Novel Topology in C-terminal Region of the Human Plasma Membrane Anion Exchanger, AE1*

Quansheng Zhu†, Diana W. K. Lee§, and Joseph R. Casey¶
From the Canadian Institutes of Health Research Membrane Protein Research Group, Department of Physiology, and Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Human AE1 performs electroneutral exchange of Cl\(^-\) for HCO\(_3^-\) across the erythrocyte membrane. We examined the topology of the AE1 C-terminal region using cysteine-scanning mutagenesis and sulphydryl-specific chemistry. Eighty individual cysteine residues, introduced into an otherwise cysteine-less mutant between Phe\(^{806}\) and Cys\(^{885}\), were expressed by transient transfection of HEK293 cells. Topology of the region was determined by comparing cysteine labeling with the membrane-permeant cysteine-directed reagent biotin maleimide, with or without prior labeling with the membrane-impermeant reagents, bromothymol blue and lucifer yellow iodoacetamide (LYLA). Phe\(^{806}\)–Leu\(^{835}\), Ser\(^{852}\)–Ala\(^{855}\), and Ile\(^{872}\)–Cys\(^{885}\) were labeled by biotin maleimide, suggesting their location in an aqueous environment. In contrast, Phe\(^{836}\)–Lys\(^{851}\) and Ser\(^{856}\)–Arg\(^{871}\) were not labeled by biotin maleimide and therefore localized to the plane of the bilayer, as transmembrane segments (TM). Labeling by qBBr revealed that Pro\(^{815}\)–Lys\(^{829}\) and Ser\(^{852}\)–Ala\(^{855}\) are accessible to the extracellular medium. Pro\(^{815}\)–Lys\(^{829}\) mutants were also labeled with LYLA. Mutants Ile\(^{872}\)–Cys\(^{885}\) were inaccessible to the extracellular medium and thus localized to the intracellular surface of AE1. Functional assays revealed that one face of each of two AE1 TMs was sensitive to mutation. Based on these results, we propose a topology model for the C-terminal region of the membrane domain of human AE1.

Human AE1, also called Band 3, is the most abundant integral membrane protein of the erythrocyte membrane (50% membrane protein, 1.2 \(\times\) \(10^6\) copies per cell) (1). AE1 facilitates the electroneutral exchange of Cl\(^-\) for HCO\(_3^-\) across the plasma membrane. AE1 also maintains the flexible biconcave disc shape of the erythrocyte through interaction with the spectrin/actin cytoskeleton, mediated by ankyrin (2). In aged erythrocytes, AE1 serves as the senescence antigen for clearance from the circulation (3, 4). In addition, AE1 participates in the adhesion of malaria-infected erythrocytes to endothelial cells (5). Mutation or deletion of the AE1 gene induces blood group antigens, variant erythrocyte morphologies, and human diseases, including the Diego\(^a\) blood group antigen (6), southeast Asian ovalocytosis (7), and erythroid spherocytosis (8). AE1-deficient mice have retarded growth, hemolytic anemia, and a high rate of neonatal death (9).

Human AE1 belongs to a multigene family consisting of three members. AE1 is found in the erythrocytes, and an N-terminal truncated form is present in the kidney; AE2 is found in a variety of tissues; and AE3 is found in the brain, retina, and heart. AE1 is a 110-kDa protein composed of 911 amino acids. AE1 has two domains: a 55-kDa membrane domain, which is highly conserved with AE2 and AE3, and a 45-kDa cytoplasmic domain. The membrane domain is responsible for anion exchange activity and is predicted to span the lipid bilayer 12–14 times (10). The structure of the AE1 cytoplasmic domain was recently determined by x-ray crystallography (11).

The C-terminal portion of the AE1 membrane domain is implicated in the anion translocation process. Two AE1 inhibitors, pyridoxal phosphate (PLP) and DIDS, both react with Lys\(^{851}\) in the C-terminal region (12, 13). Mutagenesis and methylation studies also highlight the functional importance of Lys\(^{851}\) (14, 15). The naturally occurring P868L mutation in this region substantially increased anion exchange activity (16). Also, the Asp\(^{821}\)–His\(^{834}\) region is involved in the adhesion of malaria-infected erythrocytes to endothelial cells (5). This stretch was also identified as the senescence antigen of aged erythrocytes (17). Some proteolytic sites in the AE1 C-terminal region were accessible only after treatment of erythrocytes with high concentrations of sodium hydroxide, suggesting that those regions normally are folded into the AE1 structure (18). In addition, carbonic anhydrase II binds to the LDADD motif in the cytoplasmic C-terminal tail to facilitate HCO\(_3^-\) transport activity (19).

Determination of the topology of AE1 is important because of the role of AE1 as a cell surface protein and in the anion translocation process. Although topology of the membrane domain of AE1 has been studied extensively, a clear picture of the C-terminal region remains elusive. Hydrophy analysis of the region yields ambiguous results, with unclear transmembrane segments (10). Topology models have been proposed for this region based on experimental evidence including, epitope mapping (20), N-glycosylation-scanning mutagenesis (21, 22), protease accessibility (23), and cysteine-scanning mutagenesis (24, 25). In this report, we examined the topology of the C-terminal

\* This research was supported by an operating grant from the Canadian Institutes of Health Research. The costs of publication of this article must therefore be hereby marked "in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Funded by a studentship from Canadian Blood Services.

