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Registry No. PDA, 73451-05-5; PDCoA, 93255-33-5; PDC, 93255-34-6; POPC, 6753-55-5; DPPC, 2644-64-6.

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Erythrocyte Band 3 Protein: Evidence for Multiple Membrane-Crossing Segments in the 17 000-Dalton Chymotryptic Fragment†

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ABSTRACT: We have investigated the topology of the band 3 protein of the human erythrocyte membrane by a combination of chemical labeling and proteolytic cleavage. The N-terminal third of the membrane-bound domain of band 3 is a 17 000-dalton chymotryptic fragment that is known to traverse the membrane an odd number of times. At least three lysine residues on this fragment can be labeled by reductive methylation of intact cells, under conditions that cause labeling of exofacial, but not intracellular, lysine residues. One of the labeled lysines is the one that reacts with anionic aryl isothiocyanates, and another is very close to the C terminus of the fragment. Both these are on the C-terminal 11-kilodalton CNBr peptide. The third labeled lysine is on the 6-kilodalton N-terminal CNBr peptide, which had not been previously known to have an extracellular site. Control experiments using a stilbenedisulfonate derivative demonstrate that the labeled 6-kilodalton CNBr peptide is not a degradation product of the 11-kilodalton CNBr fragment. Also, the exofacial lysine on the 6-kilodalton peptide can be labeled by reductive methylation even when the stilbenedisulfonate site is occupied by 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate, which blocks band 3 mediated transport of BH_4 into the cells. This is further indication that the labeled lysine is accessible from the extracellular water. These data are the first direct evidence that the 17-kilodalton chymotryptic fragment spans the membrane more than once.

Band 3 is a 95 000-dalton polypeptide that constitutes over half the integral membrane protein of human red blood cells (Fairbanks et al., 1971). The protein consists of an N-terminal water-soluble cytoplasmic domain of M_r 43 000 (Steck et al., 1976; Fukuda et al., 1978; Steck et al., 1978) and a glycosylated membrane-bound domain of M_r 52 000 (Markowitz & Marchesi, 1981; Jenkins & Tanner, 1977; Steck et al., 1978). The function of the membrane domain is to transport anions (Cl and HCO_3 physiologically) by way of an obligatory one-

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for-one exchange [see Knauf (1979)]. The cytoplasmic domain is not required for anion transport (Lepke & Passow, 1976; Grinstein et al., 1978); its function is to anchor elements of the red cell membrane skeleton (Bennett & Stenbuck, 1980).

The abundance of band 3 makes it one of a small number of transport proteins for which detailed structural work is possible. One goal of this work is to determine which portions of the primary structure are in contact with the extracellular water. The most direct approach to this problem is to perform irreversible chemical modification or proteolytic cleavage of band 3 in situ and then to isolate the protein (or its fragments) and localize the site(s) of modification in the primary structure [e.g., Williams et al. (1979), Drickamer (1976), and Drickamer (1977)]. In this manner, a fairly detailed model of the membrane domain of band 3 has evolved, with three regions susceptible to extracellular proteolytic cleavage thus far localized. They are approximately 17 000 daltons (Steck et al., 1976), 25 000 daltons (Jennings et al., 1984), and 33 000 daltons (Ramjeesingh et al., 1983) from the N terminus of the membrane domain. The two 8000-dalton segments defined by these cleavage sites are each believed to cross the membrane as helical hairpins, i.e., U-shaped loops (Ramjeesingh et al., 1983; Brock et al., 1983; Jennings et al., 1984).

Less is known about the structure of the 17 000-dalton segment that is defined by the major extracellular chymotrypsin cleavage site and the N terminus of the membrane domain (Steck et al., 1976, 1978; Grinstein et al., 1978). It clearly must cross the membrane an odd number of times, and it is often depicted in models as having three membrane-spanning segments [e.g., Jennings et al. (1984), Ramjeesingh et al. (1983), and Wieth et al. (1982)], but there is very little evidence that the 17-kilodalton fragment crosses the membrane more than once. There is a CNBr cleavage site about 6000 daltons from the N terminus of the 17-kilodalton segment, and several groups have reported that the C-terminal 11 000-dalton CNBr fragment can be labeled from the extracellular surface of intact cells by nonpenetrating reagents, including irreversible inhibitors of anion exchange (Drickamer, 1976, 1977; Mawby & Findlay, 1982; Ramjeesingh et al., 1980). The N-terminal third of this 11 000-dalton fragment contains an extremely nonpolar sequence of 32 residues (Mawby & Findlay, 1982). Existing data are consistent with the possibility that this hydrophobic segment is the sole membrane-crossing region of the 17-kilodalton fragment, since no extracellular site has been identified that is between the 11-kilodalton fragment and the N terminus of the membrane domain.

