Identification of Residues Lining the Translocation Pore of Human AE1, Plasma Membrane Anion Exchange Protein*

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AE1 is the chloride/bicarbonate anion exchanger of the erythrocyte plasma membrane. We have used scanning cysteine mutagenesis and sulfhydryl-specific chemistry to identify pore-lining residues in the Ser643–Ser690 region of the protein. The Ser643–Ser690 region spans transmembrane segment 8 of AE1 and surrounds Glu681, which may reside at the transmembrane permeability barrier. Glu681 also directly interacts with some anions during anion transport. The introduced cysteine mutants were expressed by transient transfection of HEK293 cells. Anion exchange activity was assessed by measurement of changes of intracellular pH, which follows transmembrane bicarbonate movement mediated by AE1. To identify residues that might form part of an aqueous transmembrane pore, we measured anion exchange activity of each introduced cysteine mutant before and after incubation with the sulfhydryl reagents para-chloromercuribenzenesulfonate and 2-(aminoethyl)ethanethiosulfonate hydrobromide. Our data identify transmembrane mutants A666C, S667C, L669C, L673C, L677C, and L680C and intracellular mutants I684C and I688C that could be inhibited by sulfhydryl reagents and may therefore form part of a transmembrane pore. These residues map to one face of a helical wheel plot. The ability to inhibit two intracellular mutants suggests that transmembrane helix 8 extends at least two helical turns beyond the intracellular membrane surface. The identified hydrophobic pore-lining residues (leucine, isoleucine, and alanine) may limit interactions with substrate anions.

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AE1 in the erythrocyte membrane (50% of integral protein (9)) has made the protein a model for the study of transport protein structure and function (10, 11).

Several residues have been implicated as part of the anion exchange mechanism of AE1. On the basis of indirect evidence, Passow has proposed a model for anion translocation that involves residues from TMs 5, 8, 10, 12, and 13, including mouse residues Glu172, Glu355, Lys509, Glu641, His703, Arg731, His735, His816, and His834 (12). Jennings and co-workers (13–15) have strong evidence to implicate the TM8 residue, Glu681, in the transport process, since labeling this residue with Woodward’s reagent K reduced with sodium borohydride resulted in altered anion exchange kinetics. The functional role of Glu681 in AE1 was confirmed in mutagenesis experiments of mouse AE1 (16) and extended to the homologous position of mouse AE2, suggesting that the mechanistic role of Glu681 is conserved among anion exchange proteins (17). Woodward’s reagent K 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 Glu681, is cotransported. Sulfate/proton cotransport takes place in both inward and outward directions, which implies that Glu681 has access to both the intracellular and extracellular sides of the membrane. Taken together, Glu681 is functionally involved in anion exchange events and may reside at the permeability barrier of AE1.

Glu681 of TM8 is the best characterized residue that interacts with anions during translocation event. We have therefore focused on TM8 in efforts to identify residues involved in anion translocation. In a previous report we analyzed a panel of introduced cysteine residues spanning the TM8 region from Ser643 to Ser690. Using accessibility to chemical modification by 3-(N-maleimidylpropionyl)biocytin and lucifer yellow iodoacetamide we identified the bilayer spanning residues as the sequence Met664–Glu681 (18).

Substituted cysteine mutagenesis and sulfhydryl chemistry have proved a fruitful approach to identify residues of the transport pathway. The method is to mutate all native cysteine residues in a protein and to systematically re-introduce unique cysteine residues into the cysteineless background. Inhibition of anion transport by sulfhydryl-specific reagents is then assessed for each mutant. The approach has been useful for studies of the bacterial transport proteins, lactose permease (19–21) and UhpT (22). Among mammalian membrane proteins, this approach has proved very successful for ion channels. Derivatization of a cysteine with a methanethiosulfonate will impair ion conductance through steric blockage of a con-

1 The abbreviations used are: TM, transmembrane segment; AE1C, cysteineless AE1; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5 and 6-carboxyfluorescein, acetoxyethyl ester; biotin maleimide, 3-(N-maleimidypropionyl)biocytin; MTSEA, 2-(aminoethyl)ethanethiosulfonate hydrobromide; pCMBS, para-chloromercuribenzenesulfonate; CFTR, cystic fibrosis transmembrane regulator.

