Trapping of Inhibitor-Induced Conformational Changes in the Erythrocyte Membrane Anion Exchanger AE1†

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ABSTRACT: AE1, the chloride/bicarbonate anion exchanger of the erythrocyte plasma membrane, is highly sensitive to inhibition by stilbene disulfonate compounds such as DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate) and DNDS (4,4′-dinitrostilbene-2,2′-disulfonate). Stilbene disulfonates recruit the anion binding site to an outward-facing conformation. We sought to identify the regions of AE1 that undergo conformational changes upon noncovalent binding of DNDS. Since conformational changes induced by stilbene disulfonate binding cause anion transport inhibition, identification of the DNDS binding regions may localize the substrate binding region of the protein. Cysteine residues were introduced into 27 sites in the extracellular loop regions of an otherwise cysteineless form of AE1, called AE1C$^-$. The ability to label these residues with biotin maleimide [3-(*N*-maleimidylpropionyl)biocytin] was then measured in the absence and presence of DNDS. DNDS reduced the ability to label residues in the regions around G565, S643−M663, and S731−S742. We interpret these regions either as (i) part of the DNDS binding site or (ii) distal to the binding site but undergoing a conformational change that sequesters the region from accessibility to biotin maleimide. DNDS alters the conformation of residues outside the plane of the bilayer since the S643−M663 region was previously shown to be extramembranous. Upon binding DNDS, AE1 undergoes conformational changes that can be detected in extracellular loops at least 20 residues away from the hydrophobic core of the lipid bilayer. We conclude that the TM7−10 region of AE1 is central to the stilbene disulfonate and substrate binding region of AE1.

AE1, also called Band 3, is the chloride/bicarbonate anion exchange protein of the erythrocyte membrane. AE1 is part of a family of anion exchange proteins that includes AE2, which is widely expressed, and AE3, expressed in brain, heart, and retina (*2*). Members of the anion exchange protein family have a two-domain structure. The divergent 43−77 kDa cytoplasmic domains are involved in binding to the cytoskeleton through ankyrin (*3, 4*). The membrane domain, which is conserved with 90% identity between AE family members, is responsible for anion transport activity and spans the membrane 12−14 times (*5*).

Stilbene disulfonates have been used widely as probes of anion exchanger structure and function (*6, 7*). Stilbene disulfonate compounds markedly inhibit anion exchange activity, with K_i values that range from 0.08 μM for DIDS[1] to >300 μM for 4,4′-diaminostilbene-2,2′-disulfonate (DADS) (*6*). DIDS and the tritiated analogue H_2DIDS were used in the functional and protein chemical identification of Band 3 as the erythrocyte anion exchange protein (*8*). Subsequently it was shown that H_2DIDS forms a covalent cross-link between K539 and K851 of AE1 (*9, 10*).

Several lines of evidence suggest the existence of extensive interactions between stilbene disulfonates and AE1. An increase of AE1 thermal stability is associated with DIDS labeling of AE1 (*11*). Indeed, DIDS labeling of AE1 in erythrocytes stabilizes cells against pressure-induced hemolysis (*12*). AE1 binding of the stilbene disulfonate BIDS was previously shown to induce a conformational change in AE1, as measured by changes in the ability to quench tryptophan fluorescence (*13*). However, that study was unable to localize the regions of AE1 that underwent conformational changes. The stilbene disulfonate binding site of AE1 is not superficial in the structure of the protein, since an anti-H_2DIDS antibody was able to immunoprecipitate denatured, but not native, H_2-DIDS-labeled AE1 (*14*).

The relationship between the AE1 binding sites for stilbene disulfonates and substrate anions has been actively studied.

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[1] Abbreviations: AE1C$^-$, cysteineless AE1; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-(5 and 6)-carboxyfluorescein, acetoxymethyl ester; BIDS, 4-benzamido-4′-isothiocyanostilbene-2,2′-disulfonate; Biotin maleimide, 3-(*N*-maleimidylpropionyl)biocytin; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; EC, extracellular loop; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; HRP, horseradish peroxidase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone; TM, transmembrane segment; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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^{35}Cl nuclear magnetic resonance studies indicated that stilbene disulfonates differ in their effect upon AE1 (*15*−*17*). The NMR data suggest that DIDS leaves the transport chloride binding site unaffected, whereas DNDS binding blocked the substrate chloride binding site. One sulfonate group of DNDS may directly occupy the chloride binding site in the protein (*15*). Experiments with the stilbene disulfonate 4,4′-dibenzamidostilbene-2,2′-disulfonate (DBDS) showed that rate of DBDS release from AE1 was influenced by the presence of transportable anions (*18*). This result indicates that anions and stilbene disulfonates bind at two separate sites. However, eosin maleimide labeling did not block anion binding, which suggests that eosin maleimide binds to an access channel that leads to the anion binding site deeper in AE1 (*19*−*21*). Since AE1 labeling with eosin maleimide blocks subsequent labeling with DIDS, the two molecules share an overlapping binding site (*22, 23*). Analysis of the kinetics of the AE1/eosin maleimide interaction suggests that eosin maleimide is sensitive to conformational changes at the anion binding site (*24*). Taken together, these studies suggest that stilbene disulfonates function as noncompetitive inhibitors of anion transport and likely inhibit anion exchange by inducing conformational changes in the protein. One sulfonate group of DNDS may directly occupy the chloride binding site of AE1.

