Topology of the Anion Exchange Protein AE1: The Controversial Sidedness of Lysine 743[†]

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Received October 23, 2001; Revised Manuscript Received January 2, 2002

ABSTRACT: The topology of the band 3 (AE1) polypeptide of the erythrocyte membrane is not fully established despite extensive study. Residues near lysine 743 (K743) have been reported to be extracellular in some studies and cytoplasmic in others. In the work presented here, we have attempted to establish the sidedness of K743 using in situ proteolysis. Trypsin, papain, and proteinase K do not cleave band 3 at or near K743 in intact red cells, even under conditions that cause cleavage on the C-terminal side of the glycosylation site (N642) in extracellular loop 4. In contrast, trypsin sealed inside red cell ghosts cleaves at K743, as does trypsin treatment of inside-out vesicles (IOVs). The transport inhibitor 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate (H_2DIDS), acting from the extracellular side, blocks trypsin cleavage at K743 in unsealed membranes by inducing a protease-resistant conformation. H_2DIDS added to IOVs does not prevent cleavage at K743; therefore, trypsin cleavage at K743 in IOVs is not a consequence of cleavage of right-side-out or leaky vesicles. Finally, microsomes were prepared from HEK293 cells expressing the membrane domain of AE1 lacking the normal glycosylation site. This polypeptide does not traffic to the surface membrane; trypsin treatment of microsomes containing this polypeptide produces the 20 kDa fragment, providing further evidence that K743 is exposed at the cytoplasmic surface. Therefore, the actions of trypsin on intact cells, resealed ghosts, unsealed ghosts, inside-out vesicles, and microsomes from HEK293 cells all indicate that K743 is cytoplasmic and not extracellular.

The chloride−bicarbonate exchange protein of the erythrocyte membrane is known as band 3 or AE1[1] (anion exchanger 1) and consists of a 40 kDa N-terminal cytoplasmic domain and a 52 kDa C-terminal membrane domain (*1*−*3*). Band 3 has long been a model system for the study of the molecular mechanism of coupled transport (*4*−*8*). As is true of most integral membrane proteins, it has not yet been possible to obtain crystals of band 3 that are suitable for X-ray crystallography. Accordingly, other approaches have been used to build a structural model of the membrane domain, including cryoelectron microscopy (*9*, *10*) and nuclear magnetic resonance spectroscopy of selected individual sequences (*11*, *12*). To use this kind of information to model the structure of the band 3 membrane domain, it will be necessary to have a firm understanding of its topology, i.e., the sidedness of each part of the sequence.

Because band 3 is so abundant in red blood cells, it is possible to study its topology by biochemical methods in the native cell (*13*−*17*). In addition, site-directed mutagenesis, in conjunction with either cell-free translation (*18*) or heterologous expression (*19*−*21*), has been used to investigate AE1 topology, as has been done for many other membrane proteins. Topological information has also been derived from identifying polymorphisms associated with blood group antigens (*22*−*28*). These divergent approaches have produced very consistent results regarding the topology of several regions of the AE1 polypeptide; there is general agreement that the loops containing residues 430−432, 551−562, 628−658, and 851−854 are exposed on the extracellular surface (*13*, *18*, *20*, *22*, *23*, *26*, *29*, *30*) and that both the N-terminus and C-terminus are cytoplasmic (*13*, *16*, *17*).

However, there has been disagreement regarding the sidedness of other parts of the protein, particularly the residues near K743. A biochemical study from this laboratory originally provided evidence that K743 is exposed on the cytoplasmic side of the membrane (*31*); the evidence was based on the finding that trypsin, sealed inside resealed red cell ghosts, cleaves the protein between K743 and A744. The cleavage is slow at physiological ionic strength but is more rapid at low ionic strengths (20−30 mM salt). This finding agrees with the cell-free translation experiments of Tanner and co-workers (*32*), who found that trypsin cleavage of microsomes containing unmutated AE1 produced an ∼20 kDa fragment (24 kDa in their gel system) that the authors attributed to cleavage at K743.

Recently, Popov et al. (*18*) performed glycosylation scanning mutagenesis on AE1 with the N642D mutation to remove the naturally occurring N-glycosylation site. An

[†] This work was supported by NIH Grant R01 GM26861-22.

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[1] Abbreviations: AE1, anion exchanger 1; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; H_2DIDS, 4,4′-diisothiocyanatodihydrostilbene 2,2′-disulfonate; SITS, 4-acetamide-4′-isothiocyanatostilbene 2,2′-disulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LYIA, lucifer yellow iodoacetamide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMG, *N*-methyl-D-glucamine; PMSF, phenylmethanesulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; SDS, sodium dodecyl sulfate; SAO, Southeast Asian ovalocytes.

10.1021/bi015879p CCC: $22.00
Published on Web 02/12/2002

additional mutation (K743N) creates an N-glycosylation site without any other substitutions, because there is a serine residue at position 745. Cell-free translation of AE1 mRNA containing both the N642D and K743N mutations produces an N-glycosylated polypeptide (although not with 100% efficiency), indicating that residue 743 is exposed to the lumen of the endoplasmic reticulum during translation and/or insertion (*18*).

Popov et al. (*19*) subsequently expressed the N642D/K743N double mutant in HEK293 cells and found that the site is not glycosylated, indicating that any copies of the protein that are glycosylated on N743 during synthesis (*18*) are subsequently degraded, presumably because they are misfolded. Popov et al. (*19*) also found that insertion of the 34-residue EC 4 loop (with glycosylation site) at position 754 gives a mixed population of protein, a substantial fraction of which is glycosylated. The insertion mutant can bind to SITS-Affigel, indicating that the inhibitor binding site is preserved. This finding suggests that Q754 is extracellular.

Fujinaga et al. (*20*) recently used permeant and impermeant labeling reagents to assess the sidedness of cysteine residues inserted at various sites in AE1 expressed in HEK293 cells. Cysteine residues substituted at positions 731, 742, 745, and 751 could be modified by the membrane impermeant reagent lucifer yellow iodoacetamide (LYIA) in intact cells, indicating accessibility from the extracellular medium (*20*). The mutated proteins (except S731C) carry out anion exchange. The cysteine scanning experiments suggest that a loop including K743 is extracellular.

