biotin maleimide. Fig. 2 shows representative data from the labeling experiments at 10 sites throughout the region. AE1C$^+$ labels with biotin maleimide to a barely detectable level, consistent with the absence of cysteine residues from the construct. Cytosolic control mutants S595C and K892C label to a similar extent to Y555C, which is a control site for the external surface of the protein. Interestingly, mutant S574C does not label with biotin maleimide, suggesting that transmembrane residues are not accessible to biotinylation, under the conditions used in this experiment. Fig. 2B shows the amount of AE1 protein loaded into each lane of the blot in A. It is apparent that all of the mutants express similar amounts of AE1, yet they label differentially with biotin maleimide.

All mutants in the Asn$^{642}$–Ser$^{690}$ region were subjected to the analysis seen in Fig. 2. The relative level of labeling with biotin maleimide and the amount of AE1 present in each sample were determined by densitometry of the biotinylation blot and the corresponding immunoblot. After normalization of each biotin signal for the AE1 expression level, the degree of biotinylation was expressed as a percentage of the labeling found for the S643C mutant, labeled in parallel. The labeling of the S643C mutant was set to 100. In this way, samples could be compared within an experiment and between experiments. Fig. 3 shows the relative amount of biotin label incorporated into each introduced cysteine mutant. Strikingly, the incorporation of biotin defines two regions, Ser$^{643}$–Met$^{663}$ and Ile$^{684}$–Ser$^{690}$, that readily label with biotin maleimide, separated by a region, Met$^{664}$–Gln$^{683}$, that labels poorly. The dashed line, representing the mean level of biotinylation for AE1C$^+$, the cysteineless mutant ($n = 8$), indicates that residues Met$^{664}$–Gln$^{683}$ have labeling that cannot be differentiated from AE1C$^+$. The filled box at the bottom of Fig. 3 shows the proposed topology for the region, on the basis of biotin maleimide accessibility data.

Relative Aqueous Accessibility of Residues—The ability to label with biotin maleimide appears to differentiate residues that are extramembraneous from transmembrane residues, as presented in Figs. 2 and 3. In the case of human AE1, we know
that the region surrounding Asn$^{642}$ is extracellular, since the site is glycosylated. However, for the introduced cysteine method to be applicable to define regions of unknown topology, a method is required to differentiate the inside of the cell from the outside. Previously this question has been addressed by labeling introduced cysteine mutants, in intact cells, with biotin maleimide and observing the ability to block this labeling by prelabeling with a membrane-impermeant reagent, stilbene maleimide (29). In our hands, however, this reagent behaves as membrane-permeant, when used at 10–200 μM, for 15 min at room temperature. Permeability may have been due to the fact that stilbenes have been reported to be weak substrates of AE1 (30). We therefore chose to use a different membrane-impermeant cysteine-directed reagent, LYIA. This reagent has been used as a fluorescent marker of the pinocytic pathway and is therefore likely to be membrane-impermeant (31).

In experiments to determine topology, HEK293 cells expressing AE1 introduced cysteine mutants were labeled with biotin maleimide, with or without prelabeling with the membrane-impermeant reagent, LYIA. LYIA reacts only with external cysteine residues and so blocks subsequent reaction with biotin maleimide. Fig. 4 shows the degree of biotinylation of representative mutants and the amount of biotinylation that is therefore likely to be membrane-impermeant (31).

Fig. 5 quantifies the degree to which LYIA is able to compete with biotin labeling of the extracellular face of AE1. Ly664–Ser690 had an accessibility ratio of 4.6 ± 1. Introduced cysteine mutants in the Ile644–Ser660 region have an average accessibility of 1.8 ± 0.2, which is clearly consistent with the value seen for the cytosolic control sites and not consistent with the extracellular surface. The bar at the bottom of Fig. 5 shows the proposed topology of the 643–690 region.

