Topology of the Membrane Domain of Human Erythrocyte Anion Exchange Protein, AE1*

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Anion exchanger 1 (AE1) is the chloride/bicarbonate exchange protein of the erythrocyte membrane. By using a combination of introduced cysteine mutants and sulfhydryl-specific chemistry, we have mapped the topology of the human AE1 membrane domain. Twenty-seven single cysteines were introduced throughout the Leu709–Val911 region of human AE1, and these mutants were expressed by transient transfection of human embryonic kidney cells. On the basis of cysteine accessibility to membrane-permeant biotin maleimide and to membrane-impermeant lucifer yellow iodoacetamide, we have proposed a model for the topology of AE1 membrane domain. In this model, AE1 is composed of 13 typical transmembrane segments, and the Asp307–His834 region is membrane-embedded but does not have the usual α-helical conformation. To identify amino acids that are important for anion transport, we analyzed the anion exchange activity for all introduced cysteine mutants, using a whole cell fluorescence assay. We found that mutants G714C, S725C, and S731C have very low transport activity, implying that this region has a structurally and/or catalytically important role. We measured the residual anion transport activity after mutant treatment with the membrane-impermeant, cysteine-directed compound, sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES). Only two mutants, S852C and A858C, were inhibited by MTSES, indicating that these mutants formed the basis for a topology model of human AE1 membrane domain (Fig. 1). Twenty-seven single cysteine mutants of human AE1 were expressed by transient transfection of human embryonic kidney; AE2 is in a wide variety of tissue; and AE3 is in the brain, retina, and heart (1). All members of the anion exchanger family consist of two domains, an N-terminal cytoplasmic domain and a 55-kDa C-terminal membrane domain. The membrane domain is highly conserved (70% overall identity), spans the lipid bilayer 12–14 times, and is able to mediate anion transport by itself (2). Hydrophathy analysis of AE membrane domains shows 10 strong peaks of hydrophobicity (1).

Our goal is to map the topology of the human AE1 membrane domain, using substituted cysteine mutants and sulfhydryl-specific chemistry. Individual cysteine residues were introduced into a cysteineless form of AE1, called AE1C that was previously shown to be fully functional (3). Two sulfhydryl-directed compounds were used to characterize the introduced cysteine mutants as follows: (i) membrane-permeant biotin maleimide that covalently labels cysteine residues with a biotin group that is readily detected by streptavidin–biotin chemistry; and (ii) LYIA, a membrane-impermeant reagent, used to block the biotinylation of cysteines by the former compound. Introduced cysteine mutants were incubated with biotin maleimide, with or without a preincubation with LYIA. Biotinylation signals obtained for these mutants were interpreted as follows: no labeling with biotin maleimide implies localization to a hydrophobic membrane environment, biotinylation signal prevented by the preincubation with LYIA implies an extracellular localization, and biotinylation signal unaffected by LYIA implies an intracellular localization.

Previously we have used this methodology to map the topology of the Ser641–Ser890 region of AE1 (4). As expected, AE1C was not labeled by the sulfhydryl-directed compound biotin maleimide. In the 45 mutants that were made between residues Ser43 and Ser890, we found a stretch of 20 amino acids (Met662–Gln682) that were not labeled, implying that these residues formed an α-helical transmembrane segment (4). By contrast, introduced cysteine residues in the aqueous phase, either intracellular or extracellular, were labeled by biotin maleimide. A preincubation of the cells with the membrane-impermeant compound LYIA had little effect on the biotinylation of intracellular cysteines but impaired the biotinylation of most of the extracellular cysteine residues (4).

