AE2 Anion Exchanger Polypeptide Is a Homooligomer in Pig Gastric Membranes: A Chemical Cross-Linking Study†

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ABSTRACT: Although considerable information is available on the oligomeric states of the AE1 (band 3) anion exchanger, little is known about the physiological state of the polypeptides encoded by the nonerythroid AE genes, AE2 and AE3. We have previously characterized the proteolytic susceptibility of native pig gastric AE2. In the course of studies in which pig gastric membranes were treated with the AE2 transport antagonist, DIDS, we noted evidence for cross-linking of AE2 proteolytic fragments to higher-order oligomeric forms. We have characterized the ability of DIDS and of selected N-hydroxysuccinimide cross-linking agents to increase the proportion of SDS-resistant oligomers of pig gastric AE2 and its proteolytic fragments. Cross-linking exhibited time and concentration dependence. N-Terminal protein sequencing proved that DIDS treatment created AE2 homodimers. Putative homotetramers were also observed. Protomers were cross-linked via residues within the C-terminal 40 kDa of AE2. Prior proteolytic cleavage of AE2 in membranes resulted in decreased yield of subsequently cross-linked products. AE2 cross-linking could not be detected in membranes pretreated by hypotonic wash and freeze−thaw. The results are interpreted in light of the deduced amino acid sequence of the transmembrane domain of pig AE2.

The three genes of the AE[1] anion exchanger gene family, AE1, AE2, and AE3, each encode several predicted polypeptide products from alternatively spliced and initiated transcripts. Recombinant polypeptide products of all three genes have been shown to mediate Cl^-/HCO_3^- exchange, but only erythroid AE1 (red cell band 3) has been proven to perform this function as an endogenous polypeptide product in its native cell environment (*4, 5*). Because AE1 is the most abundant integral membrane protein in the mammalian red cell, it has undergone extensive biochemical characterization in parallel with its thorough physiological investigation over many years (*6, 7*).

Though many studies of monovalent anion exchange by red cell AE1 have provided no evidence of cooperative kinetics, several studies of divalent anion exchange have shown kinetics that could be better modeled by cooperative than by simple Michaelis−Menten models (*8*). Consistent with these results, the majority of biochemical studies of AE1 in detergent solution have suggested that the protein is distributed among homodimeric, homotetrameric, and perhaps also higher order oligomeric forms (*9*−*12*). Assembly of detergent-solubilized AE1 into two-dimensional crystalline arrays has suggested a hexameric structure, comprised of a trimer of homodimers (*13*).

Prominent among the techniques used to evaluate the native oligomeric state of AE1 in red cell membranes has been chemical cross-linking (*14*). The ability to cross-link proteins to SDS-resistant oligomers has been among the most widely used assays for development of new protein cross-linking reagents and for testing of new methods for "nearest neighbor analysis" of membrane proteins (*15*). This method has been especially fruitful for assessment of oligomeric structure of proteins with single transmembrane span (*16*), but has also proven useful for polytopic transmembrane proteins; the purinergic ligand-gated cation channel is a recent example (*17*).

The oligomerization state of red cell membrane AE1 regulates or is influenced by its attachment to cytoskeleton. Most studies conclude that red cell ankyrin (Ank1) preferentially binds to tetrameric AE1. Tetramerization is thought to require regions of the N-terminal cytoplasmic domain of AE1 (*18*), as does ankyrin binding (*19*). In contrast, the N-terminal cytoplasmic domain is not required for dimerization, as the isolated transmembrane domain of AE1 is itself a stable dimer in detergent solution (*10, 20*). Moreover, the equilibrium between noncovalently associated AE1 dimer and tetramer in nonionic detergent, and between monomer and noncovalent dimer in SDS, can be selectively regulated by some but not by other stilbene disulfonate antagonists. Selected stilbene disulfonates can also modulate the noncovalent dimerization equilibrium between dimers of monomers

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[1] Abbreviations: AE, anion exchanger; Ank, ankyrin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptidyl N-glycosidase F; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DADS, 4, 4′-diaminostilbene-2,2′-disulfonic acid; BS^3, bis(sulfosuccinimidyl) suberate; DSS, disuccinimidyl suberate; DSP, dithiobis(succinimidyl propionate); DTSSP, dithiobis(sulfosuccinimidyl propionate); TFA, trifluoroacetic acid.

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previously cross-linked with bis-sulfosuccinimidyl suberate (BS^3) (*21*). However, the physiological importance of the conformational changes reflected by such altered reactivity is not known. Higher order oligomerization of AE1 is associated with the presence of membrane-associated Heinz bodies (oxidized, aggregated hemoglobin) (*22*) or with binding of circulating antibodies to "senescent cell antigen" thought to comprise epitopes in one or more exofacial loops of AE1 (*23*).

In parallel with our studies on the functional properties of recombinant AE2, we have initiated biochemical studies on structural aspects of native AE2. These studies should provide information about native AE2 which can be interpreted in light of progressing structural studies of two-dimensional crystals of native erythroid AE1 (*24*, *25*) and for similar projected studies with recombinant AE2 (*26*). Motivated by the moderately abundant expression of AE2 in the gastric parietal cell (*27*), we selected pig gastric mucosa as the starting point for purification of pig AE2 in SDS (*3*) and for the subsequent immunoaffinity purification of AE2 in nondenaturing detergents (*28*). We have studied the N-glycan of pig gastric AE2 (*3*), shown the likely presence of O-glycan (*28*), and mapped AE2 protease susceptibility in native membranes (*28*).

In the course of studies examining the effects of DIDS on pig gastric AE2 polypeptide, we noted that DIDS treatment of membranes increased the AE2 immunoblot signal intensity as detected by antibody to AE2 C-terminal peptide. Proteolytic treatment of these membranes to generate stable C-terminal fragments of AE2 showed that the additional immunoreactive material migrated as predicted for C-terminal fragment homooligomers. The experiments presented here prove the presence in gastric membranes of AE2 homodimers and suggest the ability to generate AE2 homotetramers by chemical cross-linking. The studies also show that AE2 in pig gastric membranes exists as at least two populations of differing susceptibility to chemical cross-linking, whose proportions in the membrane can be experimentally altered. The results are interpreted in light of the amino acid sequence of the transmembrane domain of pig AE2 as deduced from the cloned cDNA.

EXPERIMENTAL PROCEDURES

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA from freshly resected pig stomach was prepared using the RNeasy kit (Qiagen). Reverse transcription (RT) of 1 μg of total RNA was primed by oligo(dT)-12−18 and performed with the First Strand DNA Synthesis kit (Ambion). One-twentieth of the volume of the RT reaction served as template in a 50 μL reaction volume for hot start PCR (Expand High Fidelity PCR System, Boehringer) in the supplier's recommended buffer.

PCR mixes lacking only primers were preheated at 82 °C for 1 min, then corresponding primers (oligonucleotide sequences available upon request) were injected into the mix through oil. The complete reaction mixes were denatured for 3 min at 95 °C, then cycled through these conditions: denaturation for 45 s at 94 °C, annealing for 2 min at 60 °C (or 52 °C when using Qiagens Q-solution), and elongation for 2−3 min at 72 °C. Final extension of 10 min at 72 °C was terminated by rapid cooling to 4 °C after 33−35 cycles (AE2) or 25 cycles (GAPDH and β-actin as cDNA quality controls). PCR products were separated in 1% agarose gels and purified from gel with the QIAquick Gel Extraction Kit (Qiagen) as needed.