The present studies were undertaken to determine whether or not any portion of the 6000-dalton N-terminal CNBr peptide of the 17-kilodalton fragment is in contact with extracellular water. Using reductive methylation of intact cells, we show that a lysine residue in the 6-kilodalton CNBr peptide is accessible to the extracellular water. Covalent attachment of H_2DIDS[1] to the 11-kilodalton fragment does not prevent labeling of the lysine on the 6-kilodalton peptide. Of all the extracellular sites identified on band 3 thus far, this lysine is the farthest toward the band 3 N terminus. These data are the first direct evidence that the 17-kilodalton fragment crosses the membrane more than once.

Experimental Procedures

Materials

Human red cells (EDTA anticoagulant) were obtained from either the Lipid Research Laboratory or the Blood Donor Center of The University of Iowa. Enzymes were from Boehringer; CNBr was from MCB. H_2DIDS and [^{3}H]H_2DADS were prepared from DADS (Eastman) as described previously (Jennings et al., 1984). [^{3}H]H_2BADS was prepared from [^{3}H]H_2DADS by the method of Kotaki et al. (1971); the H_2BADS was separated from H_2DADS and H_2DBDS by silicic acid chromatography in pyridine–acetic acid–water (10:1:40) (Kotaki et al., 1971); purity was checked by thin-layer chromatography in the same solvent system and in 1-propanol–ammonium hydroxide–water (6:3:2) (Cabantchik & Rothstein, 1974). The [^{3}H]H_2BADS was converted to [^{3}H] H_2BIDS by vortexing with thiophosgene (Cabantchik & Rothstein, 1974); unreacted thiophosgene was removed by multiple ether extractions.

Methods

Labeling of Cells. Cells were washed 3 times in PBK6, with a 10-min incubation at room temperature before each spin to lower the intracellular pH. Cells were resuspended at 15% hematocrit in PBK6, cooled to less than 2 °C, and reductively methylated with 16 mM HCHO–5 mM [^{3}H]$NaBH_4$ essentially as described previously (Jennings, 1982), except that the extracellular pH was 8.8 (by the addition of $Na_2B_4O_7$ to 16 mM) instead of 8.4. Labeling with [^{3}H]H_2BIDS (3 μM) was at 37 °C, 30% hematocrit, for 1 h, in 150 mM $NaHCO_3$, pH 9.

CNBr Cleavage of the 17-Kilodalton Fragment. Labeled cells were washed in PBK7, treated with chymotrypsin (1 mg/mL in PBK7, 1 h, 37 °C), and membranes were isolated as described previously (Jennings, 1982). The 17-kilodalton fragment was produced by treating the unsealed membranes with trypsin or chymotrypsin[2] (50 μg/mL) in PBK7.4, for 45 min at 37 °C. Following the incubation the trypsin or chymotrypsin was inhibited by TLCK (50 μg/mL) or PMSF (30 μg/mL), respectively. Membranes were then stripped of peripheral protein with 0.1 N NaOH (Steck & Yu, 1973), washed in 5 mM $NaHCO_3$, and then solubilized by heating 3 min at 100 °C in 80 mM dithiothreitol–4% sodium dodecyl sulfate. The 17-kilodalton fragment was purified by gel filtration on Sephadex G-100S in 20 mM NaCl–1.5 mM $NaHCO_3$ (pH 8)–0.1% sodium dodecyl sulfate. Fractions enriched in the 17-kilodalton fragment were pooled, concentrated by ultrafiltration (Amicon YM10), and rechromatographed on the same column. Purity was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Coomassie blue stain and counting of slices), using 6–18% linear gradient gels run in the Laemmli (1970) buffer system. The 17-kilodalton fragment was treated with CNBr (5 mg/mL) in aqueous 0.1 N HCL–0.1% sodium dodecyl sulfate under N_2 in the dark at room temperature for 4–6 h unless otherwise specified. The