‡ Supported by a summer studentship from Alberta Heritage Foundation for Medical Research (AHFMR).

§ Senior Scholar of the AHFMR. To whom correspondence should be addressed: Dept. of Physiology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. Tel.: 780-492-7203; Fax: 780-492-8915; E-mail: joe.casey@ualberta.ca.

1 The abbreviations used are: DIDS, 4,4\'-diisothiocyanatostilbene-2,2\'-disulfonic acid; LYLA, lucifer yellow iodoacetamide; AE1C, cysteine-less AE1; BCECF-AM, 7\'-\(N\)-\(\beta\)-carboxylethyl)-1 and 6\'-carboxyfluorescein, acetoxymethyl ester; biotin maleimide, 3-(\(N\)-maleimidylpropionyl)biocytin; HEK, human embryonic kidney; qBBr, bromothymol blueimunobromide bromide; TLCK, N-\(\beta\)-tosyl-L-lysine chloromethyl ketone; TM, transmembrane segment; TPCN, N-tosyl-L-phenylalanine chloromethyl ketone; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.
region of AE1, using cysteine-scanning mutagenesis and sulf-hydryl-specific chemical labeling. Cysteine-scanning mutagenesis has been applied widely to study membrane transporters (26, 27). Mutation of individual amino acids to cysteine represents a minor structural modification. Therefore, it possesses some potential advantages over other approaches, as most mutant proteins remain functional. We constructed 80 introduced cysteine mutants at each position between amino acid Phe^{806} and Cys^{885}, spanning the C-terminal quarter of the membrane domain. Accessibility of each individual cysteine mutant protein to membrane permeant and impermeant chemical reagents was assayed. On the basis of these results, we propose a topology model for the C-terminal region of human AE1. A preliminary version of this work has been published as an abstract (28).

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were from New England Biolabs. Pwo DNA polymerase was from Roche Molecular Diagnostics. Plasmid preparation kits were from Qiagen. T4 DNA ligase, DMEM and all cell culture reagents were from Invitrogen. 3-(N-Maleimidylpropionyl) biocytin (biotin maleimide) and lucifer yellow iodoacetamide (LYIA) were from Molecular Probes. Monobromotrimethylammoniobutyrophenone-maleimide (biotin maleimide) and lucifer yellow iodoacetamide (LYIA) were from Toronto Research Chemicals. Protein A-Sepharose-CLAB, streptavidin/biotinylated-horseradish peroxidase complex, sheep anti-mouse IgG-conjugated horseradish peroxidase and ECL reagents was assayed. On the basis of these results, we propose a topology model for the C-terminal region of human AE1. A preliminary version of this work has been published as an abstract (28).

Site-directed Mutagenesis—A previously constructed human AE1 cDNA with all five endogenous cysteine codons mutated to serine (AE1C) was used as the template for site-directed mutagenesis (29). All AE1 constructs were cloned into the pRBG4 mammalian expression vector (30). Silent BatEII and MluI sites were first introduced into AE1C cDNA at codons 804 and 824, respectively, to facilitate cloning and screening of mutant DNAs. Mutagenesis was performed using a PCR-based megaprimer mutagenesis strategy (24). The mutagenic primers were designed using the Primers program (Whitehead Institute for Medical Research). PCR was performed using an ERICOMP thermal cycler and Pwo DNA polymerase. Cysteine codons were individually introduced in AE1C cDNA at each position corresponding to amino acid Phe^{806}–Cys^{885} at BatEII and EcoNI sites. Each mutant cDNA contains only a single cysteine codon. All mutants were verified by DNA sequencing.

Protein Expression—Mutant AE1 cDNAs were expressed by transient transfection of human embryonic kidney 293 cells (HEK), as previously described (25). Briefly, HEK cells were plated onto 60-mm dishes in 4 ml of DMEM, containing 5% (v/v) fetal bovine serum, 5% (v/v) calf serum (Invitrogen). 6–9 h following seeding, cells were transfected with mutant plasmids, using the calcium phosphate precipitation method (25). Cells were grown at 37 °C in a 5% CO_{2} atmosphere and harvested 48 h post-transfection. In some experiments, dishes were precoated with polylysine, to increase cell adhesion. Culture dishes were incubated with pollysine solution (0.1 mg/ml in distilled water) for 30 min in the tissue culture hood. Dishes were washed with water and dried under UV light overnight.

Biotin Maleimide Labeling Assay—Labeling with biotin maleimide proceeded as described previously (25). Transfected HEK cells were washed with 5 ml of PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na_{2}HPO_{4}, 1.5 mM KH_{2}PO_{4}, pH 7.4) and allowed to detach in 2 ml of PBS for 10 min, at room temperature. Cells were collected and sedimented by centrifugation for 5 min at 800 × g. Cells were then resuspended in 1 ml of PBSCM (PBS containing 0.1 mM CaCl_{2} and 1 mM MgCl_{2}, pH 7.0) and incubated with a final concentration of 0.2 mM biotin maleimide (added from a 20 mM stock in Me_{2}SO). After a 10-min incubation at room temperature, the sample was added to the addition of 1 ml of stop solution. Cells were then washed, collected, and lysed in IPB buffer. As a control for the labeling procedure, the intactness of cells was assessed. Mutant Arg^{808} was cloned into the AE1C background. C479S and R808C/C479S were both constructed in a wild type AE1 background and therefore have four endogenous cysteine residues.