In light of the disagreement concerning the topology of K743, we have performed further in situ proteolysis experiments to try to clarify the sidedness of this residue. Trypsin digestions of five different preparations (intact human red blood cells, unsealed ghosts, resealed ghosts, inside-out vesicles, and microsomes from HEK293 cells expressing the AE1 membrane domain) give results that are entirely consistent with a cytoplasmic location of K743 and do not provide any evidence for extracellular exposure of this residue in the mature protein. Possible explanations for the divergent experimental results regarding the sidedness of this residue are discussed.

EXPERIMENTAL PROCEDURES

Materials. Human blood (heparin anticoagulant) was obtained by venipuncture from healthy adults and stored at 4 °C before use. Papain and proteinase K were purchased from Boehringer/Mannheim (Indianapolis, IN). Bovine pancreatic trypsin was purchased from either Boehringer or Sigma (St. Louis, MO). The trypsin used in digestion of microsomes was sequencing grade from Boehringer. The trypsin used in the other experiments was either conventional analytical grade trypsin (lyophilized) from Boehringer or TPCK-treated trypsin from Sigma. We have observed no effect of the source of trypsin on our results.

H_2DIDS was prepared as described previously (*29*). The Ecdysone-inducible mammalian expression system, calcium phosphate transfection kit, and Zeocin were purchased from Invitrogen (Carlsbad, CA). G418 and muristerone A were purchased from Alexis (San Diego, CA). Site-directed mutagenesis and ligation reactions were performed with kits from Stratagene (La Jolla, CA). ECL Western blotting detection reagents were from Amersham Pharmacia Biotech (Arlington Heights, IL). Gramicidin A was purchased from Calbiochem (San Diego, CA). [^{35}S]Na_2SO_4 and [^{3}H]$NaBH_4$ were purchased from DuPont NEN (Boston, MA). All other chemicals were reagent grade or better and were purchased from Fisher Scientific (Pittsburgh, PA), Life Technologies (Gaithersburg, MD), Sigma Chemicals, or Atlanta Biologicals (Atlanta, GA).

Proteolytic Digestion of Intact Red Blood Cells at Low Ionic Strengths. Human red cells were washed three times in 150 mM KCl and 10 mM HEPES (pH 7.4), and 800 μL of cells was suspended in 8 mL of 150 mM *N*-methyl-D-glucamine-Cl and 10 mM HEPES (pH 7.4) with 10 μL of 1 mg/mL gramicidin A (in ethanol). In the presence of gramicidin, the outward K^+ gradient causes a net loss of KCl, with a half-time of less than 10 min (not shown). After 15 min at 37 °C, the suspension was diluted with 8 mL of distilled water, incubated for an additional 15 min, then diluted with 16 mL of distilled water, and incubated for 30 min at 37 °C. These incubations should be sufficient to deplete the cells of at least 90% of their original K^+ and Cl^-. The cells were then centrifuged and resuspended in 20 mM MOPS acid, 14 mM *N*-methylglucamine (NMG, pH 7.3, 33 mOsmol/kg). Because the cells had been depleted of K^+ and Cl^-, there was very little lysis in this hypoosmotic medium.

For proteolytic digestion, cells were resuspended in the same medium with 1 mg/mL trypsin, proteinase K, or papain (activated with 2 mM cysteine). After incubation for 45 min at 37 °C, cells were washed three times with 25 volumes of the same medium with 0.2% bovine serum albumin. Low-speed washes (800*g* for 2 min) were used to minimize contamination by unsealed membranes from cells that lysed during the digestions. For electrophoresis, 10 μL of a 1−2% suspension of cells was solubilized by heating at 100 °C in sample buffer and run on a 6 to 15% polyacrylamide gel (*33*). Immunoblots were performed by the method of Towbin et al. (*34*), with monoclonal antibody IVF12 (*31*) and alkaline phosphatase-conjugated secondary antibody.

Resealing of Trypsin in Ghosts. Trypsin was sealed inside red cell ghosts by methods similar to those used previously (*31*, *35*). Cells were washed in 165 mM KCl and lysed at 0 °C in 40 volumes of 4 mM $MgSO_4$ and 1.2 mM acetic acid containing 25 μg/mL trypsin (Sigma, TPCK-treated). Two minutes after lysis, ammonium acetate was added to a final concentration of 150 mM. Ghosts, still at 0 °C, were washed twice (10 min at 28000*g*) in 150 mM ammonium acetate and 10 mM sodium phosphate (pH 7.5) to remove extracellular trypsin. To measure how much trypsin is trapped, the same procedure was performed on trypsin labeled with ^{3}H by reductive methylation (*36*). After two washes at 0 °C, 2.5% of the trypsin originally in the lysis solution was in the ghost pellet; this is very close to the amount expected if trypsin occupies all the intracellular water in the ghosts.

Ghosts (~0.25 mL) with incorporated trypsin were resuspended in 7.5 mL of 200 mM sucrose, 30 mM ammonium acetate, and 10 mM sodium phosphate (pH 7.5) with 25 μg of soybean trypsin inhibitor. In one aliquot, trypsin inhibitor was omitted, and 25 μg of trypsin/mL was added to the suspension to allow extracellular digestion. Suspensions were incubated for 30−90 min at 37 °C, diluted with 30 mL of 150 mM ammonium acetate and 10 mM sodium phosphate

(pH 7.5), and then centrifuged for 10 min at 28000*g*. The pellets exhibited clear evidence of heterogeneity, with a darker pink lower pellet and a white, less tightly packed upper pellet, representing ghosts that became unsealed during the incubation (*31*). Much of the upper pellet was removed by adding 1 mL of wash medium, swirling gently, and aspirating; the remaining pellet was resuspended in 1 mL of phosphate-buffered 150 mM ammonium acetate. Phenylmethanesulfonyl fluoride (10^{-4} M) was added to inhibit trapped trypsin activity, and the membranes were solubilized in electrophoresis sample buffer.