[1] Abbreviations: H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; DADS, 4,4′-diaminostilbene-2,2′-disulfonate; H_2DADS, 4,4′-diaminodihydrostilbene-2,2′-disulfonate; H_2BIDS, 4-benzamido-4′-isothiocyanodihydrostilbene-2,2′-disulfonate; H_2BADS, 4-benzamido-4′-aminodihydrostilbene-2,2′-disulfonate; H_2DBDS, 4,4′-dibenzamidodihydrostilbene-2,2′-disulfonate; PBK6, 150 mM KCl–20 mM sodium phosphate, pH 6; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBK7, 150 mM KCl–10 mM sodium phosphate, pH 7; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane.

[2] The only difference between the fragments produced by intracellular proteolysis with chymotrypsin vs. trypsin is that the chymotryptic fragment has an N-terminal lysine residue that the tryptic peptide lacks (Mawby & Findlay, 1982). We found no difference in the extent of reductive methylation of the 17-kilodalton fragments resulting from the two different proteolysis procedures.

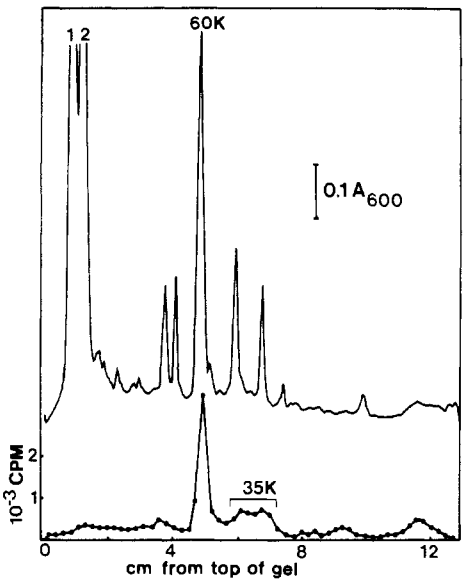

FIGURE 1: Reductive methylation of the 60- and 35-kilodalton chymotryptic fragments of band 3. Intact cells were reductively methylated with 16 mM HCHO and 5 mM B^3H_4 as described under Methods and subsequently treated wth chymotrypsin. Membranes were isolated, solubilized, and run on a sodium dodecyl sulfate–polyacrylamide gel. The Coomassie blue staining pattern (above) and radioactivity profile (below) show extensive labeling of the band 3 fragments. The spectrin peaks (1 and 2) have been truncated; peak absorbance was 1.8 times that shown.

products were identified by gel electrophoresis of the whole reaction mixture after neutralization with Tris base. The presence of sodium dodecyl sulfate throughout the procedure did not interfere with the cleavage reaction but prevented the aggregation of the 11-kilodalton product observed by Mawby & Findlay (1982). The 6-kilodalton peptide was partially purified from the CNBr digest by Sephadex LH-60 chromatography in 7:3 95% ethanol–88% formic acid (Mawby & Findlay, 1982). The CNBr digest was applied directly to the column, and fractions enriched in the 6-kilodalton fragment were dried, taken up in the running solvent, and rechromatographed on the same column.

Results

Confinement of Labeling to Cell Exterior. Our previous work (Jennings, 1982) on reductive methylation of intact red cells showed that label is confined mainly to extracellular sites when the labeling is done at pH_i 7 and pH_o 8.4. In the present studies extracellular labeling was increased by raising pH_o to 8.8, and intracellular labeling was minimized by lowering pH_i to 6.0. At the low temperature used, this pH gradient is stable for times sufficiently long to carry out the labeling (6 min). This was verified by centrifuging an aliquot of cells immediately after the reductive methylation; the intracellular pH (estimated by lysing the cells in two volumes of distilled water) was 6.0 ± 0.1 even though the extracellular (supernatant) pH was 8.8. Figure 1 shows that, under these conditions, there is even less labeling of spectrin than we found previously (Jennings, 1982). The only significantly labeled lysine residues are those in contact with extracellular water.