The known AE2 nucleotide and amino acid sequences from mouse (J04036), rat (J05166), rabbit (S45791), and human[2] (U62531) aided the design of slightly degenerate oligonucleotide primers encoding conserved regions in the aligned AE2 sequences. Although standard PCR buffers used in a variety of conditions failed to amplify cDNA products encoding the entire pig AE2 transmembrane domain from any pertinent primer pairs, shorter PCR products could be amplified. Successful amplification of some of these shorter products required the annealing modifier, Q-solution (Qiagen), likely due to extended regions of unusually elevated GC content (see Results). Purified overlapping PCR products for pig AE2 were directly sequenced using an ABI 373 DNA Sequencer. Nucleotide sequence of PCR fragments was verified by DNA sequencing of both strands, and at least two independently amplified PCR fragments were used to confirm each nucleotide position. The complete nucleotide sequence for pig AE2 transmembrane domain was assembled, and the encoded amino acid sequence was deduced. Database searches and sequence analysis were carried out with GCG suite of programs (University of Wisconsin Genetics Computing Group).

Preparation of Gastric Membranes. Pig stomachs were obtained following cardiovascular surgical experiments approved by the IACUC of Beth Israel Deaconess Medical Center, and stripped mucosae were frozen at −80 °C until use. A membrane fraction enriched in basolateral plasma membrane was prepared as previously described (*3*, *28*). For some experiments, "hypotonically washed membranes" were prepared by the following additional steps. Membranes were first washed in 10 mM sodium phosphate or Tris-HCl, pH 7.4, containing 1 mM EDTA, then frozen−thawed twice, then washed once more in the same buffer.

Chemical Cross-Linking of Gastric Membranes. Cross-linking of membranes (2 mg of protein/mL final concentration) with 1 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, Calbiochem) or 2 mM bis(sulfosuccinimidyl) suberate (BS^3), dithiobis(sulfosuccinimidyl propionate) (DTSSP), disuccinimidyl suberate (DSS), and dithiobis-(succinimidyl propionate) (DSP, Pierce) was carried out in "isotonic buffer" containing 250 mM sucrose, 1 mM EDTA, 0.1 mg/mL PMSF, and 10 mM sodium phosphate, pH 7.4, for 10−60 min at room temperature or as indicated in figures legends. Reactions with water-soluble cross-linkers were initiated by mixing equal volumes 2× membrane suspension with 2× concentrated solutions of DIDS, BS^3, or DTSSP in the same buffers. Reactions with water-insoluble cross-linkers were started by addition of a 1/50 vol of 100 mM DSS or DSP in DMSO to a 1 vol membrane suspension (2 mg/mL protein). All reactions were stopped by addition of at least 20 vol of 100 mM Tris-HCl, pH 7.6, at the times indicated. Membranes were then centrifuged at 4 °C in the TLA-45 rotor for 20 min at 109000*g* in the TL-100 ultracentrifuge and resuspended in appropriate buffer.

[2] The human AE2 cDNA sequence is that of Medina et al. (*1*), which has corrected the errors in the earlier deposited human AE2 sequence (*2*).

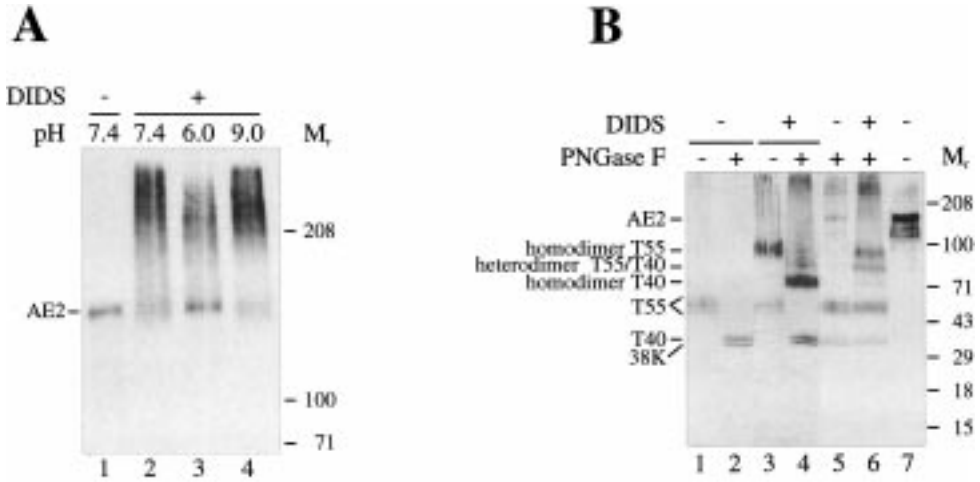

FIGURE 1: Treatment of gastric membranes with 1 mM DIDS leads to pH-dependent oligomerization of AE2. (A) Immunoblot of membranes pretreated without (lane 1) or with 1 mM DIDS (lanes 2−4) at the indicated pH values at 20 °C for 30 min. (B) Immunoblot of membranes pretreated without (lanes 1, 2, and 5) or with 1 mM DIDS (lanes 3, 4, and 6), then incubated in the absence (lanes 1 and 3) or presence of PNGase F (lanes 2 and 4−6) followed by further incubation in the absence (lane 7) or presence of trypsin (lanes 1−6). Lanes 5 and 6 show, respectively, membranes without and with DIDS-pretreatment in which AE2 was incompletely deglycosylated prior to trypsin exposure. Lane 7 represents control membranes incubated identically except for the omission of DIDS, PNGase F, and trypsin.

N-Deglycosylation and Proteolysis of Membranes. N-Deglycosylation of membranes (2 mg/mL) with 2500U/mg PNGase F[3] (New England Biolabs, Beverley, MA) was carried out in 100 mM Tris-HCl, pH 7.6, for 1h at 37 °C. The membrane suspension was then supplemented with NaCl to 150 mM, and tryptic digestion at 20 °C was carried out for 10−15 min at enzyme/protein ratio of 1:200. For chymotryptic digestion, an equal volume of water was added to yield 1 mg/mL membrane protein in 50 mM Tris-HCl, pH 7.6. Chymotrypsin was added to yield chymotrypsin/protein ratios from 1:50 to 1:1, and digestion was carried out at 37 or 20 °C for the indicated times. The primary antibody used for immunoblot was affinity-purified rabbit anti-mouse AE2 C-terminal aa 1224−1237 (*27*), shown previously to recognize pig AE2 (*3*,*28*). SDS−PAGE and immunoblot protocols were previously described (*28*), and immunoreactive bands were visualized by ECL (Amersham).

Immunoaffinity Isolation of Cross-Linked T40 Dimer. To obtain the AE2 cross-linked T40 dimer in quantity sufficient for N-terminal amino sequencing, 25/42 membranes (2 mg/mL protein, 50 mg of total protein) were treated with 1 mM DIDS (in dark) for 30 min at room temperature in isotonic buffer**.** Membranes were pelleted at 47000*g* in an SS-34 rotor for 1 h at 4 °C and resuspended to 1 mg of protein/mL in 140 mM sodium bicarbonate. Tryptic digestion was carried out at an enzyme/protein ratio of 1:200 for 1 h, and stopped by addition of PMSF to 0.2 mg/mL. Membranes were pelleted as above and washed free from trypsin three more times in 140 mg/mL sodium bicarbonate containing 0.2 mg/mL PMSF (50 mL of each wash). The final pellet was solubilized for 30 min (+4 °C) in 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.1 mg/mL PMSF. Insoluble material was removed by centrifugation, and the clarified extract was preincubated 1 h at 4 °C with 0.5 mL of GBP Sepharose (Pharmacia) for removal of endogenous antibody. This extract was further incubated 2 h at 4 °C with 50 μL of crude ascites containing monoclonal antibody ZC-5-5 against C-terminal peptide of AE2 (*28*) and then overnight with 250 μL of Poros G beads (PerSeptive Biosystems). Immunoreactive material containing the AE2 C-terminal epitope was eluted from beads, concentrated to 180 μL, and incubated overnight at 4 °C with PNGase F (500 units). The deglycosylated sample was precipitated with methanol−chloroform (*29*) and disaggregated by treatment with trifluoroacetic acid (TFA) (*30*), electrophoresed by SDS−PAGE, transferred to Immobilon −P^{SQ} PVDF membrane (Millipore)**,** and subjected to N-terminal amino acid sequencing by Edman degradation (HHMI Biopolymers Facility, MIT) as described (*28*).