In the present study, we mapped the regions of AE1 that undergo conformational changes upon binding the stilbene disulfonate DNDS. DNDS noncovalently inhibits anion exchange activity with a K_i of 4 μM (*6*). In a previous report, we characterized the ability to label AE1-introduced cysteine residues with the membrane-permeant reagent biotin maleimide (*25*). We found that this reagent only labeled aqueous-accessible sites and could not label cysteine residues in the hydrophobic core of the lipid bilayer. Therefore, we introduced cysteine residues into each extracellular loop of AE1 and examined whether binding of DNDS to AE1 changed the degree of labeling with biotin maleimide.

MATERIALS AND METHODS

Materials. Restriction endonucleases were obtained from New England Biolabs. ECL chemiluminescent reagent, horseradish peroxidase conjugated to sheep anti-mouse IgG, Hyperfilm, and Immobilon-P membrane were obtained from Amersham. BCECF-AM was obtained from Molecular Probes. Poly-L-lysine and nigericin were obtained from Sigma. Coverslips were obtained from Fisher Scientific products.

Construction of Mutant Anion Exchangers. A human AE1 cDNA construct, called AE1C$^-$, in which all five cysteine codons were mutated to serine was previously constructed (*26*) in the expression vector pRBG4 (*27*). Individual cysteine codons were introduced into AE1C$^-$ to yield mutants, each with a unique cysteine codon (*25*). Introduced cysteine mutants at amino acids 645−647 were not constructed because their codons overlap with the *Sma*I site (nucleotides 2048−2053) used to clone introduced cysteine mutants into AE1C$^-$. Mutagenesis was performed using a PCR megaprimer mutagenesis strategy (*28, 29*). PCR primers were designed using the Primers program (Whitehead Institute for Medical Research). PCR 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 293 cells (HEK) (*30*), as previously described (*31*), except that calcium phosphate-precipitated plasmid was added at 2.8 μg of anion exchanger plasmid with 4.2 μg of pRBG4 carrier per 100 mm tissue culture dish. Cells were grown at 37 °C in a 5% CO_2 environment in DMEM (Life Technologies) containing 5% (v/v) fetal bovine serum (Life Technologies) and 5% (v/v) calf serum (Life Technologies), and harvested 48 h post-transfection.

Chemical Accessibility Assays. HEK293 cells grown in 100 mm tissue culture dishes were transfected with wild-type or mutant AE1 cDNA, as described above. Forty-eight hours after transfection, cells were harvested by incubation with 1 mg/mL trypsin in PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4) for 5 min at 37 °C. Cells were collected from the plate with a pipet, sedimented by centrifugation for 5 min at 228*g*, and washed with PBS. After resuspension in 2 mL of PBSCM (PBS buffer containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.0), cells were divided into two equal samples, labeled "+" and "−". The "+" sample was made to 100 μM DNDS and incubated for 10 min at room temperature. Then, 10 μL of 2 mM biotin maleimide (in dimethyl sulfoxide) was added to both the "+" and "−" samples, and the samples were incubated with occasional mixing for 15 min at room temperature. Reactions were stopped by the addition of 0.5 mL of 2% (v/v) 2-mercaptoethanol in DMEM and incubated at room temperature for 10 min. Cells were sedimented as above and washed with PBSCM. Sedimented cells were lysed on ice for 15 min with 250 μL of IPB containing 2% (w/v) BSA, 200 μM TPCK, 200 μM TLCK, and 2 mM PMSF. Samples were immunoprecipitated with polyclonal anti-AE1 antibody 1657, as described (*25*). After immunoprecipitation, samples were electrophoresed on 8% acrylamide gels (*32*) and transferred to an Immobilon membrane (*33*). Biotinylated proteins were detected by incubation of the blot with 10 mL of 1:2500 diluted streptavidin−biotinylated horseradish peroxidase (Amersham) in TBSTB buffer [TBST buffer (0.1% (v/v) Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.5) containing 0.5% (w/v) bovine serum albumin]. After 1.5 h incubation, blots were washed with TBST. Blots were visualized using ECL reagent and Hyperfilm (Amersham).

After analyzing each sample for incorporation of biotin, samples were normalized for variations in the recovery of AE1 protein as follows. The blots from above were stripped by incubation in 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, at 50 °C for 15 min, following the protocol supplied by Amersham-Pharmacia Biotech with the ECL kit. Blots were then processed as immunoblots (above), using 10 mL of TBSTM [TBST, containing 5% (w/v) nonfat dry milk powder (Carnation)] and 3 μL of monoclonal anti-AE1 antibody, IVF12 (*34*). After washing (see above), blots were probed with 10 mL of 1:3000 diluted horseradish peroxidase conjugated to sheep anti-mouse IgG and subsequently processed with ECL reagent (see above).