CNBr Peptides of the 17-Kilodalton Fragment. Figure 2 shows the time course of CNBr digestion of the 17-kilodalton fragment. There are two identifiable products; these have apparent M_r of roughly 11 000 and 6000 in our gel system. The basis for these molecular weight estimates is that the more rapidly migrating component has mobility very similar to that of aprotinin (M_r 6500), and the larger component has slightly

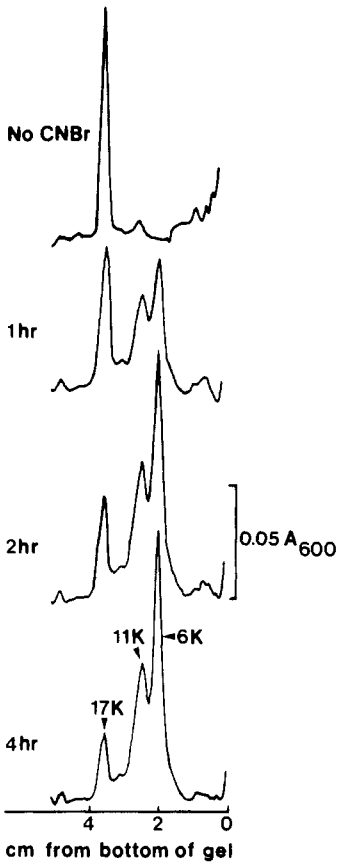

FIGURE 2: CNBr digestion of the 17-kilodalton fragment. The isolated fragment was treated for 1, 2, and 4 h with 5 mg/mL CNBr (Methods). The entire digest was run on a sodium dodecyl sulfate–polyacrylamide gel and stained with Coomassie blue. Scans of the bottom 5 cm of each lane are shown. The positions of the 17-kilodalton fragment and the two CNBr peptides (11 and 6 kilodalton) are indicated.

higher mobility than cytochrome *c*. We did not do more detailed calibration of the low M_r range on our gels, since either or both fragments may migrate anomalously. Thus, our designation of CNBr fragments as 6 kilodalton and 11 kilodalton is for the purpose of identification. We do not know the true molecular weights. These fragments are almost certainly identical with those found by Mawby & Findlay (1982), who assigned them lower molecular weights (8800 and 4700) and demonstrated unequivocally that the smaller (more rapidly migrating) fragment is N terminal. As reported by these authors, we find that the 6-kilodalton band stains much more intensely, even though the two peptides are present in equimolar amounts, and aggregation of the 11-kilodalton fragment was minimized by including sodium dodecyl sulfate in the reaction mixture.

The 17-kilodalton fragment from cells labeled by reductive methylation (Figure 1) was cleaved with CNBr under conditions identical with those in Figure 2. Under the labeling conditions used, about 1.5 methyl groups per 17-kilodalton fragment were introduced. About $^2/_3$ of the label is in the 11-kilodalton peptide, but significant radioactivity was also in the 6-kilodalton fragment (Figure 3, left, and two separate experiments not shown). The radioactivity copurifies with the 6-kilodalton peptide on Sephadex LH-60 (not shown).

Since the goal of these experiments was to determine whether or not the N-terminal CNBr fragment can be labeled from the extracellular water, it is very important to demonstrate that only two CNBr fragments are produced under our conditions. Ramjeesingh et al. (1980) showed that treatment