1D in Gel Tryptic Peptide Maps. One-dimensional in-gel tryptic peptide mapping of AE2 (in membranes) and of SDS−PAGE-isolated T40 and dimer T40 fragments was carried out with Sweadner's modification (*31*) of the method of Cleveland et al. (*32*).

RESULTS

DIDS Cross-Links AE2 in Pig Gastric Membranes. Figure 1A shows that a 30 min treatment of native gastric membranes with 1 mM DIDS at 20 °C shifted most AE2 immunoreactivity from the monomeric position (lane 1) to a heterogeneous collection of higher M_r forms (lanes 2−4). As expected for the covalent reaction between isothiocyanate groups and free amino groups on target proteins, the reaction was attenuated at pH 6.0. However, the reaction at pH 7.4 was not further enhanced at pH 9.0. The heterodisperse high M_r complex could represent both homooligomeric and heterooligomeric cross-linking of AE2.

These possibilities were further evaluated in Figure 1B, in which DIDS-treated membranes further incubated in the absence or presence of PNGase F, were then treated with trypsin. Trypsin cleaves AE2 at the Arg−Ala bond between the second and third consensus N-glycosylation sites in the exofacial loop linking TMs 5 and 6 (*28*). The resultant heterogeneous C-terminal AE2 fragment (T55, lane 1) was converted by N-deglycosylation to T40 (lane 2). [The 38K-

[3] One unit of PNGase F is defined as the amount of enzyme required to remove all of the carbohydrate from 10 μg of denatured RNAse B at 37 °C in 1 h (500 New England Biolab units = 1 IUB milliunit.)

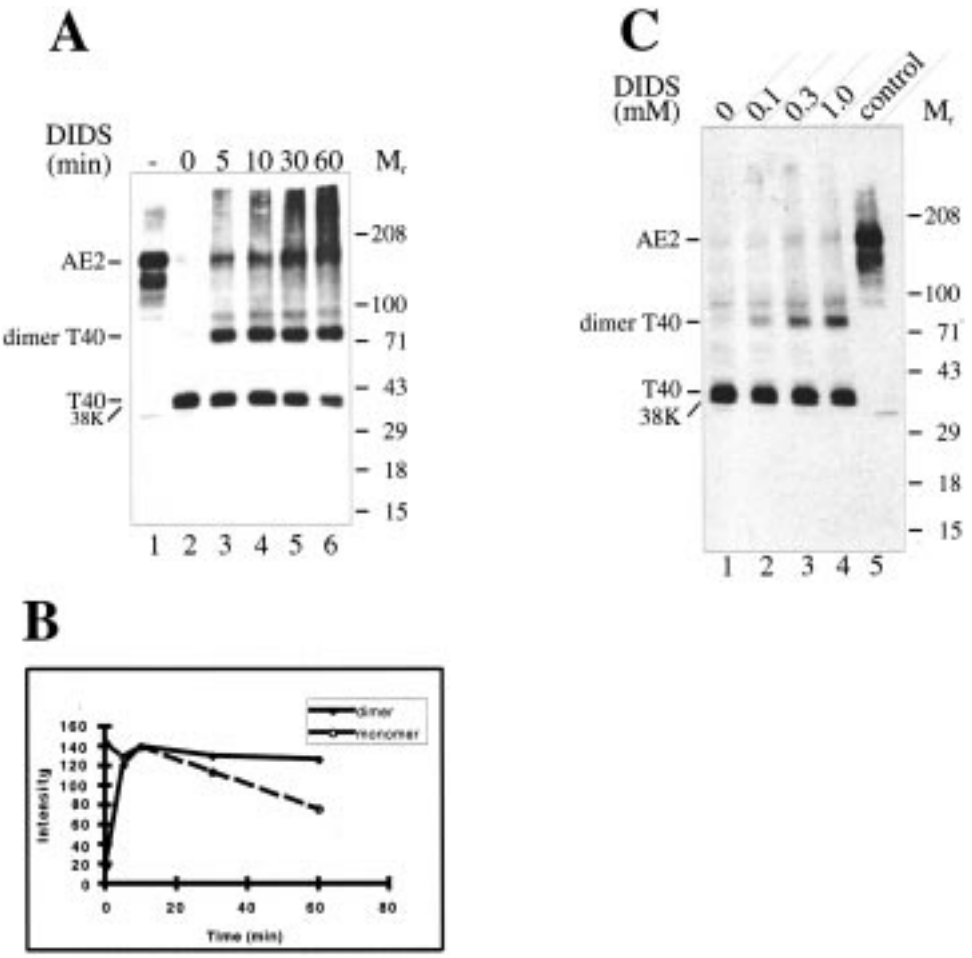

FIGURE 2: Characteristics of AE2 oligomerization by DIDS. (A) Immunoblot of gastric membranes treated with 1 mM DIDS for the indicated time periods. (B) Quantitation of monomer and dimer bands of immunoblot in panel A. (C) DIDS concentration dependence of AE2 oligomerization (30 min incubations at 20 °C).

band is the product of an endogenous protease activity active on deglycosylated AE2 (*3*)]. After DIDS treatment, additional bands at ~100 kDa (putative T55 dimer, lane 3) or 80 kDa (putative T40 dimer, lane 4) are also evident, but without evident decrease in intensity of the T55 or T40 bands. The ~100 and 80 kDa bands (putative T55 dimer and T40 dimer, respectively) were liberated by trypsin from the high M_r oligomer/aggregate depicted in Figure 1A (lane 2). (For a schematic of the AE2 proteolytic cleavage sites and C-terminal proteolytic fragments of AE2 to be discussed in this paper, see Figure 7.)

Figure 1B, lane 5, shows incompletely N-deglycosylated gastric membranes treated with trypsin, yielding both T55 and T40. DIDS-treated membranes were also partially N-deglycosylated prior to trypsinization. As evident in lane 6, in addition to putative T55 dimer and putative T40 dimer bands, a band with the intermediate M_r predicted for the T55/T40 heterodimer was also present. These results further support the hypothesis that the 80 and ~100 kDa bands represent homodimers of C-terminal tryptic peptides of AE2.

These AE2 C-terminal peptides were studied further for the information they might impart about the oligomeric status of native AE2 in pig gastric membranes. Figure 2 shows that AE2 is cross-linked by DIDS in intact membranes, as evidenced by the putative T40 dimer generated by subsequent trypsinization. Whereas the level of putative T40 dimer plateaued at 5−10 min (panels A and B), longer periods of DIDS pretreatment led to a slow decrease of T40 monomer in parallel with a slow increase in putative tetramer (comigrating with native AE2, panels A and C) and higher M_r forms (panel A). Formation of putative dimer T40 increased as a function of DIDS concentration (panel C), but DIDS concentrations greater than 1 mM did not further increase its abundance (not shown).