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-tranfection, coverslips were rinsed with serum-free DMEM and

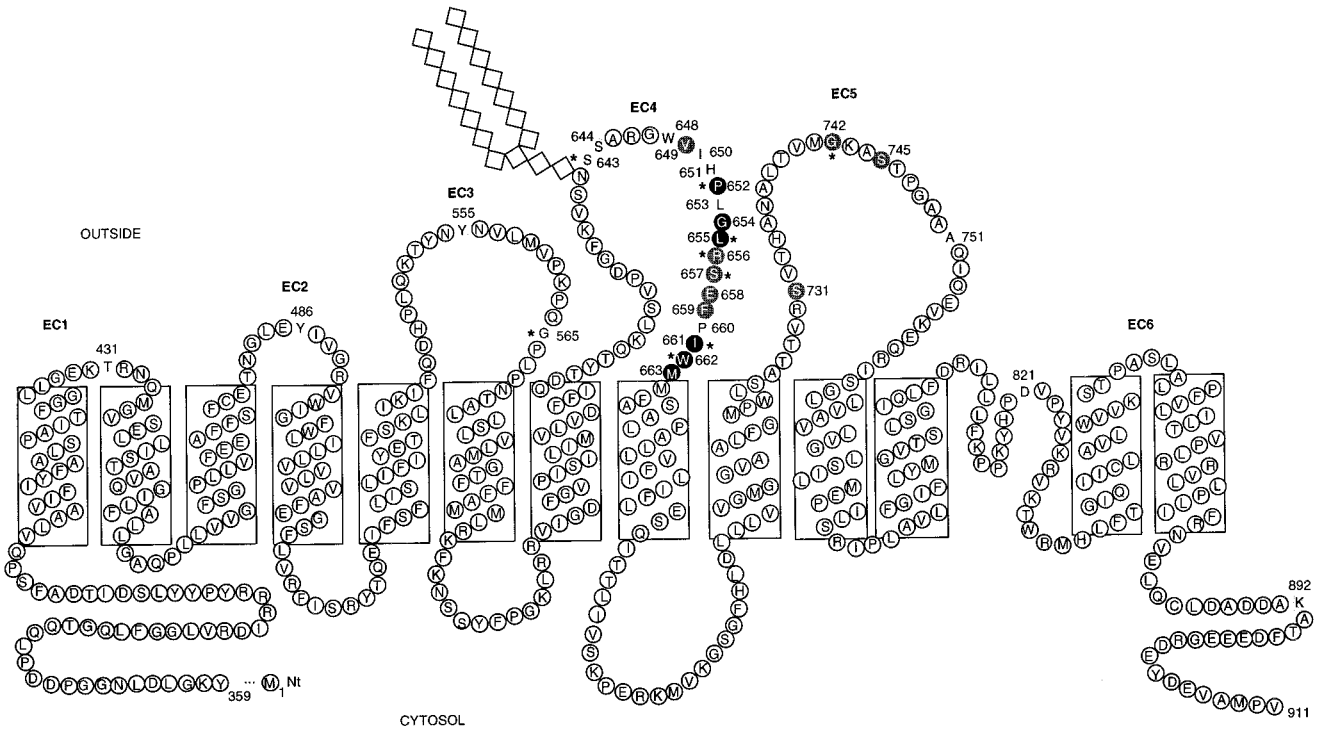

FIGURE 1: Topology model for the membrane domain of human AE1 protein. Introduced cysteine mutants are coded to indicate the effect of DNDS upon biotinylation. Light gray circles indicate little or no effect of DNDS (ratio >0.8); medium gray filled, ratio 0.4−0.8; black filled, ratio <0.4. The topology shown was established from data on accessibility of introduced cysteine residues to impermeant reagents (*25, 37*). The large branched structure represents the single chain of N-linked glycosylation at N642 (*55*). Mutants marked with an asterisk have DNDS effect ratio values that differ from 1.0, with $p < 0.05$.

loaded with BCECF-AM by incubation in 4 mL of serum-free DMEM containing 2 μM BCECF-AM for 20−30 min at 37 °C. 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 $MgSO_4$, 2.5 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM Hepes, pH 7.4) containing 140 mM sodium chloride (chloride buffer), or with 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 (*35*), 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).

Electrophoresis and Immunoblotting. Samples were electrophoresed on 8% acrylamide gels (*32*) and transferred to an Immobilon membrane (*33*). AE1 was detected by incubation of the blot with 10 mL of TBSTM [TBST buffer (0.1% (v/v) Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.5) containing 5% (w/v) nonfat dry milk powder (Carnation)] and 3 μL of anti-human C-terminal peptide antibody 1657 (*25*). After washing, blots were probed with 10 mL of 1:3000 diluted horseradish peroxidase conjugated to goat anti-rabbit IgG. After 1.5 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 (*36*).

Image Analysis. Films were scanned with a Hewlett-Packard scanner ScanJet 4C calibrated with a Kodak Q-14 gray scale. 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:

$$\text{biotinylation}_{\text{norm}} = \text{pixels biotin signal/pixels AE1 immunoblot}$$

DNDS effect was expressed as the ratio:

$$\text{DNDS effect} = \text{biotinylation}_{\text{norm+DNDS}}/\text{biotinylation}_{\text{norm−DNDS}}$$

RESULTS

We have previously introduced a series of individual cysteine mutations into the TM8 region of a cysteineless version of human AE1 called AE1C$^-$ (*25, 37*). The transmembrane disposition of the introduced cysteine residues was determined from the accessibility of each introduced cysteine to sulfhydryl reagents. In this study, we used similar methodology to measure conformational changes associated with noncovalent binding of the disulfonic stilbene anion exchange inhibitor, DNDS. Since DNDS is not able covalently react with AE1, any DNDS effect cannot be attributed to modification of a sulfhydryl.