**Fig. 1.** Representative data of labeling human AE1 introduced cysteine mutants with biotin maleimide. HEK cells were transiently transfected with human AE1 cDNAs, as indicated in the figure. Cells were harvested and incubated with 0.2 mM biotin maleimide for 10 min, at room temperature. After solubilization, samples were immunoprecipitated with anti-AE1 antibody, subjected to electrophoresis on 8% acrylamide gels and transferred to polyvinylidene difluoride membrane. Incorporated biotin was detected by horseradish peroxidase-streptavidin and ECL, as indicated. Blots were stripped and probed with monoclonal anti-AE1 antibody to detect the amount of AE1 in each sample, as indicated. A and B, individual cysteine codons were introduced into AE1C background at amino acid positions indicated at the top of each panel. C, biotin maleimide labeling of intracellular AE1 was assessed. Mutant Arg^{808} was cloned into the AE1C background. C479S and R808C/C479S were both constructed in a wild type AE1 background and therefore have four endogenous cysteine residues.
and washed consecutively with wash buffer 1 (0.1% Igepal, 1 mM EDTA, 4°C in place of preimmune serum, and the mixture was incubated at room temperature with constant rotation. Resin was then removed by centrifugation at 7,500 g for 1 min to sediment any insoluble material. The amount of biotin incorporated into each introduced cysteine mutant was detected with horseradish peroxidase-streptavidin and ECL reagent. Blots were stripped and probed with anti-AE1 antibody, as indicated.

**Anion Exchange Assays**—HEK cells were grown on polylysine-coated glass coverslips in 60-mm tissue culture dishes and transfected as described. Two days post-transfection, cells were rinsed with serum-free DMEM (Invitrogen) and incubated with in 4 ml of serum-free DMEM medium containing 2 μM BCECF-AM (37°C, 30 min). Coverslips were mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer’s buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM sodium bicarbonate, 2.5 mM NaH2PO4, 25 mM NaHCO3, 10 mM Heps, pH 7.4), containing either 140 mM sodium chloride (chloride buffer) or 140 mM sodium gluconate (chloride-free buffer). Both buffers were bubbled continuously with air containing 5% carbon dioxide. Intracellular pH was measured by measuring fluorescence changes at excitation wavelengths 440 and 502 nm and emission wavelength of 520 nm, in a Photon Technologies International RCR/DeltaScan spectrofluorometer. Intracellular pH was calibrated, using the nigericin-high potassium method (32), with three pH values between 6.5 and 7.5. Transport rates were determined by linear regression of the initial linear rate of change of pH, using Sigma plot software.

**Image Analysis and Data Analysis**—Films from immunoblots and chemical biotinylation blots were scanned with a Hewlett Packard Image Analysis and Data Analysis—Films from immunoblots and chemical biotinylation blots were scanned with a Hewlett Packard Image Analysis and Data Analysis—Films from immunoblots and chemical biotinylation blots were scanned with a Hewlett Packard
Scanjet 4C scanner, calibrated with a Kodak gray scale. Scanned film images were quantified using NIH Image 1.60 software. Images obtained using KODAK image station 440CF were quantified by KODAK ID image analysis software. Biotinylation levels were calculated according to Equation 1.

\[
\text{Biotinylation norm} = \frac{\text{Pixels biotin signal}}{\text{Pixels AE1 immunoblot}} \quad \text{(Eq. 1)}
\]

Each mutant was normalized to the biotinylation level of mutant Y555C, which was treated in parallel and electrophoresed on the same acrylamide gel. Normalized data were calculated according to Equation 2.

\[
\text{Relative Biotinylation} = \frac{\text{Biotinylation norm mutant}}{\text{Biotinylation norm Y555C}} \times 100\% \quad \text{(Eq. 2)}
\]

where qBBr and LYIA accessibility were calculated according to Equation 3 (for LYIA, LYIA replaced qBBr).

\[
\text{Accessibility} = \frac{\text{Biotinylation norm - qBBr}}{\text{Biotinylation norm - qBBr}} \quad \text{(Eq. 3)}
\]

Statistical analysis—Standard errors were calculated with KaleidaGraph 3.5 software (Synergy Software).

RESULTS

Construction of Introduced Cysteine Mutants at the C Terminal of AE1—Eighty consecutive introduced cysteine mutants were constructed between Phe806 and Cys885 in the C-terminal region of AE1, where 12 mutants were reported previously (25). Each cysteine mutant was cloned into AE1C, an AE1 mutant with all five endogenous cysteine codons mutated to serine (29). Each mutant protein contains only a single cysteine residue. Some mutants outside the C-terminal region were used as controls for the labeling protocol. Y555C was used as the extracellular surface control, since it is adjacent to the two chymotryptic cleavage sites in intact erythrocytes (33); K892C was used as intracellular surface control because it is located in the C-terminal tail of the protein, which previously mapped to the cytoplasm (20, 34). Cys201, an endogenous cysteine residue located in the N-terminal cytoplasmic domain of AE1, was also cloned into AE1C as an intracellular surface control.