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fined ion translocation pore. Pore-lining residues in the cystic fibrosis chloride channel, CFPTR (23–25), γ-aminobutyric acid receptor chloride channel (26) and acetylcholine receptor sodium channel (27, 28) have been identified in this way. Differential sensitivity to inhibition by methanethiosulfonate compounds has also identified residues of the transport pathway in the glutamate transport protein GLT-1 (29), sodium glucose cotransporter (30), and sodium/calcium exchanger (31).

We used substituted cysteine mutagenesis and sulphydryl chemistry to identify pore-lining residues in the TM8 region of human AE1 chloride/bicarbonate exchange protein. Previously we constructed a cysteineless form of human AE1, called AE1C, and characterized the protein as fully functional (32). We have systematically replaced the residues of AE1 from the glycosylation site at Asn642 through TM8 into the cytoplasmic region and identified the sequence Met664–Gln683 as spanning the bilayer (18). In this report we measure the effect of two sulphydryl reagents upon AE1 anion transport function, using BCECF fluorescence to monitor intracellular pH shifts associated with Cl-/HCO₃⁻ exchange in transfected HEK293 cells. Of the two sulphydryl reagents, pCMBS is membrane-impermeant but slowly transported by AE1, while MTSEA is membrane-permeant in its unprotonated form (33). We have identified a sequence of leucine, isoleucine, and alanine residues that lie on one face of an α-helix and, when mutated to cysteine, are susceptible to inhibition by pCMBS and MTSEA.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases were from New England Biolabs. ECL chemiluminescent reagent, horseradish peroxidase conjugated to sheep anti-mouse IgG, Hyperfilm, and Immobilon-P membrane were from Amersham Pharmacia Biotech. BCECF-AM was from Molecular Probes. Poly-L-lysine, pCMBS, and nigericin were from Sigma. Coverslips were from Fisher. MTSEA was from Toronto Research Chemicals.

Construction of Mutant Anion Exchangers—A human AE1 cDNA construct, called AE1C, in which all five cysteine codons were mutated to serine was constructed previously (32) in the expression vector pRBG4 (34). Individual cysteine codons were introduced into AE1C, to yield mutants, each with a unique cysteine codon (18). 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 (35, 36). Polymerase chain reaction primers were designed using an Oligo computer 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.

Protein Expression—Anion exchangers were expressed by transient transfection of human embryonic kidney (HEK) 293 cells (37), as described previously (38, 39), except that calcium phosphate-precipitated plasmid was 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 bovine serum (Life Technologies, Inc.) and 5% (v/v) calf serum (Life Technologies, Inc.) containing 5% (v/v) fetal bovine serum (Life Technologies, Inc.) loaded with BCECF-AM by incubation in 4 ml of serum-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) and 5% (v/v) calf serum (Life Technologies, Inc.) and 5% (v/v) fetal bovine serum (Life Technologies, Inc.) containing 5% (v/v) calf serum (Life Technologies, Inc.) and loaded with BCECF-AM by incubation in 4 ml of serum-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 5% (v/v) calf serum (Life Technologies, Inc.) and loaded with BCECF-AM by incubation in 4 ml of serum-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 5% (v/v) calf serum (Life Technologies, Inc.) and loaded with BCECF-AM by incubation in 4 ml of serum-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.).