Digestion of Unsealed Membranes or Inside-Out Vesicles with Trypsin. Red cells were washed in 165 mM NaCl, chilled on ice, and lysed at 0 °C in 20 volumes of 5 mM $NaHCO_3$ (pH 8). The membranes were recovered by centrifugation for 30 min at 40000*g* and washes under the same conditions. Inside-out vesicles were prepared by the method of Steck and Kant (*37*). Unsealed membranes or inside-out vesicles were incubated with trypsin (10 μg/mL) for 60 min at 37 °C in 20 mM NaCl or 150 mM NaCl and 5 mM $NaHCO_3$ (pH 8). Some of the samples were treated with H_2DIDS (10 μM, 90 min, 37 °C, in 100 mM $KHCO_3$, pH 9.5) either before or after treatment with trypsin. Following the digestion, trypsin was inhibited by incubation with TLCK (N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 100 μg/mL) at room temperature for 20 min. Protein was solubilized in sample buffer and run on SDS−polyacrylamide gels as described above; immunoblots were developed using ECL.

Expression of the AE1 Membrane Domain in HEK293 Cells. Plasmid pIND-AE1MD, containing an insert encoding the membrane domain of AE1 (residues 382−911), was prepared as follows, starting with pBluescript containing full-length human AE1 cDNA (a kind gift from A. M. Garcia and H. Lodish). Restriction sites for *Eco*RI (one at the position of L378 and F379 and the other in the 3′-UTR approximately 30 bases from the stop codon) were added by site-directed mutagenesis (Stratagene QuickChange). An ATG codon was introduced in place of L382. The plasmid was digested with *Eco*RI and the insert cloned into pIND; clones with the correct orientation were selected by sequencing, and two further mutations were introduced to improve the consensus for translation initiation (*38*) (GGCGC-CATGG). To remove the glycosylation site, N642 was mutated to glutamine.

Isolation of Stable Cell Lines Expressing the Membrane Domain of AE1. EcR-293 cells were grown on 150 mm plates in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 400 μg/mL Zeocin. The cells were transfected with pIND-AE1MD; 48 h after transfection, 800 μg/mL G418 was added to select for stable transfectants. Clones were isolated at the end of 2 weeks, and expression of the AE1 membrane domain in response to muristerone A (5 μM, 48 h) was assessed by Western blot analysis.

For immunofluorescence microscopy, cells were grown in poly(D-lysine)-coated four-well culture slides to ~60% confluence. They were then induced with 5 μM muristerone A added to the medium and grown for an additional 48 h. The wells were washed twice with PBS. After the wash, a freshly prepared 1:1 methanol/acetone mixture was added to the wells for 2 min and aspirated, and the cells were washed three times with PBS. Primary antibody IVF12 (*31*) at a dilution of 1:1000 was added to the wells and incubated at 37 °C for 30 min, followed by three washes in PBS. FITC-labeled goat anti-mouse IgG was the added at a dilution of 1:25. After being washed with PBS, the slide was imaged using a Zeiss Axiovert 135 microscope and a Hamamatsu C5985 chilled CCD camera.

Microsomes were prepared as described by Sumbilla et al. (*39*). Microsomes [500 μg of protein in 100 μL of 50 mM MOPS (pH 7.0) and 10% sucrose] were incubated with trypsin at room temperature for 30 min. Trypsin was inhibited with TLCK (10 μL of a 1 mg/mL solution). Microsomes were solubilized in sample buffer (2 min at 100 °C). The protein was separated on a 6 to 15% polyacrylamide gel, and Western blots were performed as described above and visualized with ECL.

RESULTS

Lack of External Cleavage at K743 by Proteolysis of Intact Cells. To examine the sidedness of K743 as exhaustively as possible, proteolytic digestions were performed with a variety of membrane preparations, starting with intact human erythrocytes. Trypsin cleavage at K743 in ghosts (*31*) and also in extracellular loop 3 (K551 and/or K562) in intact cells (*40*) is accelerated by lowering the ionic strength. To determine whether trypsin or other proteases can cleave band 3 at or near K743 in intact red cells, the ionic strength was lowered on both sides of the membrane by incubation in *N*-methyl-D-glucamine-Cl and gramicidin, which causes net efflux of K^+ through gramicidin and Cl^- through band 3 (*41*). With progressive dilution of the extracellular medium during the net KCl efflux (see Experimental Procedures), it is possible to prepare red cells that do not lyse (<1% lysis) in a medium with an osmolality as low as 33 mOsmol/kg of H_2O.

Figure 2 shows that trypsin, papain, and proteinase K (1 mg/mL) at low ionic strengths can cleave band 3 in intact cells to produce a variety of fragments that react with monoclonal antibody IVF12, the epitope of which is near the C-terminus (*31*). Most of these fragments have been observed previously. For example, the glycosylated fragments with the leading edge migrating at 35 and 30 kDa result from cleavage in EC loops 3 and 4, respectively (*29*, *40*, *42*, *43*). In addition, a new fragment was observed at ~26 kDa. The position and sharpness of this band indicate that the cleavage site is on the C-terminal side of the glycosylation site at N642 (EC loop 4); Edman degradation of this fragment (not shown) suggests that the cleavage site is between N642 and S643, but the sequence is not conclusive because of the low repetitive yield. The band runs as a doublet, indicating that there may be two closely spaced cleavage sites. Significantly, the ~26 kDa fragment is the smallest C-terminal fragment produced by proteolysis of intact cells, indicating that there are no proteolysis sites accessible under these conditions between extracellular loop 4 (residues ~625−660) and the C-terminus of band 3 (V911).