Labeling of Introduced Cysteines with Biotin Maleimide—Biotin maleimide, a membrane permeant chemical reagent, reacts with the sulfhydryl groups of cysteine residues to introduce a biotin group into the labeled protein through a thioether bond. Biotin can be detected by streptavidin conjugated horseradish peroxidase on blots. Since biotinylation occurs only in the aqueous environment, cysteine residues in the aqueous medium can react with biotin maleimide, whereas cysteine residues in the TMs cannot (24). We labeled intact HEK cells that expressed a single cysteine mutant AE1 protein with biotin maleimide. Fig. 1 presents representative data from the biotin maleimide labeling experiments. AE1C was very slightly biotin-labeled, consistent with the absence of cysteine residues in the construct and the limited reactivity of biotin maleimide toward primary amines. Extra and intracellular control mutants Y555C and K892C, were both strongly biotinylated. All mutant proteins were expressed to a similar level as shown in Fig. 1. The reaction of each individual cysteine mutants with biotin maleimide varied greatly, due to varying degrees of exposure to the aqueous medium.

The majority of AE1 expressed in transfected HEK cells is retained in intracellular membranes (24). This intracellular...
protein may be misfolded, although intracellular-retained AE1 has previously been shown to be functionally active (35). Thus, it is important to determine if any intracellular AE1 is labeled by biotin maleimide, as this protein may not have native structure. The R808C mutation has previously been shown to cause retention of AE1 in intracellular membranes (36). To determine whether intracellular AE1 is accessible to labeling by biotin maleimide we constructed the R808C/C479S double mutant in a wild type AE1 background. C479S was used as the cloning background for R808C because of availability of restriction sites. C479S AE1 (with four endogenous cysteine residues) and R808C/C479S AE1 were treated with biotin maleimide, in the same way as each of the introduced cysteine mutants (Fig. 1, A and B). AE1C/H11002 and R808C were not significantly labeled (Fig. 1C), consistent with the absence of cysteine residues in AE1C. While the four endogenous Cys residues in C479S were strongly labeled by biotin maleimide, R808C/C479S incorporated 10% as much biotin. Therefore, the R808C mutation causes intracellular retention of AE1, which causes greatly reduced labeling of the protein’s cysteine residues. We conclude that in the analysis of the Arg 806–Cys885-introduced cysteine mutants (Fig. 1, A and B), intracellular-retained AE1 contributed <10% of the biotinylation signal.

The ability to label each introduced cysteine mutant in the Phe806–Cys885 region with biotin maleimide is quantified in Fig. 2. The biotin signal of each introduced cysteine mutant was quantified by densitometry of the biotinylation blot and the corresponding anti-AE1 immunoblot. Data were then normalized to the Y555C mutant, which was used as an internal standard in each experiment. Three regions (Phe815–Leu827, Ser852–Ala855, and Val876–Cys885) stood out as labeled in a background of otherwise unlabeled or weakly labeled mutants. The strong labeling of these three regions is consistent with aqueous-accessible localization (Fig. 2, bottom). Each of these regions had a consistent pattern of labeling, with a labeling maximum and a decrease moving away from that maximum. On the margins of the strongly labeled regions were the weakly labeled stretches, Phe806–Lys814, Val828–Leu835, and Val872–Pro875. Notably Phe806–Lys814 and Ser852–Ala855, and Val876–Cys885 did not label with biotin maleimide to an extent greater than AE1C, consistent with two aqueous-inaccessible TMs, as identified previously in TM8 of AE1 (24). Two mutants (P875C and R879C) were not biotinylated, yet were found in regions that were otherwise strongly labeled. Interestingly, Phe806–Lys814 and Val828–Leu835 had labeling patterns with a suggestion of 3- and 2-fold periodicity, respectively.

Accessibility of Biotinylated Cysteine Residues to qBBBr—The intracellular or extracellular location of introduced cysteine residues was determined by differential labeling with the membrane impermeant compound, qBBBr. The bimane compound, qBBBr, has a positively charged quaternary amine group, which will not penetrate erythrocytes and cultured V79 cells with up to 1 h of incubation under physiological conditions and was not toxic (37, 38).
Accessibility to membrane impermeant qBBr was measured by the ability to block cysteine labeling by biotin maleimide. Data could not accurately be collected for mutants that labeled weakly with biotin maleimide. Therefore, only mutants that had >30% biotin maleimide labeling relative to Y555C were assayed. Fig. 3 shows the degree of biotinylation of representative mutants and the effect of qBBr on biotinylation. The lower blot shows that similar amounts of AE1 were expressed in each sample. The extracellular control mutant, Y555C, shows strong competition of biotinylation by qBBr. In contrast, the intracellular control, Cys201, shows little effect of qBBr on biotin maleimide labeling.