Effects of Other Stilbene Disulfonates and of Hydroxysuccinimide Cross-Linkers on AE2. To test the hypothesis that DIDS treatment merely enhanced the previously described SDS-resistant dimerization of AE2 (*28*), rather than covalently cross-linking AE2 monomers, the effects of the monoreactive stilbene disulfonate, SITS, were compared to those of DIDS. The experiment in Figure 3A was performed with C-terminal AE2 fragments generated by chymotrypsin treatment of gastric membranes (*28*). In contrast to DIDS (lanes 1−4), SITS treatment in identical conditions (lanes 5−8) did not lead to the presence of putative CH39 dimer and did not alter kinetics of AE2 C-terminal chymotryptic fragment generation or C-terminal epitope loss compared with control membranes (lanes 9−12). The noncovalent stilbene disulfonate AE inhibitor, DADS, was similarly without effect on AE2 cross-linking (not shown). Similar results were observed with trypsin-treated gastric membranes (not shown).

Hydroxysuccinimide reagents also cross-linked AE2 C-terminal proteolytic fragments. Exposure of hydroxysuccin-

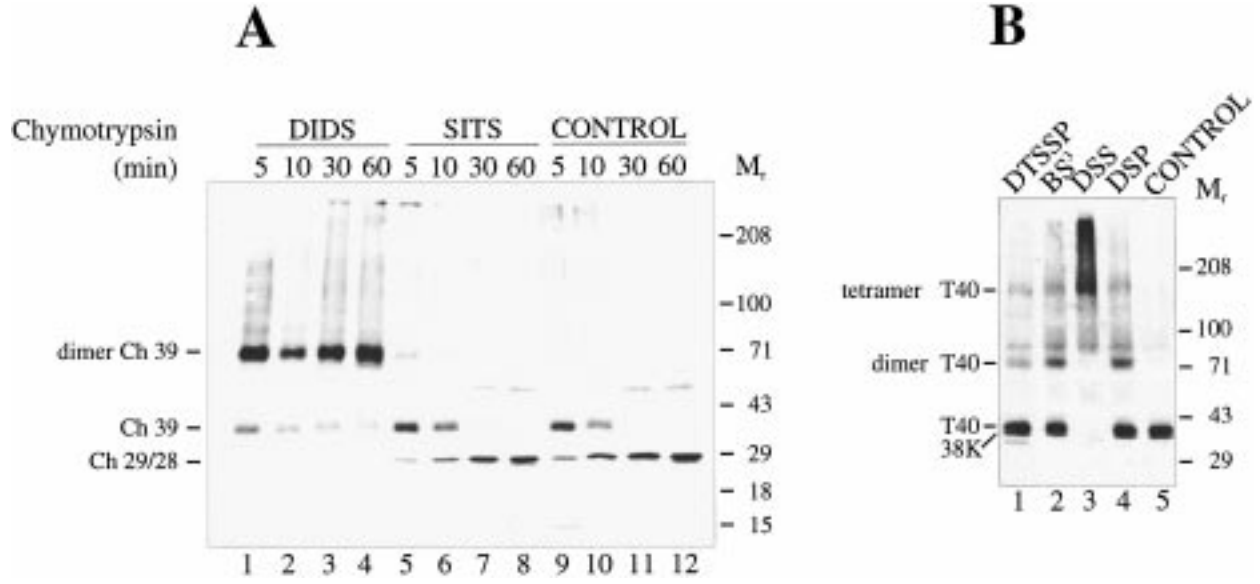

FIGURE 3: Effects of SITS and of N-hydroxysuccinimidyl ester compounds on AE2 dimerization. (A) Immunoblot of gastric membranes pretreated with 1 mM DIDS (lanes 1−4), 1 mM SITS (lanes 5−8), or without cross-linker (lanes 9−12), followed by sequential treatments with PNGase F, and with chymotrypsin for indicated times. B. Immunoblot of gastric membranes treated for 10 min at 20 °C with 2 mM DTSSP, BS[3], DSS, DSP, or without cross-linker as indicated, followed by sequential treatments with PNGase F and with trypsin.

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Pig    AE2  LRSVAHFQRQMLKKREEQGRLLPAGAGLEPKSAQDKALLQMVEAAGAVEDDPLRRTGRPFGGLIRDVRRRYPHYLSDFRDALDPQCLAAVIFIYFAALSP  100
Mouse  AE2  .....................P.................V...A.....................................................  718
Rat    AE2  .....................P.................V...A.....................................................  719
Rabbit AE2  .....................P.L...............A.....................................V.............  718
Human  AE2  .....................T...............A.....................................................  722

Pig    AE2  AITFGGLLGEKTHDLIGVSELIMSTALQGVIFCLLGAQPLLVIGFSGPLLVFEEAFFSFCTSNNLEYLVGRVWIGFWLVLLALLMVALEGSFLVRFVSRF  200
Mouse  AE2  ...........K.............V.....................S..E.............F.....................  818
Rat    AE2  ...........Q.....................................K..Q.....................................  819
Rabbit AE2  ...........Q.....................................T..S..Q.....................................  818
Human  AE2  ...........Q.............V.....................S..H.............F.....................  822

                                                  T55/40 ▼  CH55/39          CH35
Pig    AE2  TQEIFAFLISLIFIYETFYKLVKIFQEHPLHGCSVSNSSEADSGENATWARAAATTQPGNGSSAGPAGPSGQGRPRGQPNTALLSLVLMAGTFFIAFFLR  300
Mouse  AE2  ..................I...........G..D...G.SSSSNMTW.TTILV.D.S.A---S.Q...EK.....................  915
Rat    AE2  ..................I.............D.....SS.-NMTW..T.LA.D.S.A---S.---.E.....................  912
Rabbit AE2  ..................I...................T..S......G.GS.LG.A.R..----..QA.....................  915
Human  AE2  ..........................A.....V.G...M...G.RP.LG...R.L----..Q....K.....................  919

                                                 T29 ▼          T28 ▼
Pig    AE2  KFKNSRFFPGRVRRVIGDFGVPIAILIMVLVDYSIEDTYTQKLSVPSGFSVTAPEKRGWVINPLGENSPFPAWMMVASLLPAVLVFILIFMETQITTLII  400
Mouse  AE2  ...........I.....................................................D...........KT...V.....................  1015
Rat    AE2  ...........I.....................................................D...........KT...V.....................  1012
Rabbit AE2  ...........I.....................................................D...........K....V.....I...............  1015
Human  AE2  ...........I.....................................................K....V.....I...............  1019

           T24                                                    T20 ▼      T20 ▼
Pig    AE2  SKKERMLQKGSGFHLDLLLIVAMGGICALFGLPWLAAATVRSVTHANALTVMSKAVAPGDKPKIQEVKEQRVTGLLVALLVGLSLVIGDLLRQIPLAVLF  500
Mouse  AE2  ..........................................................................M.....................  1115
Rat    AE2  ..........................................................................M.....................  1112
Rabbit AE2  ..........................................................................I.....................  1115
Human  AE2  ..........................................................................I.....................  1119