Figure 1 shows the introduced cysteine mutants used in these experiments. To identify sites that undergo conforma-

tional changes upon DNDS binding, we introduced cysteine residues into each extracellular loop of AE1, and then measured changes in reactivity with the sulfhydryl reagent biotin maleimide. Therefore, we were able to assess only introduced cysteine residues that were accessible to biotinylation. In a previous study, we demonstrated that cysteine residues localized to the plane of the bilayer could not be labeled by biotin maleimide (*25*). In another study, we were unable to identify any biotin maleimide accessible residues in EC7 (*37*). This may be due to the small size of the extracellular loop here. Therefore, in the present study, we have not analyzed any mutants in the EC7 region or in any region that could not be labeled with biotin maleimide. We have previously shown that AE1C$^-$ cannot be labeled by biotin maleimide (*25*), so any observed biotinylation arises only from the single introduced cysteine in each protein.

To determine the concentration of DNDS required to affect biotinylation efficiency, labeling with biotin maleimide was performed at a range of DNDS concentrations. Figure 2 quantifies the effect of DNDS upon two mutants, R656C (panel A) and M663C (panel B). The data for both mutants follow a smooth curve except for points at low DNDS concentration. In panel A, the point at 1 μM has a low accessibility to DNDS. In panel B, the accessibility to DNDS is increased at 1 and 2 μM DNDS, then falls at higher concentrations. The DNDS concentrations for the half-maximal effect were 8 and 5 μM for R656C and M663C, respectively. This is consistent with the K_i of DNDS, 4 μM (*6*). For both mutants, the DNDS effect approaches a maximum at 100 μM DNDS. To observe a maximal effect, subsequent experiments were therefore performed at a DNDS concentration of 100 μM.

To detect changes of AE1 conformation induced by DNDS, we expressed AE1-introduced cysteine mutants in HEK293 cells. Samples of the cells were divided in two; one half was incubated with DNDS, and the other was not. Subsequently we treated both samples with covalently acting biotin maleimide. Figure 3 shows the amount of biotin incorporated into each mutant protein, in samples treated with DNDS, or untreated. To normalize samples for variation in the amount of AE1, blots were stripped and reprobed with an anti-AE1 antibody, as shown. Quantification of the data in this particular experiment is shown beneath the blot data. Since these data are from only a single experiment, it does not correspond exactly with the average values seen in Figure 4.

Figure 4 quantifies the effect of DNDS binding upon AE1 biotinylation. A value of 1.0 means that DNDS had no effect upon the ability to label a site, while a value of 0 means that DNDS fully blocked biotinylation. No mutants had a ratio value >1.0, which would indicate an increase in the ability to be biotinylated upon DNDS exposure. In this scan of the whole AE1 membrane domain, the only regions affected by DNDS binding were EC3, EC4, and EC5. The effect in EC3 was small, but statistically significant and localized to G565C. The most profoundly affected region is the EC4 region. Among the residues in the S643−M663 interval, there is a wide range in the ability to biotinylate cysteines in the presence of DNDS. While L653C and F659C have low influence of DNDS (around 0.8), S657C, I661C, W662C, and M663C have factors around 0.2. In this region, it is clear that sites closer to the membrane surface,