Fig. 4 quantifies the qBBr accessibility results. Data represent the relative biotinylation in the absence relative to presence of qBBr. Thus, an intracellular site should be unaffected by qBBr and have a ratio close to unity. An introduced cysteine residue labeled by qBBr would have access to the extracellular medium and a ratio >1.0. Introduced cysteine mutants P815C-K829C (excluding V828C) and P854C-A855C showed strong competition by qBBr labeling and therefore lie outside the permeability barrier that restricts qBBr movement across AE1. Significantly, among L873C-C885C mutants that labeled with biotin maleimide, none were affected by qBBr prelabeling, consistent with an intracellular localization of these sites.

### Table I

**Summary of anion exchange activity of introduced cysteine mutants**

| Introduced cysteine mutation | Transport activity relative to AE1C | Biotin maleimide labeling | Introduced cysteine mutation | Transport activity relative to AE1C | Biotin maleimide labeling |
|-----------------------------|-----------------------------------|---------------------------|-----------------------------|-----------------------------------|---------------------------|
| %                           | +                                 | V846C                     | %                           | +                                 | A855C                     |
| 74                          | +                                 | L847C                     | 96                          | -                                 |                           |
| 27                          | +                                 | V849C                     | 47                          | -                                 |                           |
| 79                          | +                                 | V850C                     | 26                          | -                                 |                           |
| 57                          | +                                 | K851C                     | 9                           | -                                 |                           |
| 46                          | +                                 | S852C                     | 83                          | +                                 |                           |
| 78                          | +                                 | T853C                     | 65                          | +                                 |                           |
| 63                          | +                                 | P854C                     | 76                          | +                                 |                           |
| 32                          | +                                 | A855C                     | 53                          | +                                 |                           |
| 53                          | +                                 | S856C                     | 72                          | -                                 |                           |
| 75                          | +                                 | L857C                     | 86                          | -                                 |                           |
| 60                          | +                                 | A858C                     | 65                          | -                                 |                           |
| 22                          | +                                 | L859C                     | 10                          | -                                 |                           |
| 33                          | +                                 | P860C                     | 4                           | -                                 |                           |
| 70                          | +                                 | F861C                     | 105                         | -                                 |                           |
| 90                          | +                                 | V862C                     | 142                         | -                                 |                           |
| 89                          | +                                 | L863C                     | 77                          | -                                 |                           |
| 15                          | +                                 | I864C                     | 103                         | -                                 |                           |
| 57                          | +                                 | L865C                     | 81                          | -                                 |                           |
| 92                          | +                                 | T866C                     | 86                          | -                                 |                           |
| 50                          | +                                 | V867C                     | 90                          | -                                 |                           |
| 59                          | +                                 | F868C                     | 50                          | +                                 |                           |
| 40                          | +                                 | L869C                     | 111                         | -                                 |                           |
| 72                          | -                                 | R870C                     | 4                           | -                                 |                           |
| 44                          | +                                 | R871C                     | 109                         | -                                 |                           |
| 115                         | -                                 | V872C                     | 80                          | +                                 |                           |
| 9                           | +                                 | L873C                     | 54                          | +                                 |                           |
| 11                          | -                                 | L874C                     | 36                          | +                                 |                           |
| 61                          | +                                 | P875C                     | 20                          | -                                 |                           |
| 1                           | -                                 | L876C                     | 96                          | +                                 |                           |
| 1                           | -                                 | L877C                     | 53                          | +                                 |                           |
| 89                          | -                                 | F878C                     | 7                           | +                                 |                           |
| 54                          | -                                 | R879C                     | 82                          | -                                 |                           |
| 17                          | -                                 | N880C                     | 83                          | +                                 |                           |
| 107                         | -                                 | V881C                     | 93                          | +                                 |                           |
| 98                          | -                                 | E882C                     | 3                           | +                                 |                           |
| 78                          | -                                 | L883C                     | 9                           | +                                 |                           |
| 54                          | -                                 | Q884C                     | 65                          | +                                 |                           |
| 120                         | -                                 | C885                      | 74                          | +                                 |                           |

**Accessibility of Biotylated Cysteine Residues to LYIA**—To confirm the topology data obtained with qBBr, we determined the ability to label introduced cysteine mutants with the sulf-hydryl compound, LYIA. LYIA, which is anionic and larger (MW, 620) than qBBr (MW, 409), has been previously used as a membrane-impermeant probe of AE1 topology (24, 25). Cysteine residues in the N-terminal cytoplasmic domain (C201) and cytoplasmic C-terminal tail had little labeling with LYIA, since they had only 1.1–1.2-fold more biotinylation in the absence than presence of LYIA (Fig. 5). In contrast, extracellular control mutants Y555C and S657C were strongly accessible to LYIA.