In this article, we used the same approach to study the topology of the second half of the membrane domain of human AE1. Twenty-seven single cysteine mutants of human AE1 were constructed between residues Leu709 and Val911. Analysis of these mutants formed the basis for a topology model of human AE1 membrane domain (Fig. 1). To identify amino acids important for the anion transport function, we also measured the anion transport activity of the introduced cysteine mutants, using a whole cell fluorescence assay (5). Mutants G714C, S725C, and S731C had low activity, indicating that this region has a structurally and/or catalytically important role. Furthermore, we analyzed the residual activity after cell treatment with the cysteine-directed compound MTSES. Of the 20 mutants analyzed, the compound significantly inhibited only two,
SS52C and A858C. This region contains Lys851, which is the site of labeling by the anion transport inhibitors 4,4'-disothiocyanodihydrostilbene-2,2'-disulfonate (6) and pyridoxal phosphate (7). Found here also is the naturally occurring P868L variant (Band 3 HT), which is characterized by an increased anion transport (8, 9). Our finding highlights the importance of the Lys851-Pro868 region for the anion transport function.

**EXPERIMENTAL PROCEDURES**

**Materials**—DMEM and all the tissue culture reagents were from Life Technologies, Inc. Biotin maleimide, biocytin hydrazide, BCECF-AM, lysine chloromethyl ketone, 5% fetal bovine serum, 5% calf serum. After 4–6 h the cells were seeded in a 100-mm Petri dish, in 10 ml of DMEM containing 5% fetal bovine serum, 5% calf serum and bovine serum albumin were from Sigma. Phenylmethylsulfonyl fluoride was supplied by ICN. Protein A-Sepharose CL4B was from Amersham Pharmacia Biotech. The ECL immunoblot detection reagents, streptavidin-biotinylated horseradish peroxidase, were from Toronto Research Chemicals. Poly-L-lysine, nigericin, DIDS, LYIA were from Molecular probes. MTSES and MTSEA were from Boehringer Mannheim. Mutants were verified by DNA sequencing.

**Plasmid Constructs and Site-directed Mutagenesis**—To express human AE1 in eukaryotic cells, we used the plasmid pRBG4 (10), in which an Accl-HindIII fragment containing the human AE1 cDNA (11) was cloned into the HindIII and EcoRI sites, using an AccI/EcoRI linker (3). The vector pJRRC9 coding for the wild-type human AE1 was previously cloned into the vector pJRRC26, where all five cysteine codons were replaced by serine codons (3). Individual introduced cysteine mutants were cloned in this cysteineless background to yield mutants, which were verified by DNA sequencing. Mutagenesis was performed using a megaprimer polymerase chain reaction methodology (12, 13). Polymerase chain reaction primers were designed using the Primers program (Whitehead Institute for Medical Research). Polymerase chain reaction was performed using an ERICOMP thermal cycler and either Vent DNA polymerase (New England Biolabs) or Pwo polymerase (Boehringer Mannheim). Mutants were verified by DNA sequencing.

**Transient Expression**—HEK cells were transiently transfected using the calcium phosphate technique, as described (4, 14). Briefly, 1.5 × 10^6 cells were seeded in a 100-mm Petri dish, in 10 ml of DMEM containing 5% fetal bovine serum, 5% calf serum. After 4–6 h the cells were transfected with 890 µl of a precipitate made as described (4). The cells were incubated at 37 °C, in a 5% CO_2 incubator, and harvested 48 h post-transfection.

**Topology Assay**—Assays proceeded as described previously (4). In brief, transfected HEK cells were washed with 10 ml of PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), pH 7.5, and allowed to lift up in 4 ml of PBS for 10 min at room temperature. The cells were collected, centrifuged (1000 rpm, 5 min), and resuspended in 2 ml of PBSCM (PBS supplemented with 0.1 mM CaCl_2 and 1 mM MgCl_2). Cell suspension (1 ml) was transferred into two tubes. One tube (labeled +) was supplemented with 50 µl of a LYIA solution (6.5 mg/ml in H_2O). Samples were incubated for 10 min at room temperature. Biotin maleimide (10 µl of a 10.4 mg/ml solution in Me_2SO) was then added, and cells were incubated for an additional 10 min, with occasional resuspension. The reaction was terminated by addition of 500 µl of 2% (v/v) 2-mercaptoethanol in DMEM (containing serum and antibiotics). After 5–10 min at room temperature, the cells were centrifuged and washed with 1 ml of PBSCM. The tube labeled minus was processed similarly, except that the preincubation step with the membrane-impermeant compound was omitted. The cells were lysed and AE1 immunoprecipitated, as described in a later section.