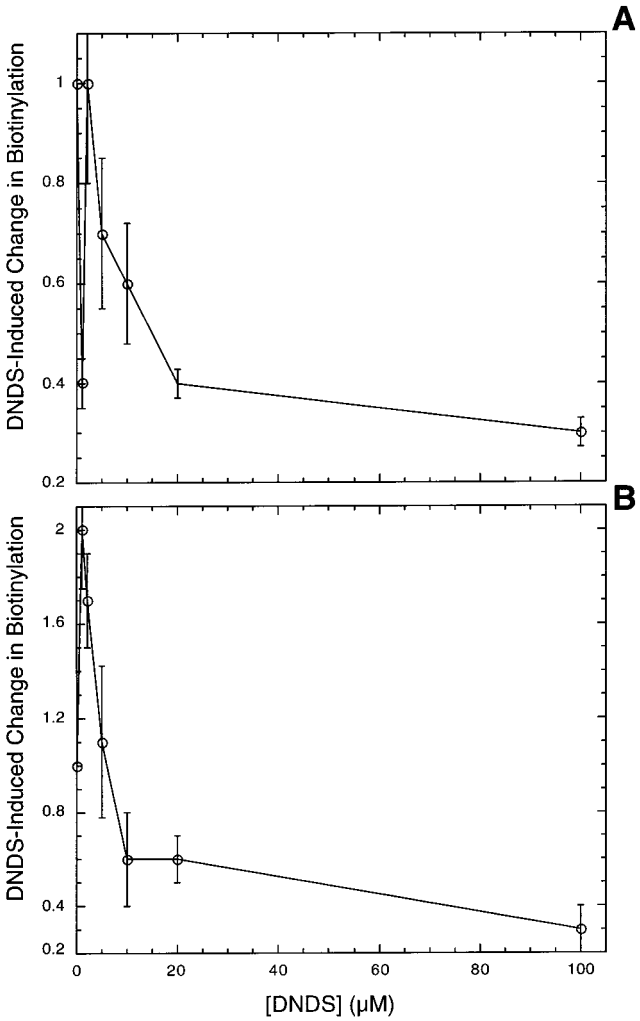

FIGURE 2: Dependence of conformational changes upon DNDS concentration. HEK cells were transiently transfected with cDNA coding for AE1 proteins. The cells were harvested and separated into two fractions. One was incubated with a range of DNDS concentrations from 0 to 100 μM, for 10 min, at room temperature. Both fractions were then incubated with 0.2 mM biotin maleimide, for 10 min, at room temperature. After solubilization, AE1 was immunoprecipitated with a polyclonal anti-AE1 antibody, subjected to SDS−PAGE, and transferred to a PVDF membrane. Labels + and − refer to the cells that were treated or untreated with DNDS, respectively. Blots were probed for the degree of biotin incorporation, using streptavidin−biotinylated horseradish peroxidase followed by ECL, as indicated. Blots were stripped and probed with the monoclonal anti-AE1 antibody IVF12, to determine the amount of AE1 present in each lane, as indicated. Data represent mean (n = 2−3) ± standard error.

determined previously to be at M664 (*25*), are more strongly affected by the presence of DNDS than sites that are more distal to the membrane. Figure 1 also presents the DNDS effect data on a topology model for the AE1 membrane domain.

Mutation of the amino acid sequence has the potential to cause a protein to misfold to a non-native conformation. To determine whether introduced cysteine mutants were functionally active, we measured their anion transport activity by following changes of intracellular pH associated with bicarbonate movement (Table 1) (*25*). Transport activities of mutants in the S643−K892 region were previously characterized (*25*, *37*), but these data are included for the sake of completeness. Among the 28 mutants examined in