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. Two days post-transfection, coverslips were rinsed with serum-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) and loaded with BCECF-AM by incubation in 4 ml of serum-free Dulbecco’s modified Eagle’s medium, containing 2 μM BCECF-AM, for 20–30 min, at room temperature. Coverslips were mounted in a fluorescence cuvette, with perfusion capabilities. Intracellular pH was monitored by measuring fluorescence at excitation wavelengths 440 and 502 nm and emission wavelength 529 nm, in a Photon Technologies International RCR spectrofluorometer. The cuvette was perfused at 3.5 ml/min alternately with Ringer’s buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM MgSO₄, 2.5 mM NaH₂PO₄, 25 mM NaHCO₃, 10 mM Hepes, pH 7.4) containing 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 calibrated by the nigericin-high potassium method (39), 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 the Kaleidagraph program (Synergy Software).

Transport Inhibition Assays—Transport experiments were performed as described above. Uninhibited rates of anion exchange were determined by perfusion with chloride Ringer’s followed by chloride-free Ringer’s, chloride Ringer’s, and chloride-free Ringer’s. The cuvette was then perfused with 10 mM chloride-free Ringer’s containing either: 5 mM MTSEA or 0.2 mM pCMBS. Cells were incubated with the inhibitor for a total of 8 min, followed by washing with chloride-free Ringer’s until the fluorescence base line was stable (350–550 s). Cells were then perfused with chloride Ringer’s, chloride-free Ringer’s, and chloride Ringer’s. Inhibition of anion exchange was determined from the initial rates of alkalization and acidification observed as buffers were changed, before and after inhibitor addition. Rates were determined by linear regression, using Kaleidagraph software. Percent residual activity was calculated as: % residual activity = rate of fluorescence change after sulphydryl reagent/rate of fluorescence change without treatment × 100.

Electrophoresis and Immunoblotting—Samples were electrophoresed on 8% acrylamide gels (40) and transferred to Immobilon membrane (41). AE1 was detected by incubation of the blot with 10 ml of TBST (TBST buffer (0.1% (v/v) Tween 20, 137 mM NaCl, 20 mM Tris, pH 7.5), containing 5% (v/v) non-fat dry milk powder (Carnation) and 3 ml of anti-human C-terminal peptide antibody 1657 (18). After washing blots were probed with 10 ml of 1:3000 diluted horseradish peroxidase conjugated to goat anti-rabbit IgG. After 1-h incubation, blots were washed with TBST and visualized using ECL reagent and Hyperfilm.

Molecular Biological Methods—Plasmid DNA for transfections was prepared using Qiagen columns (Qiagen Inc.). DNA sequencing was performed by the Core Facility in the Department of Biochemistry, University of Alberta, with an Applied Biosystems 373A DNA sequencer. All other procedures followed standard protocols (42).

RESULTS

Expression of Introduced Cysteine Mutants—A form of human AE1 in which all cysteine codons were mutated to serine codons was constructed previously and called AE1C (32). Each codon of the AE1 sequence from Asn643 to Ser680 (except Ser442–Arg457) was individually mutated to a cysteine codon and cloned into AE1C (18). This sequence begins immediately after the extracellular site of glycosylation (Asn642) and proceeds through TM8 (Met664–Gln683), into an intracellular region (18). This array of introduced cysteine mutants provides a system in which to search systematically for residues that line the transmembrane anion translocation channel.

Expression of Introduced Cysteine Mutants in HEK293 Cells—HEK293 cells expressing wild-type AE1 and a subset of AE1C mutants were pulsed with a high concentration of a low-affinity chloride channel, CFTR, 293 cells were transfected with AE1 cDNA, solution in SDS-PAGE sample buffer, and subjected to electrophoresis on a 7.5% acrylamide gel. Proteins were transferred to Immobilon-P membrane, and the immunoblot was incubated with anti-AE1 antibody, 1657 (18). Numbers indicate the position of the introduced cysteine mutation. WT is wild-type; V is vector, pRBG4-transfected cells.