**Surface Processing Assay**—Transfected HEK cells were washed twice with 10 ml of PBS and allowed to lift up in 5 ml of PBS for 10 min. The cells were centrifuged (2000 rpm, 5 min) and resuspended in 5 ml of PBSCM containing 10 mM NaIO_4 and incubated for 30 min in the dark, at 4 °C, with occasional gentle resuspension of the cells. The cells were washed twice with PBSCM, resuspended in 1 ml of 100 mM sodium acetate, pH 5.5, and transferred to an Eppendorf tube. The suspension was supplemented with 250 µl of 10 mM biocytin hydrazide in 100 mM acetate buffer, pH 5.5. The biotinylation was carried on for 30 min, at 4 °C, in the dark, with occasional resuspension of the cells. Washing the cells twice with PBSCM stopped the reaction. The cells were lysed in IPB buffer, and AE1 was immunoprecipitated.

**Cell Lysis and AE1 Immunoprecipitation**—Cell lysis and AE1 immunoprecipitation were performed as described (4). Briefly, the cells were lysed on ice in IPB buffer (1% (v/v) Nonidet P-40, 5 mM EDTA, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 10 mM Tris-HCl, pH 7.5) supplemented with 2 mg/ml bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM N-tosyl-l-phenylalanine chloromethyl ketone, and 0.1 mM N-p-tosyl-l-lysine chloromethyl ketone. After centrifugation, the supernatant was cleared up with 1.5 µl of non-immune serum.

**Topology Model for the Membrane Domain of Human AE1 Protein**—Indicated cysteine mutants are labeled according to their introduced cysteine codon.}

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**Fig. 1.** Topology model for the membrane domain of human AE1 protein. Introduced cysteine mutants are labeled according to their introduced cysteine codon.
and protein A beads. AE1 was immunoprecipitated overnight at 4 °C, using 1.5 µl of the anti-human AE1 antibody 1657 and protein A beads. Antibody 1657 was produced by injecting rabbits with a synthetic peptide corresponding to the last 13 amino acids of human AE1 (4). The beads were washed as described (4) and resuspended in 30 µl of SDS-PAGE sample buffer (4% (v/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromphenol blue, 130 mM Tris-HCl, pH 6.8). Samples were heated at 65 °C for 4 min and centrifuged at 9000 rpm for 1 min prior to SDS-PAGE.

**SDS-PAGE and Immunoblotting—**Samples were electrophoresed on 8.5% acrylamide gel (15) and transferred to PVDF membranes (16). Biotinylated proteins were detected by incubating the membranes 1 h with 10 ml of TBST (0.1% (v/v) Tween 20, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5), containing 0.5% (w/v) bovine serum albumin and 3 µl of streptavidin-biotinylated horseradish peroxidase. The membranes were washed with TBST and developed using ECL reagent followed by exposure to x-ray film. To probe blots for AE1 expression, the membranes were stripped by incubation for 10 min at 50 °C in 100 mM captoethanol, 62.5 mM Tris-HCl, pH 6.8. The membranes were washed with TBST and then incubated overnight at 4 °C in 10 ml of TBST containing 5% (w/v) non-fat dry milk and 3 µl of the mouse monoclonal anti-human AE1 antibody, IVF12. The IVF12 antibody was a kind gift from Dr. Michael Jennings (17). The membranes were washed and incubated 1.5 h with 10 ml of TBST containing 5% (w/v) non-fat dry milk and 3 µl of an antibody anti-mouse IgG, horseradish peroxidase conjugate. After washing, the membranes were developed using ECL reagents followed by exposure to x-ray film.