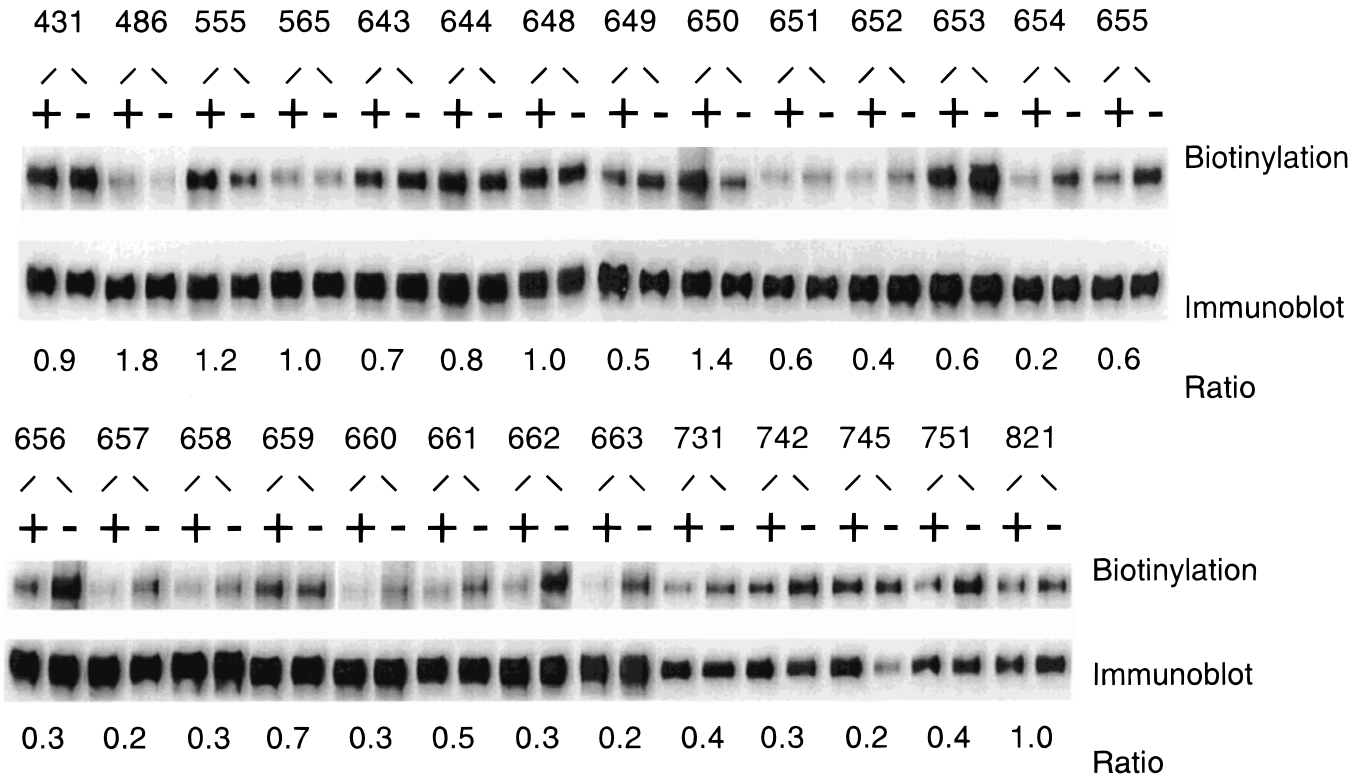

FIGURE 3: Effect of DNDS upon accessibility to sulfhydryl biotinylation. HEK cells were transiently transfected with cDNA coding for AE1 proteins. The cells were harvested and separated into two fractions. One was incubated with 0.1 mM DNDS, for 10 min, at room temperature. Both fractions were then incubated with 0.2 mM biotin maleimide, for 10 min, at room temperature. After solubilization, AE1 was immunoprecipitated with a polyclonal anti-AE1 antibody, subjected to SDS−PAGE, and transferred to a PVDF membrane. Labels + and − refer to the cells that were treated or untreated with DNDS, respectively. Blots were probed for the degree of biotin incorporation, using streptavidin−biotinylated horseradish peroxidase followed by ECL, as indicated. Blots were stripped and probed with the monoclonal anti-AE1 antibody IVF12, to determine the amount of AE1 present in each lane, as indicated. Numbers represent the ratio of the degree of biotinylation observed in the absence and presence of DNDS, in this particular experiment.

Table 1: Anion Exchange Activity of AE1-Introduced Cysteine Mutants[a]

anion exchanger	rel anion exchange act. (%)	anion exchanger	rel anion exchange act. (%)
AE1C$^-$	100	R656C	46 ± 11
T431C	73	S657C	51 ± 2
Y486C*	7	E658C	66 ± 9
Y555C	96	F659C*	1 ± 10
G565C	152	P660C	11 ± 3
S643C	83 ± 2	I661C	34 ± 10
S644C	77 ± 2	W662C*	7 ± 4
W648C*	6 ± 4	M663C	56 ± 3
V649C	72 ± 1	S731C	41
I650C*	0 ± 2	G742C	115
H651C	52 ± 16	S745C	83
P652C*	3 ± 9	A751C	96
L653C	30 ± 6	D821C	84
G654C*	10 ± 12	K892C	54
L655C*	4 ± 2		

[a] Activity values are expressed relative to AE1C$^-$ and have been corrected for the background activity of sham-transfected cells. Mutants marked with an asterisk are functionally inactive. Transport activity is expressed as mean ± standard error (n = 1−3).

this study, 8 had 10% or less residual anion transport activity, which we define as functionally inactive. Of the inactive mutants, Y486C, W648C, I650C, and P652C were not affected by DNDS while G654C, L655C, F659C, and W662C were sensitive to the presence of DNDS. The functionally inactive mutants may have compromised conformations, which inhibit function. However, since the effect of DNDS upon these compromised mutants is consistent with neighboring mutants that have significant transport activity, the compromised mutants likely report accurately on DNDS-induced conformational changes.

Membrane-impermeant DNDS would not be able to access protein, which was not processed to the plasma membrane. Therefore, we assessed the degree of cell surface processing of mutants that had low anion transport activity. Cell surface processing was measured by the ability to label AE1 mutants in whole cells with the membrane-impermeant reagent biocytin hydrazide. Among the functionally inactive mutants, all except Y486C were previously shown to be processed to the cell surface to a similar extent as AE1C$^-$ (*25*, *37*). Y486C was not previously assessed, but in the present study we found that this mutant is processed similarly to AE1C$^-$ (data not shown). Therefore, the lack of a DNDS effect on biotinylation is not due to a failure to process AE1 to the cell surface.

DISCUSSION

In this study we scanned the AE1 anion exchange protein to identify sites that are influenced by the noncovalent binding of the anion exchange inhibitor DNDS. AE1-introduced cysteine mutants were treated with the biotinylating sulfhydryl reagent, biotin maleimide, with and without prior exposure to DNDS. Decreased biotinylation in the presence of DNDS may indicate that a residue forms part of the DNDS binding site, such that DNDS binding