Fig. 1. Expression of AE1 and introduced cysteine mutants in HEK293 cells. HEK293 cells were transfected with AE1 cDNA, solution in SDS-PAGE sample buffer, and subjected to electrophoresis on a 7.5% acrylamide gel. Proteins were transferred to Immobilon-P membrane, and the immunoblot was incubated with anti-AE1 antibody, 1657 (18). Numbers indicate the position of the introduced cysteine mutation. WT is wild-type; V is vector, pRBG4-transfected cells.
chloride-free buffer (solid bar) when the cuvette was perfused with chloride containing (open bar). Fig. 2 shows an example of an anion exchange assay, in this case showing the activity of wild-type AE1, AE1C, and vector-transfected cells. Fig. 2 shows that the transport activity of AE1, AE1C, and pRBG4 are 0.49, 0.20, and 0.014 pH/min, respectively, during the alkalinization phase. Vector-transfected HEK293 cells have a background anion exchange activity around 10% of AE1C . Table I shows the transport activity determined for each of the introduced cysteine mutants, relative to AE1C . Extracellular mutants W648C, I650C, P652C, G654C, L655C, F659C, P660C, and W662C and transmembrane mutants A671C, F679C, and E681C all have greatly impaired anion exchange function. These mutants therefore were not subjected to inhibition assays with sulphydryl reagents.

**Inhibition of Anion Exchange Activity by Sulphydryl Reagents**—To begin to identify the residues that line the hydrophilic transmembrane anion translocation channel of AE1, we attempted to inhibit anion exchange activity of introduced cysteine mutants with sulphydryl reagents. The protocol we used was to mount coverslips containing transfected HEK293 cells in the fluorimeter and to assay anion exchange activity as described above. However, during one incubation with chloride-free Ringer’s buffer, the buffer was modified with either 5 mM MTSEA or Ringer’s buffer containing 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).

**Measurement of Anion Exchange Activity**—Anion exchange activity of each introduced cysteine mutant was measured using a fluorescence assay. Transfected HEK293 cells, grown on the surface of coverslips, were loaded with the pH-sensitive dye BCECF-AM and suspended in a fluorescence cuvette. The cells were alternately perfused with Ringer’s buffer containing chloride, followed by chloride-free Ringer’s buffer. The result of these manipulations is to change the direction of the transmembrane chloride gradient (34). In cells expressing functional anion exchangers chloride will move in one direction across the membrane, in exchange for bicarbonate in the other direction. As bicarbonate leaves the cell, the cell acidifies; when bicarbonate enters the cell, it alkalinizes. At the end of each experiment, fluorescence levels were calibrated with pH standards, to allow conversion of fluorescence data to absolute intracellular pH values. Transport activity is determined by measuring the rate of alkalinization and acidification after switching buffer solutions.

![Assay of anion exchange activity](image)

**Table I**

Transport activity of AE1 introduced cysteine mutants

| Anion exchanger    | Activity relative to AE1C | Anion exchanger | Activity relative to AE1C |
|--------------------|---------------------------|-----------------|---------------------------|
| AE1C               | 100                       | S667C           | 78 ± 2                    |
| S643C              | 99 ± 2                    | A668C           | 56 ± 12                   |
| S644C              | 92 ± 3                    | L669C           | 99 ± 29                   |
| A645               | ND                        | F670C           | 45 ± 14                   |
| R646               | ND                        | A671C*          | 12 ± 3                    |
| G647               | ND                        | L673C           | 55 ± 17                   |
| W648C*             | 7.1 ± 6                   | L673C           | 46 ± 8                    |
| V649C              | 86 ± 2                    | V674C           | 46 ± 8                    |
| I650C*             | 0 ± 2                     | F675C           | 52 ± 12                   |
| H651C              | 62 ± 14                   | I676C           | 70 ± 7                    |
| P652C*             | 3.6 ± 7                   | L677C           | 79 ± 5                    |
| L653C              | 35 ± 5                    | I678C           | 35 ± 11                   |
| G654C*             | 12 ± 10                   | F679C*          | 25 ± 4                    |
| L655C*             | 4.7 ± 2                   | L680C           | 57 ± 10                   |
| R656C              | 55 ± 10                   | E681C*          | 0 ± 2                     |
| S657C              | 61 ± 1                    | S682C           | 64 ± 7                    |
| E658C              | 79 ± 8                    | Q683C           | 59 ± 6                    |
| F659C*             | 1.2 ± 9                   | I684C           | 55 ± 8                    |
| P660C*             | 13 ± 3                    | T685C           | 61 ± 10                   |
| I661C              | 40 ± 9                    | T686C           | 77 ± 14                   |
| W662C*             | 8.3 ± 3                   | L687C           | 133 ± 7                   |
| M663C              | 54 ± 10                   | I688C           | 101 ± 10                  |
| M664C              | 50 ± 2                    | V689C           | 65 ± 2                    |
| F665C              | 88 ± 5                    | S690C           | 108 ± 5                   |
| A666C              | 86 ± 13                   |                |                           |