**Anion Exchange Activity of AE1 Mutants**—Mutation of structurally important residues could impair transport activity of AE1. To determine if the mutant proteins retained native structure, transport activity of each introduced cysteine mu-
Among the 80 introduced cysteine mutants analyzed, 15 were functionally inactive, defined as having no functional activity, as illustrated by R808C (Fig. 6). The recent determination of a high resolution structure for a CIC channel revealed a complex protein with transmembrane segments of varied length and tilt angle (39). If the CIC channel is a guide, we can expect the AE1 anion transporter to have a complex topology, requiring substantial biochemical characterization. In the present study, we examined the topology of the functionally important C-terminal region of AE1 (Phe806–Cys855), using the established method of introduced cysteine-scanning mutagenesis and sulfhydryl-specific chemistry (24). Labeling of an introduced cysteine mutant with biotin maleimide indicates that the residue is accessible to the aqueous environment. Sites that cannot be labeled may be in the plane of lipid bilayer or folded into an inaccessible conformation. Data on the accessibility of eighty cysteine mutants to the membrane-permeant compound, biotin maleimide, paint a clear picture of two large aqueous-accessible regions separated by two transmembrane segments and an intervening small extracellular loop. Labeling with the membrane-impermeant compounds, qBBr and LYIA, leads to the conclusion that Pro815–Arg827 is readily accessible to the extracellular medium, and the C-terminal tail region is cytosolic. The observation that Pro815–Arg827 is extracellular-accessible presents interesting implications for the folding of AE1. The data reported here were combined with findings obtained from proteolytic mapping (18), glycosylation-scanning mutagenesis (21, 22) and other introduced cysteine accessibility studies (24, 25) to develop a topology model of the C-terminal portion of human AE1 membrane domain (Fig. 8).

The second half of the AE1 membrane domain has been the subject of many topology studies. Topology of the TM8 region was established using a substituted cysteine accessibility approach similar to this study (24). The large re-entrant loop (T-loop) between TM9 and TM10 (Fig. 8) was proposed on the basis of glycosylation-scanning mutagenesis results (21, 22). The inaccessibility of G790C and S801C to labeling by biotin maleimide led to the proposal that these sites form part of a TM (25). However, we have subsequently found that introduced cysteine mutants may not label with biotin maleimide because they are folded into an inaccessible conformation, so that the identification of a TM can be made only with a sequence of biotin maleimide-inaccessible sites. Glycosylation-scanning mutagenesis showed that Phe785 could not be glycosylated but was unable to establish for certain the number of transmembrane segments between TM10 and TM12 (21). The paucity of topology data between TM10 and F806 makes it difficult to draw conclusions on the topology here.

In the present study we found that Phe806-Lys814 has a 3-fold periodicity of accessibility to biotin maleimide (Figs. 2 and 8), which is suggestive of a helical region. The NMR structure of a synthetic peptide corresponding to Gly796–Ile841 revealed Ile803–Leu810 in α-helical conformation (40). The R805C mutation, found in this region, causes hereditary erythroid spherocytosis because of a failure to process AE1 to the cell surface (36). Consistent with this finding, R805C AE1 was not functional and was not biotinylated in the present study.

The next distinct region, Pro815–Lys827, was strongly labeled by biotin maleimide, qBBr and LYIA, indicating that the region was readily accessible to the extracellular medium. Consistent with our biotinylation data, other studies have localized Phe813–Tyr824 (41), Asp821–His824 (5), Leu812–Arg827 (42), and either Lys814 or Lys817 (43) as accessible to aqueous medium. However, the accessibility of the Pro815-Lys827 region to extracellular medium is controversial. Glycosylation-scanning mutagenesis studies showed that position 820 could be partially glycosylated when translated in vitro but not glycosylated when expressed in vivo (22). These findings suggest that under some circumstances the region can be induced to face outside the cell, but normally the region is cryptic. The BRIC 132 antibody, with epitope Phe813–Tyr824 (41), can bind erythrocytes only following detergent treatment, suggesting that the epitope is not readily accessible at the extracellular surface. In contrast, binding of malaria parasite infected erythrocytes to the endothelium is dependent on the region Asp821–His824 (5), suggesting that parasitic invasion can induce exposure to the extracellular surface. Similarly, the Leu812–Arg827 region has been identified as an extracellular antigen, produced when

--

**Fig. 7. Relative anion exchange activity of introduced cysteine mutants and protein structure.** Residues are shaded to indicate effect of mutation on transport activity: unfilled, not determined; light gray, activity >60% of AE1C; dark gray, 20–60% activity relative to AE1C; black, inactive mutants with <20% activity of AE1C. A, topology model of AE1 based on the data presented in this report. B and C, helical wheel models with 3.6 residues/turn. B, helix from 836–851; C, 856–871. The first residue in each sequence is at the top of the wheel and sequence proceeds clockwise. Numbers indicate residue positions of functionally inactive mutants. Asterisks indicate residues that were inaccessible to labeling by qBBr.
marked. Pro815 resides on the extracellular portion of the membrane, which may be the last two TMs (45). Pro815 domain of AE1 revealed a mobile subunit in the protein structure. Two-dimensional crystallography of the membrane extracellular solution. This supports the marked clustering of functionally important residues on one face of helical wheel plots (Fig. 7, B and C). In both helices the functionally important residues localize to a helical face that is much more polar than the opposing hydrophobic face. This suggests that the sensitive helical surfaces may form part of the anion translocation pore, while the opposing face interacts with the rest of the protein. Interestingly, Lys851, which reacts covalently with DIDS to inhibit transport (13) and is implicated in anion translocation (15), is in the center of the sensitive face of the Phe836–Lys851 helix (Fig. 7B).