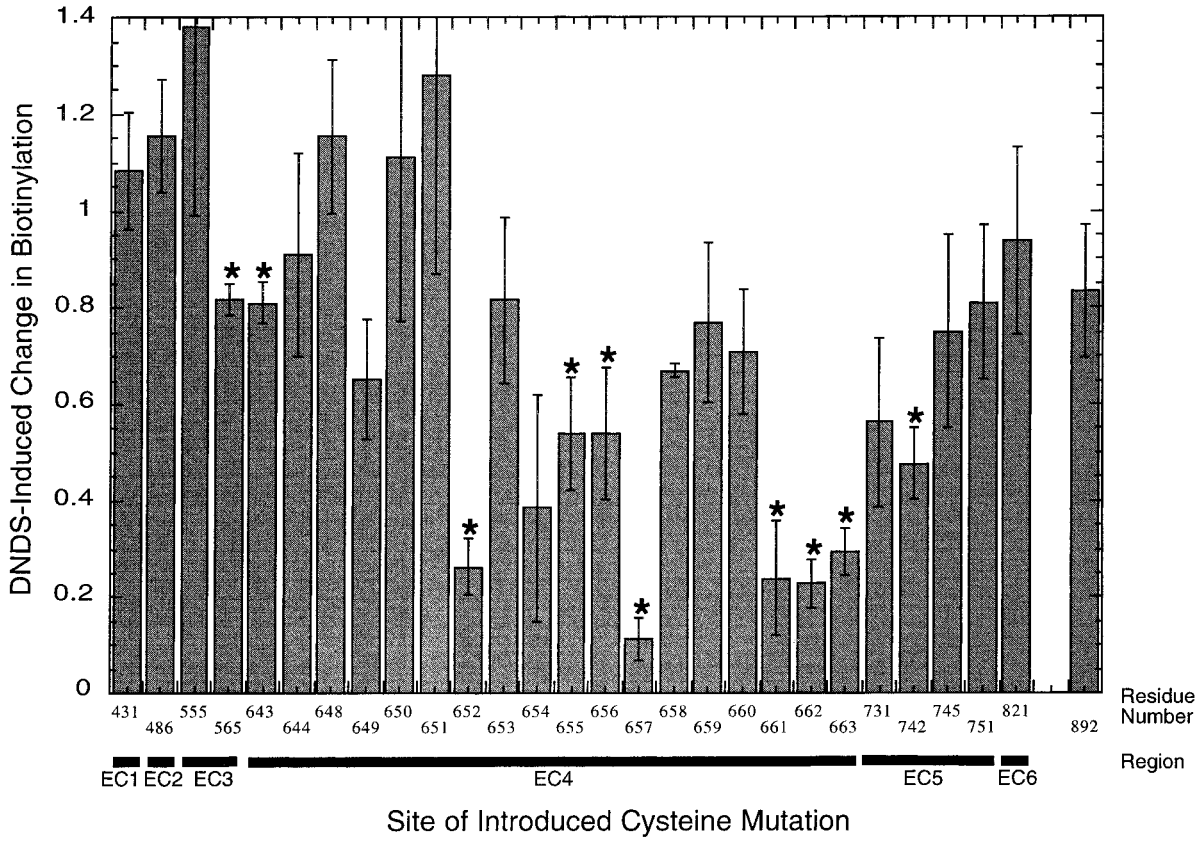

FIGURE 4: DNDS-induced changes in accessibility to biotinylation. Human AE1-introduced cysteine mutants were incubated with or without DNDS and then treated with biotin maleimide, as described. Biotin incorporation and AE1 expression level were quantified by densitometry. For each mutant, the biotin incorporation obtained with DNDS was divided by the biotin incorporation observed without the preincubation step (±ratio). The biotinylation signals are normalized by the amount of AE1 present in each lane. The ratio represents changes of accessibility to biotinylation induced by DNDS binding. Data represent mean of 3−5 independent experiments ± standard error. Mutants marked with an asterisk have values that differ from 1.0, with $p < 0.05$.

sterically blocks access of biotin maleimide. Alternatively, DNDS binding may alter the conformation of AE1, causing parts of the protein to fold in a way that makes them inaccessible to the aqueous phase. We have shown here that EC3, EC4, and EC5 showed changes in response to DNDS. We conclude that stilbene disulfonate compounds either directly interact with these regions, or alter the conformations of these regions. Conformational changes induced by DNDS binding result in changes at the anion binding site that block anion transport. Therefore, the conformationally sensitive regions of AE1 may be near the anion transport site.

We examined the effect of DNDS upon labeling cysteine residues with biotin maleimide to establish a dose−response curve for DNDS (Figure 2). The half-maximal effect of DNDS upon biotin maleimide labeling (8 and 5 μM for R656C and M663C, respectively) was consistent with the K_i observed for DNDS inhibition of AE1 transport in red cells of about 4 μM (*6*). We conclude that the effect of DNDS upon biotin maleimide labeling observed in the present report occurs by the same mechanism as the inhibition of transport. That is, the changes of biotin maleimide labeling are due to DNDS binding to AE1 in a way that inhibits transport, not to an interaction between DNDS and biotin maleimide.

At low DNDS concentrations, some deviations of the dose−response curve were observed (Figure 2). For R656C, a decrease in accessibility to biotinylation was observed at 1 μM DNDS. While this may be a spurious point, it could indicate altered behavior of AE1 when the DNDS binding site is partially occupied. Similarly, for mutant M663C, accessibility to biotinylation increased at 1 and 2 μM DNDS but decreased at higher DNDS concentrations. At DNDS concentrations below the half-maximal concentration, the DNDS binding site will not be saturated. Since the basic structural unit of AE1 is a dimer (*38*, *39*), one subunit may bind DNDS while the other is empty. The binding of DNDS at one monomer may cause a conformational change in the unbound subunit that alters accessibility of the introduced cysteine residue to biotinylation. Conformational changes transmitted between subunits of the AE1 dimer upon DNDS binding have previously been observed (*40*, *41*).

Five mutants (P652C, G654C, L655C, F659C, and W662C) had little or no anion transport activity, yet showed reduced biotinylation in the presence of DNDS. This indicates that mutation at these positions affects anion transport, but DNDS binding capacity is retained. Stilbene disulfonate binding and anion transport therefore differ in their structural requirements. This is consistent with a model in which stilbene disulfonates form extensive interactions with AE1 that cannot be significantly perturbed by single point mutations.