The methodology shown in Fig. 3 was applied to each of the...
36 functional introduced cysteine mutants in the Ser\textsuperscript{643}–Ser\textsuperscript{690} region of human AE1. Fig. 4 summarizes the results of inhibition with the positively charged methanethiosulfonate compound, MTSEA. MTSEA is membrane-permeant in its unprotonated form (33) and therefore should be able to access the entire anion translocation channel, including sites on the intracellular side of the permeability barrier. Among the 36 introduced cysteine mutants characterized, L673C, A677C, I684C, and I688C had anion exchange activity that was significantly inhibited by MTSEA treatment. Maximum inhibition was observed for mutant I684C (59 ± 3% residual activity). Neither wild-type AE1 nor AE1\textsuperscript{C} were inhibited by MTSEA, since they had respective residual anion exchange rates of 100 and 104% after MTSEA treatment. The transmembrane permeability barrier has previously been mapped to Glu\textsuperscript{681} of human AE1, so that Ile\textsuperscript{684} and Ile\textsuperscript{688} reside beyond the permeability barrier, relative to the outside of the cell.

Fig. 5 shows the effect of pCMBS treatment of AE1 introduced cysteine mutants. Mutants A666C, S667C, L673C, I684C, and I688C had impaired anion exchange activity after treatment with 0.2 mM pCMBS, while all other mutants were not impaired by pCMBS. There is a graded effect of pCMBS upon the introduced cysteine residues; the two outermost sites of inhibition (A666C and S667C) had a lower level of
inhibition than the inner sites. Two mutants, F679C and E681C, whose function is greatly impaired by cysteine introduction flank the most pCMBS-sensitive mutant, L680C. L680C is also adjacent to Glu681, which has been proposed to define the transmembrane permeability barrier. L669C and F680C could be inhibited by pCMBS, but not by MTSEA. AE1C was insensitive to pCMBS, since it had 110% residual activity after pCMBS treatment. However, wild-type AE1 had modest inhibition with 86% residual activity. Previous investigators found that AE1 in red cells could be covalently inhibited by pCMBS, but only low levels of inhibition would be expected at the low concentration and incubation time used in the present study (43). Inhibition of wild-type AE1 by pCMBS is attributed to the endogenous Cys479.

Failure to observe complete transport inhibition with both MTSEA and pCMBS reflects (i) that the assay measures only covalent inhibition, (ii) chemical reaction only occurred for 8 min, and (iii) only moderate concentrations of sulfhydryl reagents were used to prevent nonspecific effects on the cell. In studies to identify pore-lining residues of channels, a maximum inhibition of 25–40% was also observed (25, 26). Introduced cysteine mutants were defined as those inhibited by sulfhydryl reagents if residual activity after inhibition was less than 100%. That is, those mutants whose error bars did not overlap with 100% activity. Because of the large number of mutants analyzed, experiments were repeated until statistically significant only at those sites that were inhibited by sulfhydryl reagents.