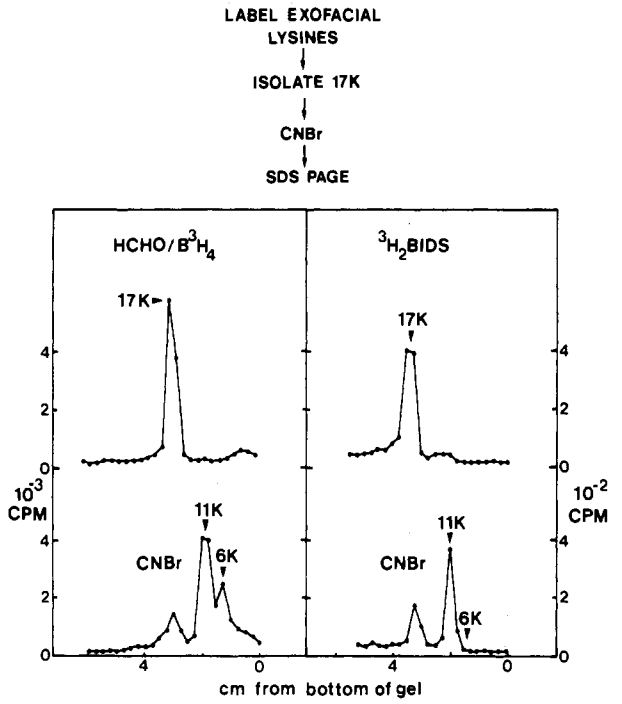

FIGURE 3: Labeling of exofacial lysines by reductive methylation (left) and [^{3}H] H_2BIDS (right). The 17-kilodalton band 3 fragment from ^{3}H-labeled cells was isolated and treated 4–6 h with CNBr, and the digest was run on a sodium dodecyl sulfate–polyacrylamide gel. The pattern of radioactivity for the bottom 6 cm of each lane is shown. The undigested preparation shows only the 17-kilodalton fragment (upper). The CNBr digest reveals label in the 6-kilodalton fragment for the reductively methylated cells but not those labeled with [^{3}H]H_2BIDS.

of the 17-kilodalton fragment with a high CNBr concentration for up to 64 h causes cleavage of the 11-kilodalton fragment. To show that this cleavage does not take place under our conditions, we labeled cells with the anion transport inhibitor H_2BIDS, which is expected to react covalently with the 11-kilodalton fragment (Drickamer, 1976; Mawby & Findlay, 1982; Ramjeesingh et al., 1980; Lieberman & Reithmeier, 1983). The products of CNBr digestion of [^{3}H]H_2BIDS-labeled 17-kilodalton fragment are shown in Figure 3 (right). The radioactivity shifts from 17 000 to 11 000 daltons, with no evidence of further cleavage. Therefore, the observed 6-kilodalton fragment is in fact the N-terminal CNBr peptide and does not contain products of degradation of the 11-kilodalton fragment.

Lack of Effect of H_2DIDS. Although reductive methylation of intact cells does not label spectrin or other intracellular proteins appreciably (Figure 1), it is possible that, since BH_4^- enters the cells as an anion via band 3 (Jennings, 1982), inward-facing lysines on band 3 could be labeled more extensively than those on, e.g., spectrin. This could be possible because the band 3 lysines on the intracellular side of the permeability barrier might have first access to the incoming $B^3H_4^-$. To examine this possibility, we treated cells with 8 μM H_2DIDS at pH 7.4, i.e., under conditions where cross-linking by H_2DIDS of the 17-kilodalton and 35-kilodalton chymotryptic fragments is not extensive (Jennings & Passow, 1979). The cells were then treated with chymotrypsin and labeled by reductive methylation as previously. The 17-kilodalton fragment was isolated[3] and treated with CNBr, and the 6-kilodalton fragment was partially purified on Sephadex LH-60. The H_2DIDS does not significantly diminish the labeling of the 6-kilodalton fragment; as previously, about 0.5 methyl group per 6-kilodalton peptide was introduced by the reductive methylation (Figure 4).

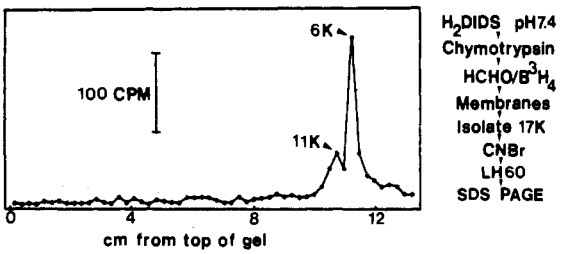

FIGURE 4: Extracellular labeling of the 6-kilodalton CNBr fragment in cells treated with H_2DIDS. Intact cells were reacted with 8 μM H_2DIDS, then reductively methylated with HCHO–B^{3}H$_4$, and then treated with chymotrypsin. The 17-kilodalton fragment was isolated and cleaved with CNBr, and the 6-kilodalton peptide was partially purified by Sephadex LH-60 chromatography and run on a sodium dodecyl sulfate–polyacrylamide gel.

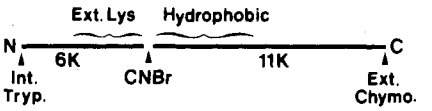

FIGURE 5: Linear map of the 17-kilodalton band 3 fragment. See the text.