In contrast, much lower concentrations of trypsin, acting on unsealed ghosts under the same conditions, produce the C-terminal 20 kDa fragment (Figure 2, lane 5), which has been shown in two separate studies to have its N-terminal sequence beginning at A744 (*31*, *44*). These experiments indicate that K743 is not accessible to proteolysis at the

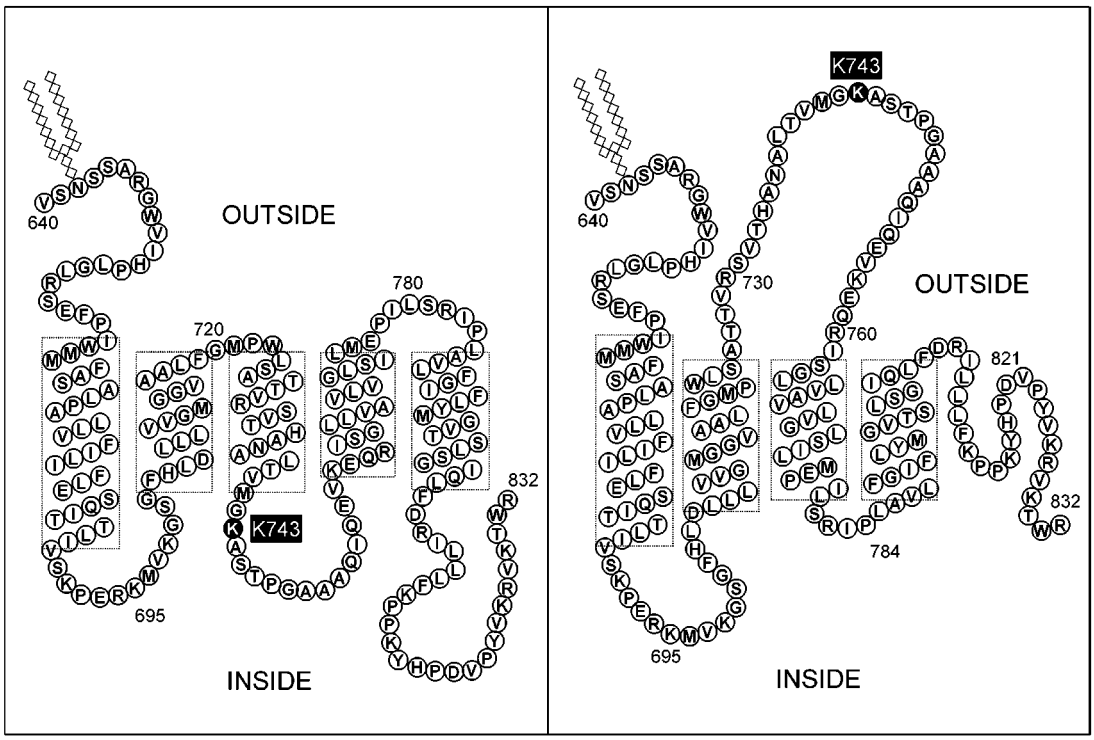

FIGURE 1: Alternative topological models of human AE1 in the region between V640 and R832. At the left is a model depicting lysine 743 exposed to the cytoplasm; the transmembrane segments that are shown are TM8−12 in the 14-TM model. At the right is a model, derived from Figure 1 of Fujinaga et al. (*20*), depicting lysine 743 exposed to the extracellular medium. The models are quite divergent starting after the intracellular cluster of positive charges near K695. The models again converge downstream of R832, which is immediately before the last two transmembrane helices.

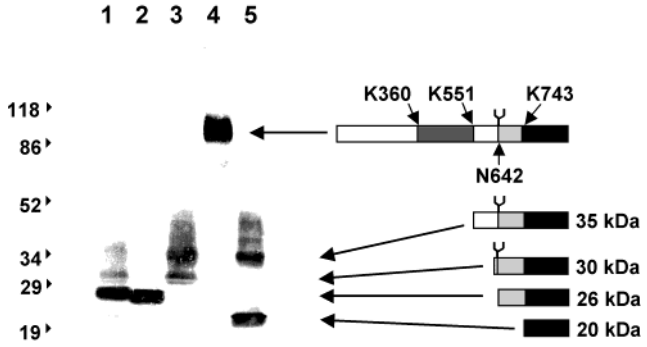

FIGURE 2: Products of in situ proteolysis of band 3 in intact cells (lanes 1−4) and unsealed membranes (lane 5), as detected by monoclonal antibody IVF12 (alkaline phosphatase secondary antibody), which binds to an epitope near the C-terminus (*31*). Intact cells were depleted of K^+ and Cl^- by incubation in NMG-Cl and gramicidin, as described in Experimental Procedures. Cells were then incubated in a medium consisting of 20 mM NMG and 14 mM MOPS (pH 7.2) with 1 mg/mL papain, proteinase K, trypsin, or no enzyme (lanes 1−4, respectively) for 45 min at 37 °C. Lane 5 represents unsealed membranes digested with 50 μg/mL trypsin for 45 min at 37 °C in the same buffer that was used with intact cells. The fragments represented by the various bands are indicated by the rectangles at the right.

extracellular surface of red cells, even under digestion conditions that are sufficiently vigorous to cause cleavage in EC loop 4 on the C-terminal side of the glycosylation site.

In Resealed Ghosts, Internal Trypsin Produces the 20 kDa but Not the 35 kDa Fragment. Despite the lack of cleavage of K743 in intact cells, the residue could still be on the extracellular surface but not accessible to proteolytic digestion. For example, it is possible that removal of the cytoplasmic domain (or associated membrane skeleton) alters the structure of the membrane domain sufficiently to allow external cleavage of K743. If this were true, trypsin would be unable to cleave this site in intact cells even if the site were extracellular. To determine the digestion pattern under conditions in which the cytoplasmic domain and membrane skeleton have been cleaved, trypsin was resealed in red cell ghosts (*31*, *35*). In agreement with earlier work (*31*), trypsin sealed inside red cell ghosts and subsequently incubated at a low ionic strength produces the 20 kDa C-terminal fragment (Figure 3). The yield of 20 kDa fragment is relatively low, as a consequence of the fact that many of the sealed membranes become leaky at low ionic strengths, allowing the trypsin to leak out and/or trypsin inhibitor to leak in. Using trypsin labeled with ^3H by reductive methylation, we found that ~75% of the trypsin initially sealed in the ghosts leaks out under the conditions described in the legend of Figure 3.

As expected, intracellular trypsin causes no cleavage in extracellular loop 3 to produce the glycosylated 35 kDa fragment (Figure 3, lanes 2 and 3). In the same preparation, trypsin (25 μg/mL) added to the external medium produces a large amount of the 35 kDa fragment (Figure 3, lane 4), with only a slight increase in the amount of the 20 kDa fragment (resulting from digestion of ghosts that become leaky during the incubation). The fact that the 20 kDa fragment is produced under conditions in which the external surface (EC loop 3) of the membrane is not cleaved (lanes 2 and 3) is further evidence for the cytoplasmic location of K743.