**Image Analysis—**Films were scanned with a Hewlett-Packard scanner ScnJet 4C calibrated with a Kodak gray scale. The quantification of the signals was performed using the program NIH Image 3.60. Biotinylation signals were normalized to the amount of AE1 present in each sample by dividing the pixels of biotinylated AE1 by the pixels of the corresponding immunoblots as follows: biotinylation norm = pixels biotin signal/pixels AE1 immunoblot.

Each mutant was normalized to the biotinylation level observed for the mutant Y555C, treated in parallel, and electrophoresed on the same gel as follows: relative biotinylation = (biotinylation norm,mutant / biotinylation norm,Y555C) × 100%. LYIA accessibility was expressed as the ratio: LYIA accessibility = biotinylation norm,LYIA / biotinylation norm,LYIA.

**Anion Transport Assay—**To measure the anion exchange activity of AE1, HEK cells were grown on 11-mm glass coverslips and transfected. Cells were rinsed with serum-free DMEM and loaded with the pH-sensitive dye, BCECF-AM (2 mM final concentration), for 30 min. The coverslip was then suspended in a fluorescence cuvette with perfusion capabilities. Experiments were performed in a Photon Technologies International RCR fluorometer, using excitation wavelengths 440 and 503 nm and emission wavelength 520 nm. The cuvette was perfused alternately with Ringer’s buffer containing 10 (or 20) mM MTSES or 5 mM MTSEA. To remove non-covalent methanethiosulfonates, cells were washed for 300–500 s with chloride-free Ringer’s buffer and anion exchange was then assayed again.

Transport rates were determined by linear regression of the initial rate of change of pH, using the Kaleidagraph program. The MTSES inhibition data were expressed as residual activity after MTSES treatment and were calculated as shown in Equation 1.

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\text{Residual activity} = \frac{\text{transport rate after MTSES treatment}}{\text{transport rate before MTSES treatment}} \times 100\%
\]

**RESULTS**

**Cysteine Accessibility to Biotin Maleimide—**The membrane-permeant compound biotin maleimide reacts covalently with sulfhydryls to introduce a biotin group, which then can be readily detected on a blot, using streptavidin-biotinylated horseradish peroxidase followed by chemiluminescence. Fig. 2A represents a typical biotinylation profile of the AE1 mutants, and Fig. 2B shows the amount of AE1 in each sample. HEK cells that express AE1C- had no biotinylation signal on the blot at the position of AE1 (Fig. 2A), consistent with the fact that the compound only reacts with cysteine residues. All proteins are expressed to similar levels (Fig. 2B), but the reactivity of each individual cysteine residue with biotin maleimide varies greatly. Fig. 3 quantifies the biotinylation signal of each mutant relative to the Y555C mutant. Of the 27 mutants, 19 have no significant biotinylation signal (L708C, G714C, S725C, S762C, A767C, L775C, S781C, G790C, S801C, F806C, K814C, T830C, G838C, C843* (where the asterisk indicates endogenous cysteine found in wild-type AE1), S852C, A858C, T866C, R871C, and R879C). Previously we have established that introduced cysteine residues located in the bilayer region are not labeled with biotin maleimide (4); therefore, we propose that these unlabeled amino acids localize to the bilayer region, as modeled in Fig. 1. Among the biotin-labeled mutants, defined as aqueous-accessible, the reactivity of each individual cysteine to biotin maleimide can vary by a factor of 10 (Fig. 3). Intracellular (Y902C) and extracellular (Y555C) control mutants were readily labeled by biotin maleimide, indicating that differences in reactivity were not related to their intra- or extracellular localization but rather to their exposure to the aqueous environment. Biotinylation data for the Y904C mutant are not shown, because this mutant is not recognized by the mouse monoclonal antibody IVF12 we are using to normalize the biotinylation signals. This lack of reactivity is interesting, since it defines the
Each introduced cysteine mutants was treated with biotin maleimide as described. Biotin incorporation and AE1 expression level were quantified by densitometry. The biotinylation signals were normalized by the amount of AE1 present in each lane. In each experiment, the level of biotinylation was compared with that of the Y555C mutant, whose labeling was set to 100. Data represent mean of 4–5 independent experiments ± S.E.