An important consideration in the use of introduced cysteines to probe topology is the maintenance of cell integrity. Cell lysis during a labeling experiment will result in entry of LYIA into cells, where it can label residues located in the cytosol. The effect of this is to misidentify cytosolic sites as extracellular. Cell lysis may have occurred during our labeling experiments, since both intracellular control sites and intracellular region iii both had LYIA accessibility factors greater than 1. To minimize cell lysis, we manipulated cells as gently as possible and minimized the number of centrifugation steps during the labeling protocol. We expressed AE1 mutants in HEK293, HEKw, and COS-7 cells to determine which cells had
In this paper, we have used chemical reactivity of introduced cysteine residues to examine the topology of transmembrane segment 8 of human AE1. This region is important to our understanding of AE1 function because it contains Glu681. Glu681 may interact with transported anions, since it provides the proton required during sulfate/proton cotransport by AE1. Since the cotransported proton can come from either side of the membrane, Glu681 may mark the transmembrane permeability barrier. We have developed a quantitative method to examine membrane protein topology that uses the membrane impermeant reagent, lucifer yellow iodoacetamide, as a probe of the environment around introduced cysteine residues. Our findings allow us to identify transmembrane segment 8 as the region from Met664 to Gin683, which places Glu681 within three residues of the intracellular surface of the protein. Use of LYIA allowed us to identify a region from Arg656 to Met665 with properties that are consistent with a vestibule region (see below). Consistent with the vestibule model is the observation that sites on the extracellular surface of AE1 were most sensitive to cysteine replacement (Fig. 8).

**Identification of the Transmembrane Region**—On the basis of the inability to label introduced cysteine residues in the Met664–Gln683 region, we propose that the region forms a transmembrane segment. In studies of the tetracycline transporter, a region of about 20 consecutive introduced cysteine residues that could not be labeled with N-ethylmaleimide was identified as a transmembrane segment (33–35). Our localization of the transmembrane segment is also consistent with recent studies on the introduction of glycosylation sites into AE1 (36), which indicated that a minimum of 14 residues are required between a glycosylation site and the N-terminal start of a transmembrane segment. Ann642 is the glycosylation site on human AE1,

![Diagram](Image 67x387 to 278x729)

**Fig. 6. Assay of AE1 anion exchange activity.** HEK293 cells transfected with human AE1 (A), AE1C–cDNA (B), or pRBG4 vector alone (C) were grown on glass coverslips and then loaded with the pH-sensitive dye, BCECF-AM. Cells were suspended in a fluorescence cuvette, and intracellular pH was monitored, as described under “Experimental Procedures.” Cells were perfused with either Ringer’s buffer containing 140 mM NaCl or Ringer’s buffer with NaCl replaced by 140 mM sodium gluconate. The bar at the top of each panel represents the time period when the cuvette was perfused with chloride-containing (solid bar) or chloride-free buffer (open bar).

The greatest resistance to lysis, but found the best labeling results in HEK293 cells.

**Anion Exchange Activity of AE1 Introduced Cysteine Mutants**—Since introduction of cysteine into sites within AE1 could alter the folding or transport activity of the protein, anion exchange activity was measured for each mutant. In this assay, the alternating chloride gradient from inward to outward results in bicarbonate movement in the opposite direction across the membrane; in chloride-free medium, chloride leaves the cell, bicarbonate enters, and the cell alkalinizes (32). In the present experiments, transport rates were determined from the initial slope of the curve as alkalinization and acidification occur. Fig. 6 shows typical anion exchange data, where AE1C retains a 52% transport rate relative to wild type AE1. This result conflicts with our previous work, which assessed anion exchange activity of AE1 and AE1C–reconstituted from microsomes of HEK293 cells expressing human AE1 (16). The impaired transport is explained by a decreased level of cell surface processing of AE1C– (see below). Negative control cells transfected with pRBG4 vector only also displayed a variable background level of transport (10–15% of AE1C– rate), as seen in Fig. 6C. Fig. 7 shows the anion exchange rate of each introduced cysteine mutant relative to AE1C–, which had a transport rate of ~0.24 pH/min during the acidification phase. Of the 45 introduced cysteine mutants analyzed, six are functionally inactive (W648C, I650C, P652C, L655C, F659C, and E681C). Inactive mutants were defined as those that had no transport activity within error of the assay. E681C, which has 0% of AE1C– activity, was expected to be inactive, since E681S was previously shown to block Cl/HCO3 exchange activity (9).