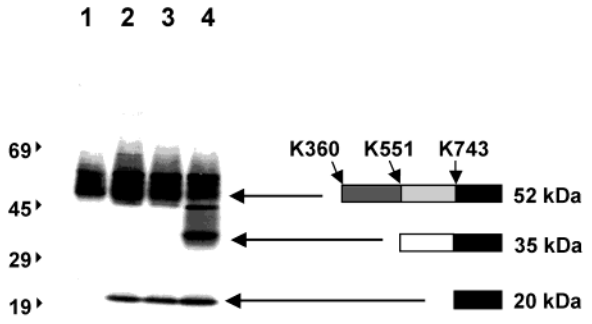

FIGURE 3: Digestion of resealed red cell ghosts by trypsin. Trypsin (25 μg/mL) was sealed inside red cell ghosts and prepared for electrophoresis and immunoblotting (developed with alkaline phosphatase) as described in Experimental Procedures. The conditions of the incubations were as follows: lane 1, high ionic strength (150 mM ammonium acetate and 10 mM phosphate at pH 7.5 for 90 min at 0 °C); lanes 2 and 3, low ionic strength (200 mM sucrose, 30 mM ammonium acetate, and 10 mM phosphate at pH 7.5 and 37 °C for 30 and 90 min, respectively); and lane 4, same as lane 3, but with 25 μg/mL extracellular trypsin.

The 20 kDa Fragment Is Not an Artifact of SDS Solubilization. In all in situ proteolysis experiments with membranes, it is assumed that the proteolytic events take place at the membrane surface. However, it is possible that the 20 kDa fragment is not actually produced by proteolysis of the protein in situ but rather is produced by trypsin just as the membranes are solubilized in SDS prior to electrophoresis. In the experimental results depicted in Figure 3 and in those previously published (*31*), soybean trypsin inhibitor was included in the extracellular medium to prevent extracellular cleavage. However, soybean trypsin inhibitor has been shown to stabilize trypsin and actually can increase trypsin activity transiently in the presence of SDS (*45*). It is necessary, therefore, to consider the possibility that the 20 kDa fragment is actually an artifact of SDS solubilization.

To determine whether the 20 kDa fragment is produced in situ, prior to SDS solubilization, unsealed membranes were incubated with 25 μg/mL trypsin in either 20 mM NaCl or 150 mM NaCl. Following the incubation, but before SDS solubilization, the ionic strength of an aliquot of the low-salt suspension was increased to 150 mM. Trypsin was then inhibited with TLCK, and membranes were solubilized in SDS. The amount of the 20 kDa fragment is small in ghosts incubated in 150 mM NaCl and is large in ghosts incubated in 20 mM NaCl, as expected (Figure 4). [Note that the production of the 20 kDa fragment is dependent on ionic strength but not on the presence or absence of Cl^-; the digestion pattern in 20 mM NMG-MOPS (Figure 2, lane 5) is similar to that in 20 mM NaCl.] The lack of the 20 kDa fragment in ghosts digested in 150 mM NaCl shows that the effects of ionic strength on proteolysis are reversible, because the membranes had been exposed to low ionic strengths during lysis. Significantly, lane 5 of Figure 4 shows that increasing the ionic strength after exposure to trypsin but before solubilization has no effect on the amount of the 20 kDa fragment. This result indicates that the 20 kDa fragment is produced at low ionic strengths in situ, before solubilization in SDS.

Protection of Digestion at K743 by Pretreatment with H_2DIDS. The membrane-impermeant transport inhibitor H_2-DIDS and related stilbene disulfonates act only from the extracellular surface of band 3 (*46*, *47*). Once bound, H_2-DIDS (a homobifunctional reagent) can react covalently with two different lysine residues (K539 and K851) to form an intramolecular cross-link within a single band 3 polypeptide (*43*, *48*, *49*). The cross-link forms much more rapidly at alkaline pH (*43*), presumably because alkaline pH promotes deprotonation of lysine side chains. As shown previously (*31*), H_2DIDS protects band 3 in unsealed membranes from trypsin cleavage at K743 (Figure 4). If H_2DIDS is added to the unsealed membranes before trypsin and incubated at 37 °C in 100 mM $KHCO_3$ (pH 9.5), the main product is the intact membrane domain (leading edge at ~52 kDa; lanes 2 and 4). There is also some uncleaved band 3 (95−110 kDa), which indicates that some of the ghosts reseal during the incubation with H_2DIDS prior to trypsin addition.

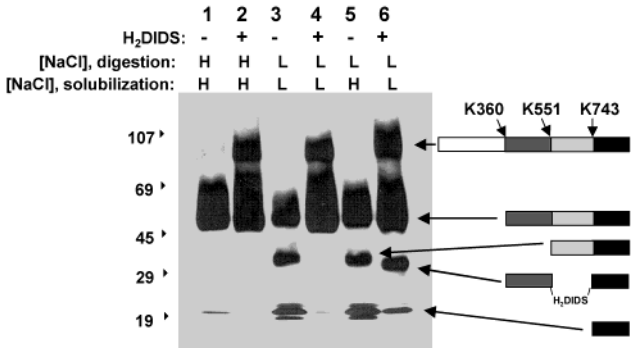

FIGURE 4: Effect of H_2DIDS on the digestion of band 3 in unsealed membranes by trypsin. Unsealed membranes were incubated with trypsin and analyzed by immunoblotting (ECL) as described in Experimental Procedures. Trypsin digestion and membrane solubilization were carried out at either high (H; 150 mM NaCl) or low (L; 20 mM NaCl) ionic strength. In lanes 2 and 4, membranes were pretreated with 10 μM H_2DIDS before exposure to trypsin. In lane 6, membranes were treated with H_2DIDS after trypsin treatment.