Transport assays revealed a cluster of important amino acids at the N-terminal end of the second last TM (Met833, His834, Phe836, Thr837, Gln840) (Fig. 7 and Table 1). Human mutations H834P and T837M both cause erythrocyte spherocytosis because of a failure of AE1 to be processed to the cell surface (36). In the present study, mutation to cysteine at each of these sites resulted in inactive protein, which was not labeled by biotin maleimide. The intolerance of positions 833, 834, 836, 837, and 840 to mutation suggests a critical role of this region in protein folding. AE1 variant P868L increased AE1 transport activity (16), but here P868C decreased AE1 activity by 50%, indicating that it is more than loss of proline that causes the increased transport rate of P868L AE1.

A short biotin maleimide-labeled region, Ser852–Ala855, lies between the two TMs. This region was also sensitive to membrane impermeant, qBr, consistent with an extracellular location. Our results therefore indicate that Ser852–Ala855 forms a short aqueous-accessible loop, connecting the last two TMs. Glycosylation-scanning mutagenesis had previously identified Pro854 as an extracellular location (22). An extracellular location for this residue is also supported by naturally occurring mutation, P854L, which induces the Diego blood group antigen (6). Our data show for the first time that P854 resides in a very small extracellular loop.

The extreme C-terminal region (Val872–Cys885) was strongly labeled by biotin maleimide, but was not accessible to the

![Fig. 8. Proposed topology model for the membrane domain of human AE1. The branched structure at Asn642 represents N-linked glycosylation. The model summarizes previous investigations (20–22, 24) and work from the present report. Arrows represent proteolytic sites found following treatment with NaOH (13). Residues were shaded to indicate the degree of labeling with biotin maleimide: unfilled, not determined; light gray, no significant labeling; dark gray, <30% labeling of Y555C; black, above 30% labeling of Y555C. Asterisk marks mutants that were accessible to qBBr, indicating that the residue was accessible to the extracellular medium. The amino (N) and carboxyl (C) termini are marked. Pro815–Leu835 has unusual structure. We could not rule out the possibility that this region is intracellular, but that Pro815–Arg827 is able to reorient to access the extracellular medium.](image-url)
membrane impermeant reagent qBBr, indicating an exposed intracellular location of the tail. This is consistent with results from epitope mapping (20) and carboxypeptidase Y digestion (34). The observed increase of biotinylation from V872C to C885 provides a high resolution determination of the last TM of AE1, which ends at Arg71. The cytoplasmic C-terminal region (Val72-Cys885) becomes progressively more accessible to the aqueous medium, moving away from the membrane.

The presence of four functionally inactive mutants in the cytoplasmic C-terminal tail is somewhat surprising. All of these mutations are close to the identified binding motif for carbonic anhydrase II on the C-terminal tail (886LDADD890) (19). Since binding of CAII is essential for full AE1 activity, the presence of four functionally inactive mutants in the C-terminal region of the membrane domain of AE1 Topology.

In summary, we individually assessed topology of each residue in the C-terminal region of the membrane domain of human AE1. We defined the last two short transmembrane segments and identified the small loop that connects them and which forms the Diegoα blood group antigen. The last two TMs each contain one helical face that is sensitive to introduced cysteine mutations, possibly because these faces form part of the transmembrane pore lining. Finally, we have presented evidence that Pro815→Arg827 is accessible to the extracellular medium. This finding combined with the biotin maleimide accessibility of Val828-Leu835 leads to the conclusion that the Pro815-Leu835 region of AE1 has unconventional structure that may be linked to the catalytic mechanism.

Acknowledgment—We thank Dr. Mike Jennings for generously providing monoclonal antibody IVF12.