**Accessibility to LYIA**—To determine the transmembrane topology of AE1-introduced cysteine residues, we measured the ability to block biotin maleimide labeling by prior incubation with the membrane-impermeant compound LYIA (4). In these experiments, biotin incorporation was measured for cells treated with and without LYIA, giving rise to “+” and “−” samples, respectively. After scanning, the signals were quantified and normalized for the amount of AE1 present in each lane, and the LYIA accessibility factor was calculated as the −/+ ratio. Preincubation with LYIA blocked biotinylation of extracellular cysteines, whereas labeling of intracellular cysteines was unaffected. For an extracellular cysteine the accessibility factor was high (the higher the factor, the more accessible to LYIA), whereas it was close to 1 for a cytosolic residue.

Fig. 4 shows a typical LYIA accessibility experiment, and the mean of four independent experiments ± S.E. is shown in Fig. 5. In the particular experiment shown in Fig. 4, D821C had an uncharacteristically low labeling with biotin maleimide, but the average value for several experiments is seen in Fig. 5. Control mutants with a known cysteic localization were S95C and K892C, the former being present in the cytosolic loop between TM 6 and 7, whereas the latter is present in the cytoplasmic C-terminal tail (19–21). Control extracellular mutants were Y555C and S643C, which are located in the loop that carries the external chymotrypsin sites (22) and the loop that is glycosylated in AE1 (1). There was a clear difference between inside and outside residues; the LYIA accessibility values for the inside controls were 1.4 and 1.5 for the K892C and S95C mutants, respectively (Fig. 5). The ratios obtained for the outside controls were always higher, typically around 5.0 for the Y555C mutant (Fig. 5), which is similar to values found previously for the S643C mutant (4). This shows that the assay distinguishes between an intracellular or extracellular localization of the introduced cysteine residue.

Mutants S731C, G742C, S745C, A751C, and D821C had LYIA accessibility similar to the outside control Y555C (Figs. 4 and 5). In contrast, cysteine residues in position C885* and K892C were relatively insensitive to LYIA treatment, as expected due to the intracellular localization of the C-terminal tail of AE1. On the basis of LYIA accessibility data, residues S731, G742, S745, A751, and D821 were mapped to an extracellular site (gray circles in Fig. 1), whereas C885* and K892C were mapped to intracellular sites and (hatched circles in Fig. 1).

**Anion Exchange Activity of AE1 Mutants**—Anion exchange activity was measured by monitoring intracellular pH changes associated with Cl−/HCO3− exchange in a whole cell fluorescence assay (10). The anion exchange activity of cells expressing human AE1 proteins was well above the background, typically 6.5 times higher than vector-transfected cells. The activity of AE1C− was around 0.2 pH/min. Anion exchange activity of each AE1 mutant was expressed relative to AE1C− (Fig. 6). Six of the 27 mutated AE1s retained less than 40% of the activity of AE1C−: G714C, S725C, S731C, S762C, G790C, and F806C (Fig. 6). Three mutants with highly reduced transport activity were found in the region Gly714–Ser731, which may indicate that this region is important for anion transport function.

**Surface Processing of AE1 Mutants**—The anion exchange assay measures transport across the plasma membrane, so that only proteins present in the plasma membrane are assayed. Impaired anion exchange activity may therefore result either from mutation of a functionally or structurally important amino acid, or intracellular retention of AE1 proteins. To distinguish between these possibilities, cell-surface expression was analyzed for the mutants that had reduced anion exchange activity. In the cell-surface expression assay, we measured the biotinylation of oxidized cell-surface carbohydrates by a membrane-impermeant compound, biocytin hydrazide (4). The level of biotin incorporation was normalized to the amount of AE1 present in each sample. Results were then expressed relative to AE1C−, as seen in Table I. As a control, we examined the processing of an aberrantly processed mutant of mouse AE1, shown previously to be retained intracellularly (14, 23), and no cell-surface expression could be detected (4). Of the six mutants that were characterized, only F806C was processed to a lesser extent than AE1C− (73 ± 23%, Table I). However the reduction in surface processing does not account entirely for the loss of transport activity. Therefore the low activity of the F806C mutant is due to a combination of reduced surface processing and a negative structural change. Since mutants G714C, S725C, S731C, S762C, and G790C were processed to the same extent as AE1C−, these mutations impair transport function.