Pig    AE2  GIFLYMGVTSLNGIQFYERLHLLLMPPKHHPDVTYVKKVRTLRMHLFTALQLLCLALLWAVMSTAASLAFPFILILTVPLRMVVLTRIFTEREMKCLDAN  600
Mouse  AE2  ..........................................M.....................................................  1215
Rat    AE2  ..........................................M.....................................................  1212
Rabbit AE2  ..........................................................................................  1215
Human  AE2  ..........................................................................D.....................  1219

Pig    AE2  EAEPVFDEREGVDEYNEMPMPV  622
Mouse  AE2  ........C.............  1237
Rat    AE2  ........C.............  1234
Rabbit AE2  ......................  1237
Human  AE2  ......................  1241
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FIGURE 4: Primary structure of the transmembrane domain of pig gastric AE2. Alignment of the AE2 transmembrane domain deduced polypeptide sequences from pig (numbered from its start, deposited under Genbank Accession no. AF120099), mouse (J04036), rat (J05166), rabbit (S45791), and human (U62531). Dots indicate identities with the pig sequence. Dashes indicate gaps. Hydrophobic regions (putative transmembrane spans) of the pig sequence are underlined; the most C-terminal of these is long enough to span the membrane lipid bilayer twice. Protease cleavage sites defined or referred to in this work are indicated above the pig sequence. Arrows mark sequenced cleavage sites (T55/T40 and T20 sites) and deduced cleavage sites (T29, T28). Shaded boxes mark hypothesized regions within which the cleavages occur to generate the indicated C-terminal fragments.

imide-treated membranes to trypsin promoted appearance of putative T40 dimer and putative T40 tetramer (Figure 3B). Both the hydrophilic reagents, DTSSP (cleavable by reducing agents) and BS[3] (noncleavable), and the lipophilic reagents,

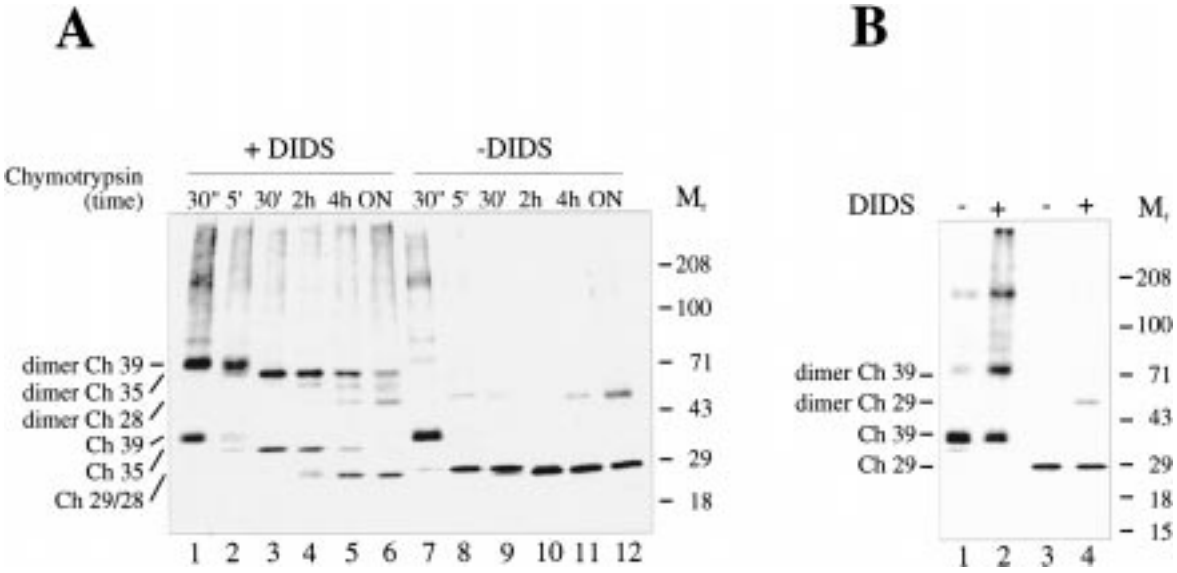

FIGURE 5: DIDS cross-links AE2 both prior to (A) and after chymotryptic cleavage (B). (A) Immunoblot showing time course of chymotryptic cleavage of native (lanes 7−12) and DIDS-cross-linked AE2 (lanes 1−6) in gastric membranes (1:1 w/w chymotrypsin:membrane protein, 50 mM Tris-HCl pH 7.6, 20 °C). (B) AE2 immunoblot of gastric membranes treated with chymotrypsin to produce either CH39 (lanes 1 and 2) or CH29 (lanes 3 and 4), then washed free of protease and further incubated in the absence (lanes 1 and 3) or presence of DIDS (lanes 2 and 4).

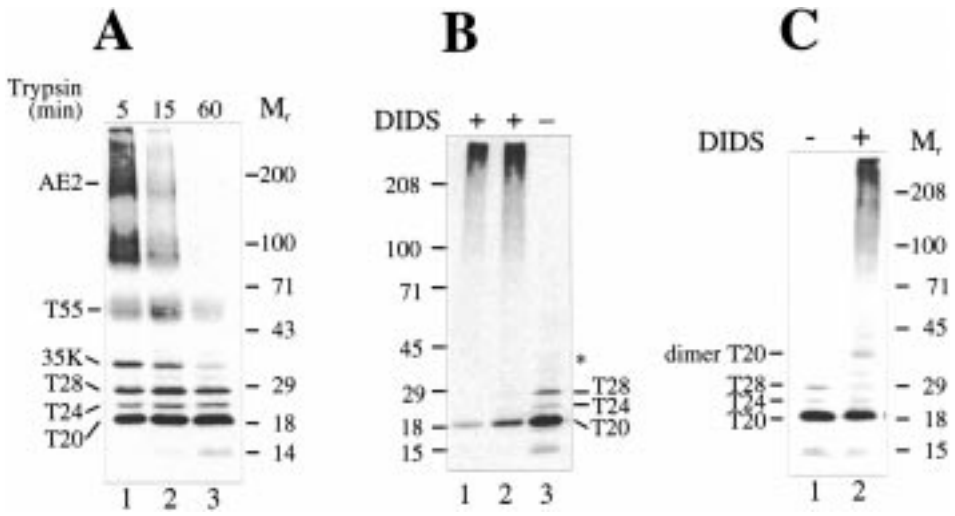

FIGURE 6: Tryptic susceptibility and DIDS cross-linking of AE2 are altered in membranes prepared in hypotonic buffers. (A) Immunoblot showing time dependence of tryptic digestion of native AE2 in membranes prepared in hypotonic buffer. In addition to T55 (see Figure 1B), tryptic digestion generated T28, T24 (putative 8−9 endo-loop), and T20 fragments. (35K is a product of an endogenous membrane protease). (B) Immunoblot of membranes prepared in hypotonic buffer, then incubated 10 min at 20 °C in the presence (lanes 1 and 2) or absence of DIDS (lane 3) at pH 8.3, and subsequently trypsinized (1:100 w/w) at 37 °C for 30 min. AE2 C-terminal tryptic fragments in these membranes were not cross-linked to dimers, but rather to heterodisperse, higher-order oligomeric structures whether in the presence of 150 mM sodium bicarbonate (lane 1) or in PBS (lane 2). Asterisk marks expected position of T20 dimer. (C) Immunoblot of membranes prepared in hypotonic buffer, then trypsinized and subsequently incubated in the absence (lane 1) or presence of DIDS (lane 2). Although the T20 C-terminal tryptic fragment of AE2 was cross-linked to high M_r forms, T20 dimer accumulated to detectable levels.