REFERENCES
1. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2906–2917
2. Jay, D. G. (1996) Cell 86, 853–854
3. Beppu, M., Mizukami, A., Nagoya, M., and Kikugawa, K. (1990) J. Biol. Chem. 265, 3220–3225
4. Kay, M. M., Wyant, T., and Goodman, J. (1994) Annu. N. Y. Acad. Sci. 719, 419–447
5. Crandall, I., Collins, W. E., Gysin, J., and Sherman, I. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 4703–4707
6. Bruce, L. J., Anstee, D. J., Spring, F. A., and Tanner, M. J. (1994) J. Biol. Chem. 269, 16155–16158
7. Jardim, P., Palek, J., Amaral, A., Hassan, K., Sapak, P., Nurse, G. T., Robin, H. L., Zhai, S., Sahr, K. E., and Liu, S. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11022–11026
8. Bruce, L. J., and Tanner, M. J. (1999) Baillieres Best Pract. Res. Clin. Haematol. 12, 637–654
9. Peters, L. L., Shvidrasani, R. A., Liu, S. C., Hanspal, M., John, K. M., Gonzalez, J. M., Brugnara, C., Gwynn, B., Mohandas, N., Alper, S. L., Orkin, S. H., and Lux, S. E. (1996) Cell 86, 917–927
10. Kopito, R. R., and Lodish, H. F. (1985) Nature 316, 234–238
11. Zhang, D., Kiyatkin, A., Bolin, J. T., and Low, P. S. (2000) Blood 96, 2925–2933
12. Kawanou, Y., Okubo, T., Tokunaga, F., Miyata, T., Iwanaga, S., and Hamasaki, N. (1988) J. Biol. Chem. 263, 8222–8238
13. Okubo, K., Kang, D., Hamasaki, N., and Jennings, M. L. (1994) J. Biol. Chem. 269, 1918–1926
14. Garcia, A. M., and Lodish, H. F. (1989) J. Biol. Chem. 264, 19607–19613
15. Jennings, M. L. (1988) J. Biol. Chem. 257, 7554–7559
16. Bruce, L. J., Kay, M. M. B., Lawrence, C., and Tanner, M. J. A. (1993) Biochem. J. 293, 317–320
17. Kay, M. M., Marchaloniis, J. J., Hughes, J., Watanabe, K., and Schuler, S. F. (2000) Proc. Natl. Acad. Sci. U. S. A. 87, 5734–5738
18. Hamasaki, N., Okubo, K., Kuma, H., Kang, D., and Yae, Y. (1997) J. Biochem. (Tokyo) 123, 577–585
19. Reithmeier, R. A. F. (2001) Blood Cells Mol. Dis. 27, 85–89
20. Wainwright, S. D., Tanner, M. J., Martin, G. E., Yendle, J. E., and Holmes, C. (1989) Biochem. J. 258, 211–220
21. Popov, M., Tam, L. Y., Li, J., and Reithmeier, R. A. F. (1997) J. Biol. Chem. 272, 18325–18332
22. Popov, M., Li, J., and Reithmeier, R. A. F. (1999) Biochem. J. 338, 269–279
23. Kuma, H., Shinde, A. A., Howren, T. R., and Jennings, M. L. (2002) Biochemistry 41, 3380–3388
24. Tang, X. B., Fujinaga, J., Kopito, R., and Casey, J. R. (1998) J. Biol. Chem. 273, 22545–22551
25. Fujinaga, J., Tang, X. B., and Casey, J. R. (1999) J. Biol. Chem. 274, 6626–6633
26. Lee, T. W., and Clarke, D. M. (2000) J. Biol. Chem. 275, 39272–39278
27. Kimura-Someya, T., Iwaki, S., Konishi, S., Tamura, N., Kuba, Y., and Yamaguchi, A. (2000) J. Biol. Chem. 275, 18692–18697
28. Zhu, Q., and Casey, J. R. (2001) PASSER J 15, 4502
29. Casey, J. R., Dang, Y., and Kopito, R. R. (1995) J. Biol. Chem. 270, 8521–8527
30. Lee, B. S., Gunn, R. B., and Kopito, R. R. (1991) J. Biol. Chem. 266, 11448–11454
31. Jennings, M. L., Anderson, M. P., and Monaghan, R. (1986) J. Biol. Chem. 261, 9092–9010
32. Thomas, J. A., Buchsbaum, R. N., Zimniak, A., and Racker, E. (1979) Biochemistry 18, 2210–2218
33. Tanzer, M. J. A. (1989) Methods Enzymol. 173, 423–432
34. Leberman, D. M., and Reithmeier, R. A. F. (1988) J. Biol. Chem. 263, 10022–10028
35. Ruzet, S., Lindsey, A. E., Ward, C. L., and Kopito, R. R. (1993) J. Cell Biol. 121, 37–46
36. Quilty, J. A., and Reithmeier, R. A. F. (2000) Traffic 1, 987–998
37. Kosower, N. S., Kosower, E. M., Newton, G. L., and Ranney, H. M. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3383–3386
38. Newton, G. L., Aguilar, J. A., Fahey, R. C., Ward, J. F., Radkowsky, A. E., and Kosower, E. M. (1992) Anal. Biochem. 201, 30–42
39. Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) Nature 415, 287–294
40. Ashkin, D., Bloomberg, G. B., Chambers, E. J., and Tanner, M. J. (1998) Biochemistry 37, 11670–11678
41. Wainwright, S. D., Mawby, W. J., and Tanner, M. J. A. (1990) Biochem. J. 272, 265–272
42. Kay, M. M., and Lin, F. B. (1990) Gerontology 36, 293–305
43. Kuma, H., Abe, Y., Ashkin, D., Bruce, L. J., Hamasaki, T., Tanner, M. J., and Hamasaki, N. (2002) Biochemistry 41, 3311–3320
44. Wang, D. N., Kuhlbrandt, W., Sarabia, V., and Reithmeier, R. A. F. (1993) EMBO J. 12, 2233–2239
45. Greaves, J. D., and Tanner, M. J. (1999) Biochem. J. 344, 699–711
46. Reithmeier, R. A. F., and Deber, C. M. (1992) in The Structure of Biological Membranes (Yeagle, P., ed), pp. 337–393, CRC Press, Boca Raton
47. Toye, A. M., Bruce, L. J., Unwin, R. J., O’Connor, D., and Tanner, M. J. (2002) Blood 99, 342–347