**Effect of Sulfhydryl Reagents on Anion Exchange Activity**—The ability of the membrane-impermeant, cysteine-directed compound MTSES to inhibit transport was investigated for all functional mutants. Fig. 7 shows that MTSES had no influence on anion exchange activity of T866C, whereas A858C was inhibited by the reagent. Residual activity after MTSES treatment was measured for each mutant and is shown in Fig. 8. For most of the mutants, the incubation of the cells with a 10 mM concentration of MTSES had no effect on anion exchange activity. However, S852C and A858C had 72 ± 6 and 56 ± 12% residual transport activity, after MTSES treatment, respectively. The inhibition of S852C and A858C was greater after 8 min incubation with 20 mM MTSES, approximately 50% inhibition (data not shown). The observed level of inhibition is particularly significant, since an 8-min incubation with 0.3 mM DIDS, a potent anion exchange inhibitor, resulted in 24% residual activity. To confirm the methanethiosulfonate inhibition data, S852C and A858C were also treated with 5 mM MTSEA; the residual activities were 78 and 112%, respectively. In Fig. 1, these mutants are located close together in TM 12 and 13. Clearly, these results define this region as being involved in anion transport.
In this paper we have mapped the topology of the second half of the membrane domain of human AE1, using introduced cysteine mutants and sulfhydryl reagents. The ability to label with biotin maleimide defines residues that are located in an aqueous environment, either intra- or extracellular. Sites that could not be labeled by biotin maleimide were defined as transmembrane sites, in the plane of the bilayer. Accessibility to LYIA, a membrane-impermeant compound, defines aqueous-accessible residues that are located on the extracellular side of the transmembrane permeability barrier. LYIA may also access aqueous sites within the plane of the lipid bilayer, if they are part of a sufficiently large pore. On the basis of the data presented in this article, we propose a new model for the topology of the membrane domain of AE1 (Fig. 1). This model contains 13 α-helical transmembrane segments, and not 14, as seen in previous models (1). As well, it contains one transmembrane region with a non-helical, extended conformation.

The cysteine-directed approach we have used to map the topology of the membrane domain of AE1 was validated previously in our study of 45 consecutive single cysteine mutants of human AE1 (4). Among these cysteine mutants, only a stretch of 20 amino acids (TM 8) was not labeled with biotin maleimide.
A similar approach to study topology of the tetracycline transporter of *Escherichia coli* used [14C]N-ethylmaleimide as cysteine labeling reagent (24, 25). A stretch of 24 consecutive amino acids was not labeled because it forms an α-helical transmembrane segment (25). As well, among 27 mutants located in extramembranous loops, either intracellular or extracellular, only one mutant could not be labeled with [14C]N-ethylmaleimide (24). Similarly, in human P-glycoprotein, introduced cysteines located within the plane of the lipid bilayer or in its immediate vicinity (within two amino acids) were not labeled using 200 μM biotin maleimide for 30 min; all cysteines in the aqueous phase were significantly labeled (26).

We conclude that in most cases the failure of a cysteine residue to react with maleimide is due to the localization of that residue in the plane of the lipid bilayer.

The region studied in this paper begins with Ile684 (following TM 8) and ends at Val911 (the C terminus). In a previous study, we established that the region Ile684-Ser690 is located in the cytoplasm (4). The first three introduced cysteine mutants prepared for the current study were L708C, G714C, and S725C. Since none of these could be labeled by biotin maleimide, they are modeled as transmembrane regions.