Decreases, but not increases, in the ability to biotinylate introduced cysteine residues were observed in the presence of DNDS. The decreases in biotinylation may represent a conformational shift of the cysteine side chain to a more hydrophobic environment, since cysteine residues in the hydrophobic core of the lipid bilayer could not be labeled

by biotin maleimide (*25, 37*). DIDS labeling has been shown not only to stabilize the protein from thermal denaturation (*11, 42*) but also to protect the protein from proteolysis (*43*). Both observations are consistent with a tightening of the protein structure upon stilbene disulfonate binding. This is consistent with our observation that sites become less accessible to biotinylation upon binding DNDS. The observation that DNDS-induced changes are transmitted to extramembranous regions suggests that the AE1 is sufficiently flexible to be able to undergo relatively large conformational changes. This is consistent with the observation that AE1 has a large volume of activation, or large conformational changes associated with transport (*44*).

Proximity to the membrane surface is a determinant of the magnitude of conformational change at a particular site. We previously showed that M664 is the first residue in the hydrophobic core of the lipid bilayer in the EC4 region, on the basis of a failure to label an introduced cysteine mutant at this position with biotin maleimide (*25*). EC4 best illustrates the importance of membrane proximity. Although the density of sites that show DNDS-dependent conformational changes is greatest close to the membrane surface, sites distant from the membrane surface are also sensitive. For example, S643C is at least 17 amino acids away from the membrane surface on the basis of 2 independent determinations of AE1 topology in this region (*25, 45*). AE1 is thought to have a large outward-facing vestibule leading to the anion translocation channel, and the EC4 region may form a part of this vestibule (*25*). In the sequence G654C−M663C, only P660C is not influenced by DNDS. Since this mutant retains only 11% residual transport activity, it is possible that the P660C mutation induces a misfolding that causes the site to be insensitive to DNDS.

The EC4 and -5 regions of AE1 undergo the largest changes upon DNDS binding. We interpret this to mean that these sites are close to the DNDS binding region. Consistent with this interpretation, we have shown that the TM8 region forms part of the anion translocation pore (*46*). Similarly, the EC5 region has been proposed to form part of the channel lining in an extended "T-loop" conformation (*45*). Independently, we have shown that the EC5 region is accessible to aqueous reagents from the outside of the cell and might form part of the translocation pore (*37*).

EC3 also was only slightly sensitive to DNDS. Within EC3, the lack of DNDS effect on Y555C and small, but statistically significant effect at Y565C may be surprising since these sites are closest to the H_2DIDS-reactive lysine residues at K539 and K542. Recently, TM6 and -7 were shown not to be essential for AE1 anion transport activity (*47*). This suggests that TM6 and -7 are not part of the anion translocation machinery. Since the stilbene disulfonate binding site overlaps with the Cl^- binding site (*48, 49*), it also suggests that TM6 and -7 do not form part of the stilbene disulfonate binding site. Our results are consistent with this model. Also consistent is work that showed that chymotrypsin digestion of AE1 in the EC3 region did not affect stilbene disulfonate binding, but papain treatment of AE1, which cuts EC4, did alter stilbene disulfonate binding (*50*). Y555 is adjacent to the site of external chymotryptic cleavage (*51*) and is therefore clearly part of an aqueous accessible extracellular loop.

The lack of DNDS effect upon biotinylation is also informative. Mutants T431C (EC1) and Y486C (EC2) are distant in the linear sequence from the sites that showed DNDS sensitivity. Both sites are found within the region of the first four TM. These TM are more highly conserved than the remainder of the membrane domain (*51*). During biosynthesis, TM1−4 must be stable as a unit in the bilayer, and it has been suggested that they form a tightly associated bundle (*51*). Similarly, TM1−3 have been assigned as a bundle of transmembrane helices that associate with the membrane through protein/protein contacts on the basis of their susceptibility to proteolysis following alkaline denaturation (*52*). The lack of a DNDS effect on the introduced cysteine mutants in the TM1−4 region suggests that this bundle is not part of the stilbene disulfonate binding site of AE1. Consistent with a localization of this region distant from the anion translocation pore, eosin maleimide covalently reacts with K430 (*53*), without blocking access to the external anion binding site (*19, 20, 24, 54*).

The site of cross-linking with H_2DIDS is K851 (*10*). We previously constructed and characterized two mutants (S852C and A858C) close to K851 (*37*). In the present study, we could not analyze these mutants because neither was labeled with biotin maleimide (*37*). Similarly, T830C, G838C, C843, T866C, R871C, and R879C could not be labeled with biotin maleimide. The two introduced cysteine mutants closest to K851 that could be labeled with biotin maleimide were D821C and K892C. Yet, DNDS influenced the biotinylation of neither of these mutants. We conclude that stilbene disulfonates interact with this region, but they do not greatly change the region's conformation. The interaction of H_2DIDS with the region may be limited to the compound's reactive isothiocyanate group.

We introduced cysteine residues into AE1 protein in a scan of the extracellular loop regions. Only three regions changed conformation upon binding of the anion exchange inhibitor DNDS. Introduced cysteine residues in the EC3, EC4, and EC5 regions showed decreased labeling with biotin maleimide in the presence of DNDS. We therefore assign these regions as close to the DNDS binding site, or regions that undergo a large conformational shift upon binding the inhibitor. Introduced cysteine mutants in other parts of the protein were insensitive to the addition of DNDS. We conclude that DNDS binding causes large changes in AE1 conformation, which are localized to the TM7−10 region of AE1. These conformationally sensitive regions may form the anion translocation pore of AE1, whose function is blocked by DNDS binding.

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