Our original evidence (Jennings, 1982) for band 3 mediated BH_4 transport was that H_2DIDS pretreatment inhibits intracellular (presumably hemoglobin) labeling of intact cells by reductive methylation. In order to ensure that this is true under the present conditions of H_2DIDS pretreatment and reductive methylation, we measured the radioactivity in the first lysis supernatant of cells incubated with or without 8 μM H_2DIDS at pH 7.4, washed 4 times, and then reductively methylated as in the Figure 4 experiment. The H_2DIDS pretreatment lowered the intracellular labeling by a factor of 24 (from 0.13 to 0.005 mol of C^3H_3/mol of Hb monomer[4]). Therefore, H_2DIDS inhibits intracellular labeling under conditions in which labeling of the 6-kilodalton fragment is not substantially affected. This is strong evidence that the labeled lysine is accessible from the extracellular water. Moreover, the lysine is not sterically or electrostatically protected by occupancy of the stilbenedisulfonate site with H_2DIDS.

In the experiment shown in Figure 4, there is significant labeling of the 11-kilodalton CNBr fragment, even though one of its lysines is occupied covalently with H_2DIDS. This is not surprising, since it is known that a lysine residue not involved in stilbenedisulfonate binding is the third residue from the C terminus of the 17-kilodalton fragment (Jennings & Adams, 1981). This lysine is known to be susceptible to extracellular reductive methylation (Jennings, 1982).

Discussion

The major finding in this paper is that the N-terminal CNBr peptide of the membrane domain of band 3 can be labeled from the extracellular water. This labeling site is farther toward the N terminus than any extracellular site on band 3 thus far demonstrated. Since the 6-kilodalton fragment contains an extracellular site and the 11-kilodalton fragment has an extremely hydrophobic segment sufficiently long to span

[3] To avoid cross-linking the 17- and 35-kilodalton chymotryptic fragments during alkali stripping of the ghosts, the H_2DIDS-treated membranes were stripped of peripheral protein with 0.2 N acetic acid.

[4] The Hb labeling of cells that were not treated with H_2DIDS is about 5-fold higher than we reported previously (Jennings, 1982). We believe the increased labeling is caused by the higher concentrations of both HCHO and B^{3}H$_4$ used in the present experiments.

the membrane as an α-helix (Mawby & Findlay, 1982), the 17-kilodalton fragment very likely spans the membrane more than once (Figure 5). It has been suggested previously that there are three membrane-spanning segments in the 17-kilodalton fragment (Jennings et al., 1984; Ramjeesingh et al., 1983; Wieth et al., 1982), but existing data had been consistent with the possibility that the only crossing segment was the very hydrophobic N-terminal sequence of the 11-kilodalton fragment.

Although the most likely arrangement of the 17-kilodalton polypeptide is three crossing segments, a detailed model requires more sequence information. The N-terminal sequence of the 17-kilodalton (and the 6-kilodalton) fragment is not easy to determine because the repetitive yield drops after the first five cycles of Edman degradation (Markowitz & Marchesi, 1981; Mawby & Findlay, 1982; M. L. Jennings, unpublished results). Until the sequence is known, it is not possible to say with any certainty how many residues are in the 17-kilodalton fragment or in either of the CNBr peptides. The apparent M_r of the 17-kilodalton fragment ranges from 14 500 (Mawby & Findlay, 1982) to over 20 000 (Williams et al., 1979) depending on the gel system. The number of residues is therefore roughly between 120 and 170. Since an α-helical membrane-crossing strand plus a hydrophilic connector loop at the membrane surface requires only about 30 residues, it is conceivable that the 17-kilodalton fragment crosses the membrane 5 times.

The site on the 6-kilodalton peptide labeled by reductive methylation of intact cells is extracellular in the sense that it can be reached by hydrophilic reagents added from extracellular medium, under conditions where there is negligible labeling of intracellular lysines. The reagents, however, are quite small and would be expected to have access to a narrow hydrophilic channel that opens to the outer membrane surface, if such a channel exists. Thus, the labeled site, though topologically on the extracellular side of the permeability barrier, may not reside literally at the outer surface of the bilayer. In any case, the labeled lysine does not appear to be associated closely with the stilbenedisulfonate site, since covalent H_2DIDS attachment does not inhibit labeling of the 6-kilodalton fragment. The labeled lysine on the 6-kilodalton peptide has no known role in the transport function of band 3.

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