DSS (noncleavable) and DSP (cleavable), appeared effectively to cross-link AE2. The ability of the lipophilic cross-linker DSS to promote the preferential accumulation of putative T40 tetramer and higher M_r forms (lane 3) may reflect its penetration inside membrane vesicles, cross-linking additional AE2 lysines. Unfortunately, neither intact AE2 nor AE2 C-terminal proteolytic fragments entered SDS polyacrylamide gels in the absence of reducing agent (not shown), thus preventing full exploitation of the advantages of the reversible (cleavable) cross-linkers, DTSSP and DSP. [Analogous observations led to development of the nonreducible cross-linker DSS (*33*)]. Nevertheless, in the presence of reducing agent (Figure 3B), the abundance of T40 monomer exceeded those of putative T40 dimer and tetramer in DSP cross-linked membranes, whereas in DSS cross-linked membranes, putative T40 tetramer and higher-order oligomers predominated. Comparison of the cleavable DTSSP and its noncleavable analogue BS^3 suggests a similar, though much less impressive, pattern. Incomplete reduction of DTSSP-cross-linked and DSP-cross-linked oligomers has also been noted previously for human AE1 by Staros and Kakkad (*34*). These results further support the hypothesis that the putative dimer and tetramer bands represent or (at least) include covalently cross-linked AE2 species.

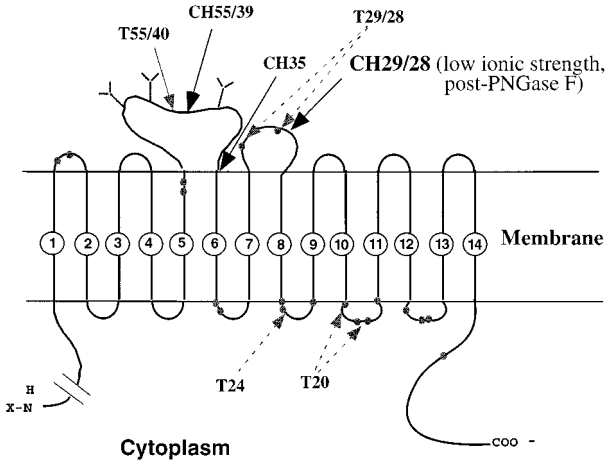

FIGURE 7: Schematic of AE2 transmembrane domain. The pig AE2 transmembrane domain is shown (14 TM model). Locations of tryptic cleavage sites (gray arrows) and chymotryptic cleavage sites (black arrows) and of Lys residues (gray circles) are noted. The two T20 cleavage sites (*28*) correspond to residues 454 and 463 (numbered according to Figure 4). Dashed gray arrows are tryptic cleavage sites accessible only in membranes prepared by the hypotonic freeze−thaw protocol. Blocked N-terminus of AE2 (*28*) is indicated by X. Since uniform sidedness and vesicular integrity of membranes in these experiments was not demonstrated, the present work does not allow topographical predictions for the transmembrane domain of AE2 to be compared to those proposed for hAE1 (*51*−*53*).

cDNA Sequence of the Transmembrane Domain of Pig AE2. Proof of the identity of the components of putative T40 dimer required determination of its N-terminal amino acid sequence(s). To facilitate analysis of the amino acid sequence, cDNA encoding the complete transmembrane domain of pig AE2 was amplified, sequenced, and deposited with Genbank under accession no. AF120099.

Figure 4 aligns the deduced amino acid sequence of pig AE2 transmembrane domain with corresponding sequences from other species, showing a very high degree of identity. As noted previously (*28*), the largest exofacial loop connecting TMs 5 and 6 in pig AE2 (Z-loop) contains a three residue insertion not present in AE2 of other species. The nucleotide sequence encoding the Z-loop and surrounding regions exhibited unusually high GC content, even in comparison to other AE2 cDNAs, including a stretch of 64/77 (84%) total (G + C). The presence in pig AE2 of only two Lys residues in the exo-loop between TM7 and TM8, contrasts with the three Lys residues in other species, and allows precise deduction of the tryptic cleavage sites giving rise to the fragments T28 and T29 (see below).

N-Terminal Amino Acid Sequence of T40 and T40 Dimer Fragments of AE2. Table 1 compares the N-terminal amino acid sequences (obtained by Edman degradation of protein electroblotted onto PVDF) of the T40 fragment and of the T80 fragment (putative dimer T40) purified from DIDS cross-linked gastric membranes treated with trypsin and subsequently N-deglycosylated. Since the N-terminal sequence of T80 was uniform and identical to that of T40 (*28*), the T80 fragment indeed comprised T40 homodimer (dimer T40). These data suggess the existence of stable AE2 homodimers in native pig gastric membranes. Pairwise comparison of amino acid yields through five cycles indicated that 12−27% of total AE2 polypeptide was cross-linked to dimer (Table 1).

Attempts to Define the Most C-Terminal AE2 Lys Residue-(s) Cross-Linked by DIDS. To locate more precisely the site-(s) of cross-linking within the AE2 T40 fragment, we compared in native and DIDS-treated membranes the M_r of AE2 C-terminal fragments generated by cleavage at sites progressively closer to the AE2 C-terminus under conditions that preserve the C-terminal epitope. Chymotrypsin treatment of N-deglycosylated membranes at low ionic strength cleaves AE2 rapidly in the exo-5−6 loop ("Z loop") to produce CH39 (Figure 5A, lane 7), and more slowly in the exofacial 7−8 loop to produce CH 29/28 [Figure 5A, lane 8; Figure 3A (*28*)].

Interestingly, DIDS pretreatment slowed considerably the kinetics of chymotryptic cleavage both of CH39 and of CH39 dimer to CH28/29 and its dimer (Figure 5A). Moreover, cleavage of DIDS-treated AE2 gave rise to the novel CH35 fragment (lanes 2−5), arising from AE2 cleavage at the C-terminal part of the Z-loop (exofacial 5−6 loop), at or adjacent to the papain cleavage site yielding the P35 fragment (*28*). These changes show that DIDS binding rendered the 7−8 exofacial loop of AE2 resistant to chymotrypsin even at high concentration and reduced ionic strength. AE2 cross-linking was not required to manifest this resistance. Nonetheless, prolonged chymotrypsin treatment of DIDS-cross-linked membranes led to conversion of putative AE2 CH39 dimer to fragments most easily explained as homodimers of CH35 and CH28, as well as fragments of intermediate M_r plausibly consisting of heterodimers of CH28 with CH39 or CH35 (lanes 4−6). These data are consistent with DIDS-mediated cross-linking of AE2 via Lys residues beyond the CH35 cleavage site.

However, the slow accumulation of SDS-resistant noncovalent CH28 dimers in control membranes (lanes 11 and 12) shows that the CH28 homodimers apparent in DIDS cross-linked membranes (lanes 5 and 6) may not be covalently linked dimers. This finding leaves uncertain the ability of Lys residues between the CH35 and the CH28 cleavage sites of AE2 to undergo cross-linking by DIDS. Therefore DIDS cross-linking was carried out following prior to chymotrypsin treatment of membranes (Figure 5B). In this setting, DIDS treatment convincingly enhanced (presumably covalent) dimerization of CH29 (lanes 3 and 4), though cross-linking was less extensive than observed for CH39 (lanes 1 and 2).

We then examined one-dimensional in-gel peptide maps of T40 and T40 dimer isolated by SDS−PAGE. We hypothesized that AE2 should show enhanced protease susceptibility in SDS and that any proteolytic fragments cross-linked via Lys residues C-terminal to the T40 cleavage site might be revealed by their increased M_r. Trypsin susceptibility of AE2 was indeed enhanced by digestion during SDS−PAGE, producing the additional C-terminal fragments T35, T29/28, T24, T20, and T14 (not shown, but similar to those shown in Figure 6A).