If H_2DIDS is added after trypsin cleavage (Figure 4, lane 6), a new band appears, with mobility slightly higher than that of the 35 kDa fragment, exactly as observed previously (*31*). This band represents the 20 kDa C-terminal fragment cross-linked to the ~17 kDa fragment consisting of the first five transmembrane segments. The fact that neither this cross-linked band nor the 20 kDa band is detected in the membranes pretreated with H_2DIDS (lanes 2 and 4) indicates that there has been no cleavage at K743 in H_2DIDS-treated membranes. The protection by H_2DIDS against trypsin cleavage in unsealed membranes does not, by itself, have any implications about the sidedness of K743; a bound ligand can prevent a proteolytic nick even though the ligand and proteolysis site are physically separated from each other (*50*). Therefore, externally bound H_2DIDS could protect either extracellular or intracellular proteolysis by stabilizing a proteolysis-resistant conformation.

Proteolysis of Inside-Out Vesicles. To investigate further the sidedness of the K743 cleavage event, inside-out vesicles (IOVs) were treated with trypsin in 20 or 150 mM NaCl. Figure 5 shows that trypsin treatment of IOVs produces the 20 kDa fragment, consistent with the idea that K743 is cytoplasmic. Significantly, H_2DIDS treatment of IOVs does not protect K743 from trypsin digestion (Figure 5, lane 4). If the 20 kDa fragment had been formed from trypsin action on contaminating ROVs or unsealed vesicles, H_2DIDS would

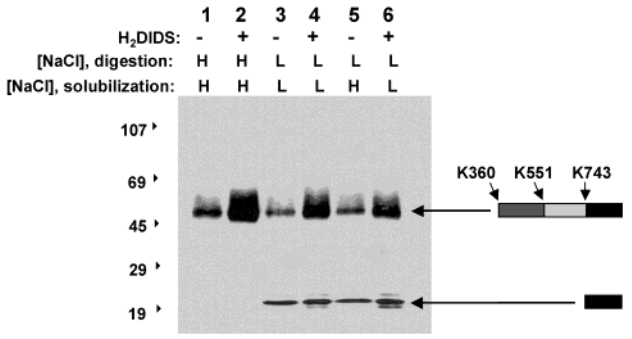

FIGURE 5: Trypsin digestion of inside-out vesicles (IOVs). IOVs were prepared and incubated at high (H; 150 mM NaCl) or low (L; 20 mM NaCl) ionic strength as indicated and then prepared for electrophoresis and immunoblotting as described in the legend of Figure 4. Vesicles were incubated with 10 μM H_2DIDS either before (lane 4) or after (lane 6) exposure to trypsin. The production of the 20 kDa band is not prevented by H_2DIDS.

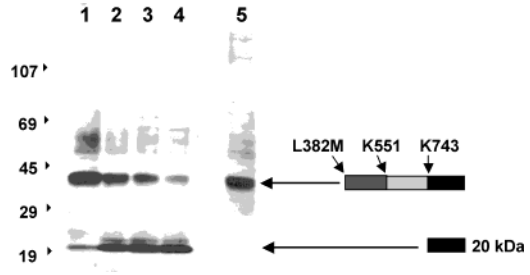

FIGURE 6: Trypsin digestion of microsomes prepared from EcR-293 cells expressing the AE1 membrane domain without the glycosylation site. Lanes 1−4 represent microsomes incubated with 0, 2, 4, and 8 μg of trypsin, respectively [30 min at 25 °C in 50 mM Na-MOPS (pH 7.0) and 10% sucrose]. Lane 5 represents a crude cell homogenate without incubation. The positions of the intact membrane domain and the 20 kDa fragment are indicated. The small but detectable amount of 20 kDa fragment in lane 1 was not seen in every experiment; it is not clear whether this fragment is a loading artifact or if it represents the product of digestion by an endogenous protease.

have prevented the cleavage. The fact that H_2DIDS protects against the cleavage in unsealed membranes (Figure 4), but not in IOVs (Figure 5), argues strongly that the cleavage is on the cytoplasmic side of the membrane.

Trypsin Cleavage of Microsomes from HEK293 Cells Expressing the AE1 Membrane Domain. As an additional test of the idea that K743 is cytoplasmic, the membrane domain of AE1 (residues 382−911) was expressed in HEK293 cells under the inducible control of the Ecdysone receptor, as first done with whole band 3 by Timmer and Gunn (*51*). The native sequence of the AE1 membrane domain (with a translation initiation site replacing L382) moved normally to the surface membrane, as indicated by immunocytochemistry (not shown). If the glycosylation site at N642 is replaced with glutamine, the polypeptide is retained in intracellular organelles (not shown). Microsomes from these cells therefore contain the AE1 membrane domain in intracellular membranes, presumably with the cytoplasmic side facing outward. Digestion of these microsomes with trypsin produces the 20 kDa fragment (Figure 6), again indicating a cytoplasmic orientation of this site. H_2DIDS does not prevent the digestion (not shown), indicating that the luminal side of the membrane is inaccessible and that the cleavage site is on the cytoplasmic side.

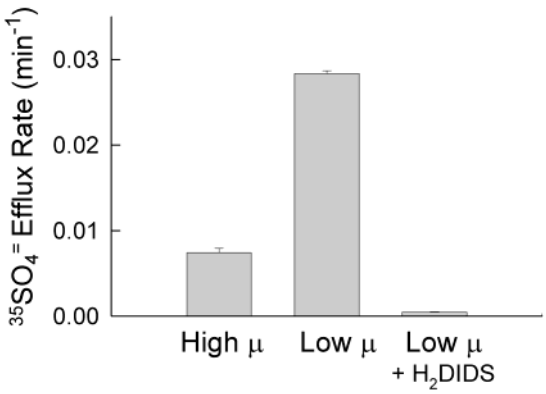

FIGURE 7: Rate constant (min^{-1}) for efflux of $^{35}SO_4^{2-}$ from red cells pretreated with gramicidin as in Figure 2 and loaded with $^{35}SO_4^{2-}$ in 20 mM NMG, 14 mM MOPS acid, and 1 mM Na_2SO_4. Efflux was measured in the same medium at 25 °C with either 150 mM ammonium acetate to increase the ionic strength on both sides of the membrane (High μ), no added salt (Low μ), or 10 μM H_2-DIDS added to the low-ionic strength medium (Low μ + H_2DIDS). The mean and range of two determinations (three to four time points each) are shown.