S731C, G742C, S745C, and A751C define an aqueous-accessible region, since all of these could be labeled with biotin maleimide. LYIA accessibility of these mutants indicates an extracellular localization of the Ala726-Ile761 region. Recently, Popov et al. (27) showed that AE1 mutants with a single N-glycosylation site in the loop Ala726-Ile761 could be N-glycosylated when expressed in an in vitro transcription/translation system. Following the 12 + 14 rule described for the glycosylation of an external loop by the oligosaccharyltransferase (28), this extracellular aqueous loop appears to contain the amino acids Trp723-Leu764 and was called the “T-loop” by the authors (27). To accommodate the data concerning a low ionic strength internal tryptic site in position Lys743 (17, 22), Popov et al. (27) proposed that this region was folded back into the membrane, with Lys743 close to the cytosol. Our topology data agrees with Popov et al. (27) concerning the extracellular localization of the

**Fig. 7.** Measurement of the residual activity after treatment with the cysteine-directed, membrane-impermeant compound, MTSES. HEK cells transfected with the cDNA coding for T866C (A) and A858C (B) were grown on glass coverslips. The coverslips were incubated with the pH-sensitive dye BCECF-AM and then suspended in a fluorescence cuvette. The cells were perfused either with chloride-free Ringer’s buffer (white bar), chloride-containing Ringer’s buffer (gray bar), or chloride-free Ringer’s buffer containing 10 mM MTSES (black line). Anion exchange rates were measured from the initial slopes observed after buffer changes. Residual activity is expressed as a percentage of the value obtained before MTSES treatment. Both alkalization and acidification slopes were taken into consideration to calculate the residual activity.
loop Ala$^{726}$-Ile$^{761}$. These residues may form an extracellular loop or the loop could enter the membrane. However, if it does so, the loop must be in an open, aqueous-accessible structure, large enough to allow access of LYIA.

Following the T-loop region are seven mutants that are inaccessible to biotin maleimide, and which we have modeled as part of two transmembrane segments. Following these transmembrane segments, three introduced cysteine mutants were made in the Asp$^{807}$-His$^{834}$ region. The Asp$^{807}$-His$^{834}$ region is not predicted to be a transmembrane region by conventional hydropathy analysis (1), since it is composed of both hydrophobic and charged amino acids. However, the data presented here showed that this region did not behave like an aqueous loop, since only D821C could be labeled by biotin maleimide. Since Asp$^{821}$ is LYIA-accessible, it is on the extracellular side of the permeability barrier. We do not have sufficient data to determine the conformation of the protein in this region. The length of the sequence (25 amino acids) is sufficient to form a helical transmembrane segment. If the region forms a helix, then Asp$^{821}$ would have to face an aqueous pore, outside the permeability barrier, and Lys$^{851}$ and Thr$^{830}$ would face away from the pore, inaccessible to biotin maleimide and LYIA. This region could also form an extended structure, as we have shown in the model (Fig. 1). Consistent with an extended structure is the presence of several prolines and glycines (high turn propensity amino acids).

Our model of topology for the Asp$^{807}$-His$^{834}$ region needs to be reconciled with experiments using the monoclonal antibody Bric132, which recognizes the Phe$^{813}$-Tyr$^{824}$ sequence of human AE1 (21). The authors have two lines of evidence for an intracellular localization of this epitope as follows: (i) there is no direct and/or indirect agglutination of intact human erythrocytes, using this antibody, and (ii) Bric132 antibody can immunoprecipitate AE1 only in leaky erythrocytes (29). Preparation of leaky erythrocytes exposes membranes to low ionic strength medium, and AE1 conformation changes in low ionic strength medium (17, 30). We propose that the Bric132 epitope is buried in isotonic conditions, but in low ionic strength solutions, the region becomes accessible either because it moves or some part of AE1 that covers it moves out of the way. Consistent with this interpretation, this region is not accessible to proteases under native conditions, but after alkaline treatment proteolytic cleavage is observed (6). Asp$^{821}$ could form a salt bond that is disrupted under altered conditions of ionic strength, leading to alternate conformations. The D821C mutation could induce a similar change of accessibility; however, the change must not be dramatic since the D821C mutant has nearly full anion exchange function. An intracellular localization of Asp$^{821}$ has also been reported on the basis of chemical modification studies (31), which underscores the need for further study of this region.