**Cell Surface Processing of AE1 Mutants**—Impaired anion exchange activity of introduced cysteine mutants may result from either some effect on the protein’s structure or from a reduced processing of the protein to the cell surface. Since the anion exchange assay only measures the functional activity of protein in the plasma membrane, intracellular retention of the protein would appear as nonfunctional protein. Therefore, we measured the cell surface expression of mutants that had low anion exchange activity (Table I). The basis of the cell surface expression assay is to express AE1 mutants in HEK293 cells, oxidize their cell surface carbohydrate with sodium metaperiodate, and react the oxidized carbohydrate with biocytin hydroride. Thus, only protein at the cell surface incorporates a biotin label. The level of biotin incorporation was quantified relative to the amount of each mutant that was expressed. Surface processing of each AE1 protein was then compared with AE1C–. Cell surface expression of AE1C– is only 66% as high as wild type AE1. As a negative control, we examined the processing of mouse AE1, shown previously to be retained intracellularly (22), and no cell surface expression could be detected. Among the nonfunctional mutants, only W648C and E681C have reduced processing to the cell surface. However, in neither case is the processing sufficiently impaired to account for the loss of transport activity (Table I). Therefore, reduced levels of processing to the cell surface do not explain loss of transport function observed for the six mutants listed above.

**DISCUSSION**
which places our proposed start of the transmembrane segment 21 residues from the glycosylation site. Experiments using introduced glycosylation sites mapped the N-terminal end of transmembrane segment 8 to Met663, within one residue of our determination (36).

One current view of AE1 is a funnel with a large outward facing mouth that narrows to a constricted permeability barrier and then opens slightly toward the cytosolic surface of the protein (37). In this model, anions proceed to the permeability barrier via a diffusional access channel (6, 38, 39). This model would suggest that a pore-lining region of AE1 would be both open and aqueous, at least in the outward facing region, outside the permeability barrier. The lack of labeling in the Met664–Gln683 region suggests that the structure of the substrate pathway is either much smaller (such that 524-Da biotin maleimide will not enter) or more hydrophobic (to prevent deprotonation of a cysteine side chain) than one might expect.

The other possibility is that the Met664–Gln683 region does not form one of the walls of the anion translocation channel. Although there is strong evidence for a direct involvement of Glu681 with anions moving across the membrane (6, 7, 9), there is no evidence that any other residues in the region interact with anions. In this interpretation, residues Met664–Gln683 would be a structural transmembrane segment, while Glu681 would extend into the aqueous channel. However, our data show that none of the Met664–Gln683 residues, including Glu681, gets labeled by biotin maleimide. This lack of reactivity is puzzling, since Glu681 reacts with Woodward’s reagent K (WRK) (5–7). However, WRK is smaller (253 Da) than biotin maleimide and has a negatively charged sulfonate group that would favor localization of the compound in the anion binding site of AE1. Taken together, Met664–Gln683 forms a transmembrane helix of AE1, and Glu681 may form part of the anion translocation channel.

Structure and Proposed Role of Extramembranous Regions—The regions of AE1 that are accessible to labeling by biotin maleimide may be structurally subdivided into three parts by their reactivity with LYIA: (i) residues Arg656–Met663 show a graded accessibility to LYIA, from high accessibility at Arg656 to low accessibility at Met663; (ii) residues Ser643–Leu655 show low accessibility to LYIA, with the exception of His651, which has high accessibility; and (iii) residues Ile684–Ser690 are poorly accessible to membrane-impermeant LYIA (Fig. 8).