Effect of Low Ionic Strength on Band 3-Mediated Anion Exchange. The digestion experiments described above were performed at low ionic strengths, and it is important to address the question of the extent to which the folding of the protein is different in 150 versus 20 mM salt. Published data on Cl^- transport indicate that low ionic strength does not cause detectable changes in the function of band 3, other than the lack of saturation of substrate binding sites at low salt concentrations (*52*). However, those experiments were performed at 0 °C in the presence of 20 mM KCl, and our proteolysis experiments were carried out at higher temperatures and often in the absence of Cl^-. We felt that it was important to assess transport under conditions comparable to those of the proteolysis experiments. Figure 7 shows that, in media very similar to those used in Figure 2, there is an easily measurable $^{35}SO_4^{2-}$ efflux that is strongly inhibited by 10 μM H_2DIDS. Addition of 150 mM ammonium acetate (which rapidly permeates the membrane as NH_3 and acetic acid) reduces the SO_4^{2-} efflux by a factor of ~4, which is expected on the basis of the known inhibitory effects of acetate on $SO_4^{2-}-SO_4^{2-}$ exchange (*53*). The rate constant for of $SO_4^{2-}-SO_4^{2-}$ exchange in Figure 7 at low ionic strengths (0.028/min at 25 °C and pH 7.2) is close to that measured by Lepke and Passow (*54*) (~0.04/min at 27 °C and pH 7.2) in a Cl^--free solution containing 50 mM SO_4^{2-}. Therefore, the rate of anion exchange at low ionic strengths is similar to that expected from measurements at physiological ionic strengths; there is no evidence from these flux data or those of Funder and Wieth (*52*) that low ionic strength produces large amounts of a nonfunctional conformation of the protein.

DISCUSSION

The sidedness of the region of AE1 near K743 has now been studied using proteolysis of the native protein as well as mutagenesis of the heterologously expressed protein. In contrast to the cysteine scanning (*20*) and some of the glycosylation scanning results (*18*, *19*), we find here that trypsin digestions of AE1 in intact red blood cells, resealed ghosts, unsealed ghosts, inside-out vesicles, and microsomes

from HEK293 cells all indicate that K743 is exposed to the cytoplasmic side of the membrane at low ionic strengths. Therefore, the conditions of either in situ proteolysis or heterologous expression and mutagenesis cause abnormal topology in this part of the protein.

Before the sidedness of the sequence near K743 is discussed, it is useful to summarize established facts about the topology of the rest of the protein. There is general agreement regarding the topology of most[2] of the first eight TM segments (residues ~400−690) of AE1. Following TM8 is a cluster of positive charges between S690 and S700, consistent with an intracellular orientation (*55*); moreover, biochemical labeling experiments have shown that K691 is cytoplasmic (*56*). Therefore, residues 690−700 are clearly intracellular (IC loop 4). Our results indicate that K743 is on the cytoplasmic side of the membrane, implying that the polypeptide crosses the membrane either twice or not at all between residues 700 and 743. The next sites of known sidedness are K851, which reacts with extracellular H_2DIDS (*49*), and P854, which is the site of a blood group antigen and is therefore extracellular (*22*). Other known sites of blood group antigens in band 3 are in extracellular loops 1, 3, and 4 (*23*−*28*), but none has yet been identified within 80 residues of K743. This is consistent with the idea that K743 is cytoplasmic.

K743 as a Nick Site. We now consider the possibility that the apparently cytoplasmic location of K743 in our experiments is an artifact of in situ proteolysis. There are known conditions that can cause helical hairpin loops of folded membrane proteins to change topology, but these generally involve elevated temperature, extremes of pH, or extensive proteolysis (*57*−*60*). One such example is band 3 itself; partial denaturation with 10−100 mM NaOH causes the appearance of numerous sites of proteolysis in the membrane domain that are not cleaved in the native protein and also causes release of hydrophobic fragments from the membrane (*59*, *61*). The conditions used in the experiments presented here are much milder (20−30 mM salt, neutral pH) and cause selective cleavage of the K743−A744 peptide bond. The only other known sites of limited proteolysis in the nondenatured band 3 membrane domain are in extracellular loop 3 (*13*), in extracellular loop 4 (*29*), and in the tether connecting the membrane domain and cytoplasmic domains (*13*, *62*).

Common features of sites of limited proteolysis (nick sites) in proteins of known structure are surface exposure and flexibility (*50*). Many nick sites for serine proteases require local unfolding of as many as 10 residues for the cleaved bond to reach the substrate binding pocket of the protease (*50*, *63*). We believe that this property of nick sites is shared by K743. That is, K743 is reasonably close to the cytoplasmic surface under normal conditions, and at low ionic strengths, there is sufficient local unfolding to allow the K743−A744 bond to be cleaved by trypsin. It is noteworthy that, following trypsin cleavage at K743, the 20 kDa C-terminal fragment can still be cross-linked to TM1−5 by low concentrations of H_2DIDS (ref *31* and Figure 4 above), indicating that the structure of the protein is reasonably well preserved even after cleavage at K743 has taken place. If the cleavable conformation were severely abnormal, with the cleavage site on the opposite side of the membrane from its normal orientation, the cleaved protein would not be expected to refold. Moreover, the anion transport data (Figure 7) give no evidence of gross unfolding of band 3 at low ionic strengths. Therefore, we believe that the conditions of proteolysis used here do not alter the sidedness of K743.

Topogenic Properties of Sequences Proximal to K743. The membrane insertion of the polypeptide starting in IC loop 4 (after TM8, at M696) does not appear to depend on the presence of TM1−8, because TM1−8 and the complementary C-terminal fragment of M696−V911 (TM9−14 in the 14-TM model) can be expressed from separate mRNAs and then assemble into a functional transporter (*64*). The topogenic properties of the sequence starting after TM8 are therefore of interest in discussing the sidedness of K743. Ota et al. (*65*) recently showed, using cell-free translation, that the T685−V756 sequence (TM9−10) acts as a type II signal-anchor (*66*) sequence; i.e., it directs the polypeptide (prolactin) fused to its C-terminus to the ER lumen.