We have modeled the Leu$^{835}$-Arg$^{879}$ region as two TM, but our data cannot rule out other models. The C terminus of the region is well defined by the ability to label Cys$^{885}$ with biotin maleimide. However, the inability to label any of seven introduced cysteine residues in this region with biotin maleimide does not allow us to define the other ends of the helices in this region. The presence of such a long stretch of amino acids that cannot be labeled by biotin maleimide suggests that the protein is compactly folded in the membrane bilayer. Any extramembranous loop may be too small to be labeled by biotin maleimide.

Our LYIA accessibility data indicate that the C terminus of AE1 is cytosolic, consistent with previous results (19–21). Since C885*, K892C, and Y904C could be labeled by biotin maleimide, these residues are in an aqueous environment. The lack of labeling of the R879C mutant suggests that the cytosolic C-terminal tail begins somewhere between Asn$^{860}$ and Cys$^{885}$. Previous work using carboxypeptidase Y digestion and epitope mapping was unable to define the length of the exposed C-terminal tail (19, 21, 32).

Results obtained by proteolytic digestion of erythrocytes membranes pretreated with 100 mM NaOH (6, 33, 34) fit remarkably well with our model (Fig. 1). When placed on our model, all of the proteolytic sites are found in extramembranous regions, except those surrounding Asp$^{821}$. The proteolysis data suggest that transmembrane segments are inaccessible to proteolysis. However, we propose that the region surrounding Asp$^{821}$ forms an extended structure that may be removed from the membrane upon alkaline treatment. Alkaline treatment is known to disrupt protein–protein contacts and denature proteins (33).

We analyzed the effect of the cysteine-directed compound MTSES on the anion exchange activity of our introduced cysteine mutants. MTSES and related methanethiosulfonate compounds have been used to probe the function of introduced cysteine residues in ion channel proteins (35, 36). We infer that inhibition by MTSES results from steric blockage of a small anion access channel, at the site of the cysteine residue. Our results identified only two of 21 active mutants, S852C and A858C, whose anion exchange activity was sensitive to sulfhydryl reagents. Therefore we interpret S852C and A858C mutations to reside in a spatially restricted area, possibly part of the anion translocation channel. The membrane impermeability of MTSES indicates that S852C and A858C lie outside the transmembrane permeability barrier. These mutants are located within the last two TM of the anion exchanger, thereby defining this region as important for the anion transport. This region also contains Lys$^{851}$, which is the lysine residue that reacts with DIDS (6) and pyridoxal phosphate (7), two potent inhibitors of the anion exchange function. As well, this region contains the P868L mutation found in naturally occurring Band 3 HT variants, which are characterized by an increased anion transport (8, 9). Taken together, these results show the importance of the Lys$^{851}$-Pro$^{868}$ region for anion transport function.

In this study we have added 27 topological constraints to...
models of the C-terminal region of AE1. The lack of labeling with biotin maleimide has placed limits on the boundaries of TM 9–11. We have confirmed the presence of an extracellular localization for the Ala834 sequence, for which we could not assign a clear topology. The inability to label the Leu835-Arg879 region with biotin maleimide indicates that the region has a compact structure, whose topology cannot be clearly modelled by our data. However, the presence of two MTSES-sensitive sites in the Leu835-Arg879 region suggests that these sites line the translocation channel of AE1. The most unusual region analyzed was the Asp807-Arg879 region, which suggests that these sites line the translocation channel of AE1. The lack of labeling with biotin maleimide has placed limits on the boundaries of the Ala726-Ile761 sequence, consistent with recent results observed by glycosylation scanning mutagenesis. In summary, introduced cysteine mutants and chemical modification have provided evidence for the topology model proposed in Fig. 1. Absolute determination of the topology of AE1 awaits a crystal structure of the protein.

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