The LYIA accessibility of region i is consistent with a vestibule or extramembranous “funnel” that forms a wide mouth to allow anion access to the transmembrane region. Consistent with the model is the dramatic drop in LYIA reactivity at the start of the transmembrane region. The declining reactivity may be explained by either increased hydrophobicity in the

### Table I

| AE1 mutant | Relative cell surface processing |
|------------|---------------------------------|
| W648C*     | 67 ± 17%                        |
| I650C*     | 113 ± 21%                       |
| P652C*     | 121 ± 5%                        |
| G654C      | 96 ± 3%                         |
| L655C*     | 104 ± 21%                       |
| F659C*     | 121 ± 14%                       |
| A671C      | 107 ± 9%                        |
| E681C*     | 61 ± 23%                        |

Fig. 7. Anion exchange rate of introduced cysteine mutants. Anion exchange activity was assayed as seen in Fig. 5. Transport rates were corrected for the background rate of vector (pRBG4)-transfected cells. Rates are expressed as a percentage of the rate of AE1C ± S.E. Mutants marked with an asterisk were not constructed.
region surrounding the cysteine residues, or steric exclusion of LYIA (649 Da). Either explanation is suggestive of a narrowing of the openness of the protein structure, moving from Arg656 to Met663. It is also possible that the Arg656 to Met663 region lies beneath the surface of the membrane bilayer, forming part of a transmembrane region, with a relatively open structure. Consistent with this interpretation is the observation that chymotrypsin/trypsin-treated erythrocyte membranes retained a peptide that spanned Ser657–Thr661, which was interpreted to represent a transmembrane segment (40). A functional role for region i is suggested by the transport activity of introduced cysteine mutants (Fig. 8). Of the eight residues from 656 to 663, three have lost transport activity and one other is greatly impaired. Region i is more sensitive to introduction of cysteine residues than the transmembrane region.

Residues Ser643–Leu655, in region ii, have lower accessibility to LYIA than might be expected for a sequence adjacent to the glycosylation site at 642. We propose that this region has a relatively inaccessible conformation, perhaps buried beneath the structure of region i. Region ii may support the structure of the vestibule. Alternatively, region ii inaccessibility to LYIA could be explained if the carbohydrate structure at Asn642 were hydrogen-bonded to the surface of AE1, sterically blocking access of LYIA. Precedent for hydrogen bonding of carbohydrate to the surface of a membrane protein comes from the crystal structure of viral hemagglutinin protein (41). In contrast to its low LYIA accessibility, region ii has a uniformly high accessibility to biotin maleimide. This would be explained by the membrane permeability of biotin maleimide, which would permit the compound to enter hydrophobic regions of protein/protein contact where the bulkier, more hydrophilic LYIA would be excluded. Introduced cysteine mutant H651C is the only exception to the observation that region ii is poorly accessible to LYIA. Since this mutant retains 68% transport activity, it is unlikely that H651C takes on a non-native conformation, so the localization of the introduced cysteine probably represents that in the native AE1 structure.

Conclusions—In this paper, we have developed an introduced cysteine method to determine membrane protein topology and applied this method to residues Ser643–Ser690 of human AE1. Central to the method is the use of LYIA to differentiate intracellular from extracellular sites. The method is able to differentiate transmembrane residues from extramembranous sites by virtue of an inability to label transmembrane cysteines with biotin maleimide. Our method may be widely applicable to the study of membrane protein topology. It has the advantage that structural perturbation of introduced cysteine mutations is milder than in other methods commonly used to determine topology.

Our data indicate that the amino acid sequence preceding transmembrane segment 8 of human AE1 may form a vestibule with an external open structure that leads to a constriction at the membrane surface. The vestibule may be supported by an underlying stretch of amino acids that is surprisingly close to the site of glycosylation. The extracellular portion of the protein may have a role in the anion exchange process. The intracellular extramembranous region forms an aqueous accessible intracellular loop. The transmembrane region spans 20 amino acids, and the Woodward’s reagent K-reactive glutamic acid residue, Glu681, is located close to the cytosolic face. Since
Glu$^{681}$ is 3 residues from the end of the transmembrane segment, it is located as close as 5 Å from the inner surface of the membrane if each residue translates 1.5 Å in a helical conformation (42). This places the permeability barrier much closer to the inner face than the outer. Such an asymmetric location for a permeability barrier was recently also identified in the structure of a bacterial K$^+$ channel, where the barrier was 12 Å from the intracellular face of the protein (43).

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