However, the topogenic properties of the preceding sequence do not imply that K743 is extracellular in the mature protein, because the final topology may depend on both upstream and downstream sequences. Groves et al. (*67*) have shown that the polypeptide representing TM9−14 cannot be stably expressed in *Xenopus* oocytes unless it is coexpressed with TM1−8. In contrast, the polypeptide consisting of TM8−14 is stable by itself. Groves and Tanner (*68*) recently performed extensive cell-free translation studies of AE1 fragments and showed that the fragment representing TM9−12 (with an additional N-terminal segment containing a glycosylation site) has a luminal N-terminus, whereas the corresponding construct containing only TM9−10 has a cytoplasmic N-terminus. Taken together, these fingings indicate that the topology of TM9−10 is affected by interactions with TM8 and TM11−12, and that interactions with TM8 stabilize an N_{in} orientation of TM9, and interactions with TM11−12 stabilize the opposite orientation.

The topology of TM6 of the cystic fibrosis protein CFTR is strongly influenced by the downstream sequences encoding the NBD1 and R domains, possibly because these domains fold rapidly (*69*). In the case of K743, mutations that would be expected to delay folding cause an increase in the degree of glycosylation of the K743N mutant in a cell-free translation system (*19*), indicating that this part of the protein folds rapidly, thereby limiting the accessibility of K743N to the glycosyltransferase. In intact cells, K743N is not glycosylated (*19*), presumably because the copies that are glycosylated during synthesis do not fold properly. The picture that emerges from the topogenesis (*65*) and glycosylation studies is that there are topogenic signals directing K743 to the ER lumen, but there is only transient exposure of K743 to the ER lumen during synthesis and/or insertion, as suggested by Popov et al. (*19*). Transient exposure of K743 to the ER lumen during insertion is compatible with our finding that K743 is cytoplasmic in the mature protein.

Expression Studies Indicating an Extracellular Location of K743. Recent cysteine scanning experiments by Fujinaga et al. (*20*) indicate that, in AE1 heterologously expressed in

[2] There is disagreement about the sidedness of Y486. Chemical labeling studies in red cells and IOVs (*80*) indicate that Y486 is cytoplasmic. However, experiments in which complementary fragments of band 3 are expressed and assemble as a functional transporter (*32*) provide strong evidence that Y486 is extracellular. Moreover, insertion of EC loop 4 in this position results in a glycosylated protein, indicating an extracellular orientation of this site (*19*).

HEK cells, several residues near K743 are extracellular. Specifically, cysteine residues substituted in positions 731, 742, 745, and 751 can be labeled in intact cells by an impermeant reagent (*20*). Also, although K743N is not glycosylated in intact cells, Popov et al. (*19*) found that insertion of EC loop 4 (with the glycosylation site) in position 754 produces a glycosylated protein. We believe that the most likely explanation for these results is that the part of the protein near K743 is relatively unstable, because topogenic signals direct it to the ER lumen, but other factors cause K743 to be cytoplasmic in the folded protein. We propose that, because the topogenic signals cause K743 to be transiently extracellular, cysteine substitutions or insertion of an EC loop may cause a sufficient perturbation to lock this part of the polypeptide in an abnormal topology.

In this connection, it is worth pointing out that, in the same expression system, a cysteine residue substituted for aspartate at position 821 can be labeled from the extracellular medium (*20*), a finding that disagrees with evidence from monoclonal antibody binding (*70*), glycosylation scanning (*18*), and chemical modification experiments (*71*), all indicating that D821 is cytoplasmic. Therefore, there appear to be sites in band 3 that, when mutated to cysteine, can be labeled with extracellular LYIA in a mammalian heterologous expression system, although the site is cytoplasmic in the native protein. These regions of the protein (near K743 and near D821) may therefore have labile topologies during synthesis and insertion; minor changes in sequence, or the insertion of a glycosylation site, can cause the polypeptide to change its orientation in the translocon. Dynamic reorientation of loops of the polypeptide within the translocon has been demonstrated recently in a model system by Spiess and co-workers (*72*).

If the cysteine substitutions or EC loop insertions induce conformations of the protein with abnormal topology, why then should the protein exhibit qualitatively normal function, as measured by anion transport (*20*) and binding to SITS-Affigel (*19*)? The answer may be that, despite the abnormal topology of the loop containing K743, the expressed protein still exhibits some functional characteristics. Recently, Groves et al. (*73*) showed that coexpression of TM1−5 and TM8−14 (i.e., missing TM6−7) in *Xenopus* oocytes is sufficient to produce a functional (stilbene disulfonate-sensitive) Cl^- transporter. The fact that an assembly that is missing two transmembrane helices can give significant anion exchange activity suggests that a misfolded form of band 3 could exhibit detectable function.

Dual Sidedness of K743? One potential explanation for the differences in sidedness of K743 between our results and those in other laboratories is that, in the normal functioning of the protein, K743 could participate in conformational changes that alter its sidedness, as appears to be true of another residue, E681, in TM8 (*74*, *75*). There is good evidence that E681 is closely associated with the transport pathway (*76*−*78*) and appears to lie at the bottom of an access channel that can be reached by small hydrophilic molecules from extracellular water (*21*). Moreover, E681 is very well conserved in AE1 of different species and among the three immediate members of the anion exchanger family (*79*). In contrast, the sequence from K742 to T746 is poorly conserved (*79*), and it is not likely that K743 has an essential role in transport. A reversible transition between the intracellular cleavable conformation and a conformation in which K743 can be reached by extracellular labels is formally possible but in our view improbable.

In summary, the study of the sidedness of the region of AE1 near K743 has provided an opportunity for a comparison of the results of heterologous expression with those obtained on the native protein. Misleading results are of course possible with both approaches, and it is conceivable that our proteolysis experiments have produced a change in the topology of band 3. However, we feel that the weight of evidence indicates that certain point mutations in this part of band 3 can cause an abnormal topology. For the vast majority of membrane proteins, topological studies can be performed only in heterologous expression systems, and comparisons with the native protein are not possible. In such systems, it is especially important to consider the possibility that a given mutation alters the topology of the protein.

ACKNOWLEDGMENT

We thank Richard Timmer and Robert Gunn for valuable advice regarding the use of the EcR-293 cells as an expression system.

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BI015879P