Fig. 6 places the sulphydryl reagent inhibited residues in models of the transmembrane segment 8 region. In a helical wheel plot of residues Met664–Ile688 (Fig. 6A), it is clear that sulphydryl-sensitive sites cluster on one face of the helix. Only a single hydrophilic site (Ser667) was sensitive to inhibition. The remaining sensitive sites are all aliphatic residues: leucine, isoleucine, or alanine residues in wild-type AE1. Interestingly the only two introduced cysteine mutants (A671C and F679C) that have greatly impaired transport activity localize on the face of the helix directly opposite from the sulphydryl-sensitive residues we have identified. The helical face containing Ala671 and Phe679 has been identified as the most hydrophobic surface of the helix and is predicted to face lipid (10).

The transmembrane topology diagram (Fig. 6B) illustrates the topological disposition of residues in the region, as determined by accessibility to labeling by biotin maleimide (18). The residues we have identified as sensitive to inhibition by MTSEA and pCMBS form a clear line through the transmembrane region. Interestingly, the sequence of sulphydryl reagent-sensitive residues starts beneath the predicted surface of the bilayer and extends beyond the surface of the membrane. Since helical periodicity of anion exchange activity is maintained up to

![Graph showing percentage of residual anion exchange activity after MTSEA treatment vs. position of introduced cysteine mutation.](image-url)
Ile\textsuperscript{688}, Fig. 6B models the Ile\textsuperscript{684}–Ser\textsuperscript{690}, shown previously to be extramembranous (18), as a helical region.

**DISCUSSION**

In this paper we have examined the amino acid sequence from Ser\textsuperscript{643}–Ser\textsuperscript{690} of human AE1, to identify residues that line a transmembrane anion translocation pore. We have focused on this region because it contains Glu\textsuperscript{681}, shown previously to be accessible to membrane impermeant Woodward’s reagent K, from either side of the membrane (13–15). This residue may therefore reside at the transmembrane permeability barrier. Because Glu\textsuperscript{681} is accessible to Woodward’s reagent K and has been implicated as one residue that interacts with anions, at least during sulfate transport, we decided to examine the region surrounding Glu\textsuperscript{681} to find other residues that might form the anion translocation channel. We recently determined the topology of the Ser\textsuperscript{643}–Ser\textsuperscript{690} region and localized Glu\textsuperscript{681} to within four residues of the cytosolic surface of the protein (18).

Our results have identified a sequence of introduced cysteine mutants (Ala\textsuperscript{666}, Ser\textsuperscript{667}, Leu\textsuperscript{669}, Leu\textsuperscript{673}, Leu\textsuperscript{677}, Leu\textsuperscript{680}, Ile\textsuperscript{684}, and Ile\textsuperscript{688}) in the transmembrane segment 8 region of human AE1 that can be inhibited either by the sulfhydryl reagents pCMBS or MTSEA. The locations of the mutants have a helical periodicity and reside on one face of a predicted α-helix, which extends at least one turn beyond the surface of the membrane. Seven of the eight identified residues are found within a 100° arc of a TM8 helical wheel plot, indicating that the region forms an α-helix. The sensitive helical face is directly opposite the most hydrophobic face of TM8 and is adjacent to Glu\textsuperscript{681}, which was shown previously to interact with anions during transmembrane translocation. The simplest explanation for our data is a covalent interaction of introduced cysteine residues with the sulfhydryl compounds; at sites that line the pore, these reagents block the pore and inhibit anion transport. On this basis we propose that Ala\textsuperscript{666}, Ser\textsuperscript{667}, Leu\textsuperscript{669}, Leu\textsuperscript{673}, Leu\textsuperscript{677}, Leu\textsuperscript{680}, Ile\textsuperscript{684}, and Ile\textsuperscript{688} line the transmembrane translocation channel and interact with substrate anions as they cross the bilayer. An alternate possibility is that the inhibited residues form a conformationally active surface of AE1, part of a cleft where two helices meet and whose movements are blocked by sulfhydryl modification. However, this model is unlikely since such a cleft would not be likely to have access to pCMBS and MTSEA, two hydrophilic reagents.