This finding encouraged a trial of in-gel proteolysis of gel fragments containing the AE2 tryptic fragments T40 and T40 dimer prepared from DIDS-treated membranes. However, none of the above-mentioned smaller C-terminal tryptic fragments were generated under these conditions (not shown). Because DIDS might have modified the AE2 Lys residues otherwise subject to tryptic cleavage, the in-gel chymotryptic susceptibility of T40 dimer prepared from

Table 1: Yield of Amino Acid Residues (pmol) in Each Cycle of Edman Degradation[a]

	1[b]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sequence	Ala	Ala	Ala	Thr	Thr	Gln	Pro	Gly	Asp	Gly	Ser	Ser	Ala	Gly	Pro
T80	1.3	5.0	5.2	1.6	3.9	5.3	3.7	4.8	2.7	1.6	0.7	0.7	1.1	2.0	1.5
T40	9.7	13.3	16.2	6.6	13.0										

[a] From 12 to 27% of all AE2 molecules were cross-linked in this experiment judging from pairwise comparison of the amino acid yields of the first five cycles [calculated as picolmoles of T40 per picomoles of (T40 + T80)]. Results confirm the hypothesis that T80 is dimer T40. [b] Cycle number.

DIDS-treated membranes was also examined. T40 dimer was indeed resistant to in-gel chymotryptic digestion (not shown), consistent with the chymotrypsin resistance of intact AE2 in DIDS-treated membranes (Figure 5A).

Membrane Preparation Conditions Modulate Subsequent AE2 Cross-Linking by DIDS. Tryptic cleavage of membranes previously exposed to hypotonic conditions (see Experimental Procedures) provided another approach to defining sites of DIDS cross-linking beyond the T40 cleavage site. This protocol yielded membranes in which AE2 susceptibility to trypsin was increased, as shown by the numerous C-terminal tryptic fragments evident in Figure 6A. The major stable fragment, T20, resulted from AE2 cleavage in the putative 10−11 endo-loop, as previously shown by amino acid sequencing of a similar fragment produced by trypsin treatment of purified AE2 in nonionic detergent (*28*). The T28 fragment resulted from AE2 cleavage in exo-loop 7−8. The still undefined T24 cleavage site likely resides in endo-loop 8−9 (see Figure 7).[4] Thus, hypotonic treatment of gastric membranes exposed three additional tryptic sites in three different hydrophilic loops of AE2. Cleavage at these sites generated three stable AE2 C-terminal fragments.

If DIDS treatment of routinely prepared membranes preceded the hypotonic wash protocol, then subsequent trypsinolysis and deglycosylation yielded only T40 and dimer T40, without AE2 fragments of lower M_r (not shown). However, if membranes prepared by the hypotonic wash protocol were first treated with DIDS and then trypsinized (Figure 6B), no AE2 fragment dimers were observed; instead, most immunoreactivity attributable to cross-linked products was retained at the top of the gel. In contrast, dimer T20 appeared reproducibly in membranes prepared by the hypotonic protocol that were first trypsinized and then treated with DIDS (Figure 6C), although most immunoreactive material still remained at the top of the gel. T20 dimer was generated at the same low yield whether DIDS treatment was carried out in phosphate pH 7.4 or 8.3, in ammonium bicarbonate, pH 7.8, or in sodium bicarbonate, pH 8.3 (not shown).

DISCUSSION

Oligomeric States of AE1 and AE2. The erythroid AE1 (band 3) polypeptide is thought to exist in the red cell membrane as a mixture of laterally mobile dimers and of more highly restricted tetramers preferentially bound to the cytoskeleton (*18*). In two-dimensional crystals, eAE1 appears to be arranged as a trimer of dimers (*25*). The first experimental evidence for dimeric organization in AE1 was obtained from disulfide cross-linking (*35*) and during a more general "protein nearest neighbor analysis" of the red cell membrane (*36*). Subsequently, various forms of homooligomeric structure has been detected by many techniques, including analytical ultracentrifugation (*11, 20*), gel filtration (*10, 37*), radiation inactivation analysis (*38*), and electron microscopy (*13*). Though the AE1 mutation of Southeast Asian Ovalocytosis has been observed to display altered cytoskeletal attachment and, perhaps, oligomerization (*39*), much remains to be learned about the physiological significance of AE1 homooligomerization.

Still less is known about the quaternary structure of other polypeptide members of the AE anion exchanger gene family. In the course of experiments on limited AE2 digestion in native gastric membranes, we observed SDS-resistant dimerization of proteolytic fragments derived from the C-terminal half of the AE2 membrane domain, suggesting that native AE2 may exist as dimers or higher-order aggregates in membranes (*28*). However, SDS−PAGE electrophoresis of gastric membranes after exposure to the chemical cross-linker DIDS showed a heterodisperse smear in the high molecular weight region (Figure 1), implying either that AE2 was cross-linked to other adjacent proteins, or that the degree of homomeric cross-linking was itself heterogeneous, or both. We postulated that study of defined AE2 proteolytic fragments might render AE2 oligomers more readily detectable, and this proved to be true (Figure 1B).

The observed AE2 bands of doubled M_r that appeared or increased in abundance after exposure to chemical cross-linkers were AE2 homodimers by two criteria: the ability to create T40/T55 heterodimers in incompletely deglycosylated membranes and the identity of the homogeneous N-terminal amino acid sequences of T40 and T80. The homodimerization was shown to result from covalent cross-linking by three results. (1) The pH dependence for DIDS cross-linking was consistent with that of the reaction between isothiocyanate and amines. (2) The monovalent DIDS analogue, SITS, did not replicate DIDS-mediated cross-linking. (3) Treatment of cross-linked T80 with TFA before amino acid sequencing did not dissociate T80 into monomers, in contrast to the ability of TFA to reverse noncovalent SDS-resistant aggregation (*30*).

The study of AE2 cross-linking in native membranes, rather than with purified protein in detergent, presented both advantages and disadvantages. AE2 in the membrane represents the "native state" or a near approximation, whereas nondenaturing detergents alter oligomeric state in a time and temperature-dependent fashion, and often lead to SDS-resistant aggregation evident as smearing on SDS−PAGE

[4] The 35 K-band preexisted in this batch of membranes (see also Figure 3A, lane 1) and arose from AE2 cleavage by endogenous membrane protease activity during membrane isolation (*3*). Unstable fragments included T55 (cleavage in exo-loop 5−6) and the novel fragments T90/100 (likely representing the entire transmembrane domain).

(Zolotarev, unpublished results). However, covalent cross-linking of nearly all detectable AE2 to tetramers by the noncleavable, lipophilic DSS further supports a native quaternary structure of tetramers and/or dimers (Figure 3B). Of course, these conclusions apply with certainty only to that fraction of AE2 polypeptide in which the AE2 C-terminal epitope remains both resistant to proteolytic digestion and accessible to antibody detection on immunoblot.