Our observation that the hydrophobic amino acids leucine, isoleucine, and alanine line the TM8 portion of the anion translocation channel at first seems surprising. The alkyl side chains of these amino acids are not hydrophilic and cannot contribute hydrogen bonds to line an aqueous channel. However, hydrophobic residues have been found to form part of the lining of the cystic fibrosis chloride channel, CFTR (23, 24), the γ-aminobutyric acid receptor channel (26), and the acetylcholine receptor Na\textsuperscript{+} channel (28). The transmembrane carbohydrate translocation channel of the maltoporin protein is lined...
with hydrophobic residues (44). Similarly, the crystal structure of a bacterial K+ channel illustrated that the ion translocation pore is lined with hydrophobic amino acids (45). The authors speculated that the hydrophobic lining provides the most energetically favorable surface for movement of ions. Interactions with the wall of the pore would only impede ion transit through the pore. A hydrophobic lining of the AE1 anion translocation pore could be important to facilitate the high turnover rate (105 ions/s) (46), which is an order of magnitude slower than the pore-lining face of the helix, while S667C is 60° removed from the rest of the pore-lining residues. All other sites cluster on one face of the helix, which is adjacent to Glu681, the presumed permeability barrier.

Mutant S667C stands apart from the other sulphydryl reagent-sensitive sites. This is the only hydrophilic site observed among the pore-lining residues. All other sites cluster on one face of the helix, which is 60° removed from the rest of the mutants, on a helical wheel plot. S667C is close to Pro670, which may kink and twist the TM8 helix. This would result in exposure of a discontinuous helical face to line the pore. That is the TM8 helical region above and below Pro680 may not be directly aligned.

Our results may provide insight into the structure of the anion translocation pore. We observed that AE1 introduced cysteine mutants are only partially (∼40%) inhibited by pCMBS. Similarly, in studies of the CFTR chloride channel, pCMBS was only able to inhibit to 25–75% (24). In contrast, translocation pathway mutants of the bacterial glucose 6-phosphate transporter, UhpT, were nearly fully inhibited by pCMBS (22). This difference in sensitivity to pCMBS may reflect the size of the anion translocation pore, since glucose 6-phosphate is larger than chloride. The CFTR channel is estimated to have a pore diameter of at least 6 Å (24), which provides at least an approximation for the size of the AE1 pore.

One prevailing model of AE1 suggests that the anion translocation pore forms an outward facing funnel and narrows to a permeability barrier at Glu681 (47). Our data may provide some support for the model. The first sulphydryl reagent-sensitive site (Ala666) is located after the start of the TM8 (Met664) (18). Since both pCMBS and MTSEA are expected to inhibit anion exchange by steric blockage of a pore, the observation that the inner portion of TM8 is more sensitive to sulphydryl reagents than the outer suggests a more open outer region and a more closed pore in the inner region. In line with this model, we found that L669C was sensitive to pCMBS, but not MTSEA, reflecting the larger steric bulk of pCMBS. Furthermore, A666C and S667C, the outermost sensitive mutants, both had lower pCMBS sensitivity than the inner mutants. We found that the sequence of pCMBS-inhibited residues ended at Leu680, which is consistent with the identification of Glu681 as the site of the permeability barrier. Since pCMBS can permeate the membrane only via AE1, it will not accumulate on the cytosolic surface to any extent. Conversely, MTSEA is membrane permeant in its deprotonated form (33) and therefore can access the anion translocation pore from both the extracellular and intracellular surfaces. Finally, our data show that the greatest sensitivity to pCMBS is found with mutant L680C, which is adjacent to Glu681, the presumed permeability barrier. The high sensitivity at this site may reflect a smaller pore diameter.

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