Jennings and Passow showed in 1979 that human eAE1 protein could be *intramolecularly* cross-linked with 10 μM H_2DIDS (*40*), but no intermolecular cross-linking was observed. The cross-linked Lys residues 539 and 851 are situated in the transmembrane domain of the AE1 polypeptide chain (*41*). We have presented above several lines of evidence proving that pig AE2 polypeptides were *intermolecularly* cross-linked (dimerized) by DIDS via their transmembrane domains. Intermolecular cross-linking of AE2 required a much higher concentration of cross-linker than did intramolecular cross-linking of AE1, and involved different Lys residues. Consistent with this observation, pig AE2 residues corresponding to hAE1 Lys 539 and Lys 542 (an alternate binding site) are absent from the dimers of pig AE2 C-terminal proteolytic fragments studied here (Table 1) and the AE2 residue corresponding to hAE1 Lys 851 is Met in AE2 of all mammalian species sequenced to date (including pig).

Sites of DIDS-Mediated Cross-Linking of AE2. Figure 7 displays the locations of Lys residues in the 14-TM model of AE2 membrane domain. Fifteen Lys residues present between the T55/T40 tryptic cleavage site and the AE2 C-terminus are potential sites of the cross-linking detected in this study. We hypothesized that progressively more extensive proteolytic cleavage of cross-linked membrane might identify which Lys residues might be required for or dispensable for AE2 cross-linking. However, all three approaches used (chymotryptic cleavage, tryptic or chymotryptic cleavage in SDS, and tryptic cleavage of hypotonically prepared membranes subjected to multiple freeze−thaw cycles) turned out to be limited by the increased resistance to proteolysis conferred by DIDS on the C-terminal 40 kDa portion of the AE2 transmembrane domain.

Therefore, the ability to cross-link previously cleaved AE2 fragments was also examined. DIDS treatment of protease-treated membranes promoted dimerization of the AE2 fragments Ch39 and Ch29 (Figure 5B) and T20 (Figure 6C), though the degree of cross-linking decreased in parallel with fragment size. Interpretation of these experiments is complicated by the ability of newly generated α-amino groups to serve as additional cross-linking sites. Thus, the successful cross-linking of T20 indicates either the presence of cross-linked residues C-terminal to the T20 cleavage site, that the T20 α-amino group is cross-linked, or both. In either case, however, T20−T20 interactions participate in formation of native AE2 dimers, likely via interactions that do not require integrity of the exofacial and endofacial loops of AE2 (*42*).

The AE2 residue corresponding to hAE1 Lys 551, shown to bind BS^3 in the intermolecular cross-linking of hAE1 (*43*), is absent from all the above cross-linked C-terminal fragments of AE2.

Influence of Heterogeneity of Vesicle Sidedness on AE2 Cross-Linking. Treatment of native gastric membranes with 1 mM DIDS produced an unexpected increase in total immunoreactive proteolytic fragments of AE2. Within 5−10 min of DIDS treatment, cross-linked AE2 dimer was evident at peak levels (as dimer T40). However, appearance of dimer T40 was unaccompanied by the expected decrease in level of AE2 monomer (T40 in Figure 2A).[5]

This result could be explained by the presence of two populations of AE2 in these gastric membranes. One population, resistant to rapid cross-linking by DIDS, was detected by immunoblot without or with prior DIDS treatment of membranes. The yield of proteolytic fragments from this population was lower than expected from the level of uncleaved AE2 in control membranes (Figure 1B, compare lanes 1 and 2 with lane 7), consistent with proteolytic loss of the C-terminal epitope from a fraction of the polypeptides. DIDS treatment of membranes allows immunoblot detection of a second population of AE2 polypeptides (Figure 1B, compare lanes 3 and 4 with lane 7). This second population is susceptible to rapid cross-linking by DIDS, evident only as cross-linked dimers, and suggests that the C-terminal epitope of the dimer was more resistant to proteolytic attack. The combined immunoblot signal intensities of the two populations together approximated the total AE2 content of control membranes.

These two AE2 populations, defined by differing susceptibility to cross-linking and to C-terminal epitope proteolysis, may reflect heterogeneity of membrane vesicle sidedness (*44*). The AE2 T55/T40 and CH55/39 fragments derive from cleavage in the exo 5−6 loop (Z-loop) of AE2 in rightside-out vesicles [∼30% of total membranes (*44*)], in which the AE2 C-terminal epitope required for immunodetection is protected from proteolytic cleavage. In inside-out vesicles (∼70% of total membranes), the AE2 Z-loop is inaccessible to protease, whereas the C-terminal epitope is exposed to protease. Thus, most T55/40 and CH55/39 on immunoblots likely originate from rightside-out vesicles with AE2 epitope protected from cleavage.

We propose that AE2 in rightside-out vesicles is resistant to intermolecular cross-linking and that proteolytic yield of T55/40 or CH55/CH39 is minimally changed by prior DIDS treatment. In contrast, AE2 in inside-out vesicles is susceptible to cross-linking by DIDS. DIDS cross-linking renders this population of AE2 (and its C-terminal epitope) more resistant to proteolytic cleavage, potentially explaining the increase in total AE2 detected by immunoblot after DIDS cross-linking.

Protease-resistance of the C-terminal epitope could also arise by a different mechanism. The procedure by which DIDS cross-linking was quenched with Tris, involving pelleting and homogenization/resuspension in moderately hypotonic conditions (see Experimental Procedures), might have been accompanied by reorientation of sidedness in a subpopulation of vesicles. In particular, a change from inside-out to rightside-out orientation might be evident as operational stabilization of the AE2 C-terminal epitope. If cross-linked T40 dimer represents only 20% of total detectable T40 protomer (Table 1), and if the original membranes comprised ∼30% rightside-out vesicles (*44*), then this

[5] Levels of dimerization-resistant AE2 declined slowly in the presence of DIDS (over hours), in parallel with accumulation of AE2-containing aggregate of very high M_r (Figure 2A).

formulation would require only ~10% of inside-out vesicles to reverse sidedness to account for the increase in total immunoblot-detectable AE2.

DIDS Modification of Additional Lys Residues in AE2. In native membranes, 1 mM DIDS very likely modifies Lys residues in addition to those involved in intermolecular cross-linking. These additional Lys residues are modified both in the dimerized and the nondimerized AE2 populations, as evidenced by the increased resistance to proteolysis of the exo 7−8 loop shared by AE2 dimer and monomer. Two findings suggest that, among these additional Lys residues, at least one likely contributes to the "DIDS binding pocket" whose occupancy results in inhibition of anion transport. First, 1 mM DIDS can extensively and irreversibly inhibit AE2 activity in all expression systems tested (*45*−*48*). Second, intramolecular cross-linking of hAE1 by H_2DIDS (*49*) and by DIDS (*50*) (covalently fixed within this binding pocket) each decreased proteolytic susceptibility of the AE1 exo 7−8 loop. Our data with chymotrypsin treatment of AE2 resemble these results.

The proposed DIDS-modified AE2 Lys residues that do not contribute to intermolecular cross-linking may contribute to intramolecular cross-linking. Circumstantial support for this hypothesis comes from the monovalent isothiocyanates, SITS, and sulfophenylisothiocyanate, which themselves neither dimerized AE2 nor inhibited AE2 dimerization by DIDS, nor decreased proteolytic susceptibility of AE2 monomer or dimer (not shown). However, intramolecular cross-linking of AE2 fragments T40 or CH39 to a complementary AE2 fragment encoding TM spans 1−5 was never detected. Such cross-linking might have been expected, since H_2DIDS and DIDS can cross-link the hAE1 fragments CH17 (comprising TM 1−5) with CH35 [comprising TM6 to the C-terminus (*40*)]. This difference suggests that any intramolecular DIDS cross-links within AE2, if present, must differ from those of cross-linked hAE1, residing entirely within the T40 fragment.

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