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Intrinsic Segments of Band 3 that are Associated with Anion Transport across Red Blood Cell Membranes*

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Summary. After treatment of red cell ghosts with chymotrypsin, the predominant intrinsic peptides remaining in the membrane fraction are 15,000 and 9,000 daltons mol wt. After partial extraction with Triton X-100, the residual membrane vesicles have almost no other stained peptides and such vesicles are reported to carry out anion transport activities sensitive to specific inhibitors. In vesicles derived from cells treated with DIDS(4,4′-diisothiocyano-2,2′-stilbene disulfonic acid), an irreversible inhibitor of anion transport that is highly localized in an abundant intrinsic protein known as band 3, the probe is largely recovered in the 15,000 dalton peptide. The part of band 3 from which it is derived is a previously reported 17,000 transmembrane segment (Steck, T.L., Ramos, R., Strapazon, E., 1976, *Biochemistry* **15**:1154). The 9,000-dalton peptide is present in the vesicles in a one-to-one mole ratio with the 15,000-dalton peptide, suggesting that both are derived from the same protein. This conclusion is supported by the finding that the 35,000-dalton C-terminal end of band 3, derived by chymotrypsin treatment of cells, is further proteolysed if the cells are converted to ghosts and its disappearance coincides with the appearance of the 9,000-dalton fragment. Evidence is presented that the 9,000-dalton fragment crosses the bilayer and that it is closely associated with the 15,000-dalton peptide.

We are pleased to pay tribute to Professor W. Wilbrandt because he has contributed so substantially to the concept that "carriers" in membranes play an essential role in the transport of ions and hydrophilic molecules. Carriers, at the time of his important studies, were hypothetical entities proposed to explain the kinetic behavior of transport phenomena (Wilbrandt & Rosenberg, 1961). In more recent times the carrier behavior that he formalized has been attributed increasingly to specific membrane proteins in which conformational changes may underlie the transport phenomena. An example that has been intensively studied is the anion exchange system of the red blood cell, the protein in this case being an abundant peptide of 95,000 daltons mol wt known as band 3 (*see* reviews of Rothstein, Cabantchik & Knauf, 1976; Cabantchik, Knauf & Rothstein, 1978; Knauf, 1979).

Because band 3 spans the membrane and is partially exposed at both the outside and cytoplasmic surfaces, it is susceptible to proteolytic attack from either side. Cleavage at the outside of intact cells by chymotrypsin results in a single cleavage producing two membrane-bound segments, one of 60,000 and the other of 35,000 daltons (Cabantchik & Rothstein, 1974*b*; Steck et al., 1976; Jennings & Passow, 1979; Rao, 1979). The former contains the N-terminus and the latter, the C-terminus of intact band 3. Cleavage at the cytoplasmic face using inside-out vesicles or resealed ghosts, releases about 40,000 daltons of soluble peptide containing the N-terminus, leaving a segment of about 55,000 daltons associated with the membrane (Lepke & Passow, 1976; Steck et al., 1976). Cleavages at both sides of the membrane result in the appearance of a 17,000-dalton transmembrane segment (Steck et al., 1976; Grinstein, Ship & Rothstein, 1978; Rao & Reithmeier, 1979). The inside and outside cleavages when produced by chymotrypsin or trypsin do not substantially diminish the anion transport capacity (Cabantchik & Rothstein, 1974*a*; Lekpe & Passow, 1976; Passow et al., 1977; Grinstein et al., 1978). Thus the soluble, cytoplasmic 40,000-dalton N-terminal segment is not essential for transport, and cleavage of the intrinsic portion of band

* *This paper is dedicated to the memory of Walther Wilbrandt.*

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3 at the outside, into two segments is tolerated. On the other hand, treatment of cells with papain or pronase can result in inhibition of transport (Cabantchik & Rothstein, 1974*b*; Passow et al., 1977; Jennings & Passow, 1979).

Specific covalent inhibitors of anion transport are highly localized in the 17,000-dalton transmembrane segment (Cabantchik et al., 1978). One of them, DIDS (4,4′-diisothioycano-2,2′-stilbene disulfonic acid) appears to inhibit by competing with Cl^- for binding to the transport site (Shami, Rothstein & Knauf, 1978). Another, NAP-taurine N-(4-azido-2-nitrophenyl)-2-aminoethyl sulfonic acid, appears to inhibit noncompetitively by interaction with another anion binding site, called the modifier (Knauf et al., 1978). Thus this segment appears to contain two sites directly related to transport activities.

Of the membrane-bound portions of band 3, the 17,000-dalton segment can be generated either by proteolytic cleavage at the outer surface in intact cells, followed by a second cleavage at the cytoplasmic surface using inside-out vesicles, or by a single cleavage in leaky ghosts (Steck et al., 1976; Grinstein et al., 1978; Rao & Reithmeier, 1979). The 35,000-dalton, C-terminal segment is generated by cleavage at the outer surface of intact cells (Cabantchik & Rothstein, 1974*b*; Steck et al., 1976; Jennings & Passow, 1979; Rao, 1979), but little or none is observed when ghosts are subjected to proteolysis (Steck et al., 1976; Rao & Reithmeier, 1979). A detailed assessment of the 35,000-dalton segment has been technically difficult because it is a broad, poorly stained band on SDS-acrylamide gels and it may be proteolysed during its extraction and electrophoresis. Its reported recovery is variable, and in a number of papers in which cells were treated with chymotrypsin, its presence has not been reported at all. Nevertheless, Jennings and Passow (1979) have recently demonstrated that even when cells are treated with high concentrations of chymotrypsin for prolonged periods of time, the segment does not undergo further cleavage. To summarize, the segment is produced by proteolysis of cells, but has not been reported in proteolysed ghosts, suggesting that it may be susceptible to further proteolysis.

In the present study the proteolysis of band 3 in intact membranes as compared to leaky ghosts is further assessed using an electrophoretic procedure that allows better visualization of the proteolytic fragments. The predominant membrane-bound fragments remaining in vesicles derived from extensive proteolysis of ghosts are 15,000 and 9,000 daltons, the former being derived from the 17,000-dalton transmembrane segment and the latter from the 35,000-dalton C-terminal segment produced by proteolysis of intact membranes. Such vesicles retain some capacity to transport anions (Rothstein, Ramjeesingh & Grinstein, 1980).

Materials and Methods

Nonradioactive DIDS and tritiated dihydro-DIDS ($^3H_2$DIDS) were synthesized from their diamino analogs as previously described (Cabantchik & Rothstein, 1974*a*). Cellex-D, having an exchange capacity of 0.80 meq/g, was obtained from Bio-Rad. Triton X-100 was purchased from New England Nuclear.

Recently outdated blood bank cells were washed three times at 0–5° with phosphate-buffered saline (PBS: 150 mM NaCl, 5 mM sodium phosphate, pH 8). In some experiments, the washed cells (50% hematocrit) were labeled with $^3H_2$DIDS by exposure to 10 μM of the probe for 30 min at 37 °C. The probe is highly localized in band 3 (Cabantchik & Rothstein, 1974*a*; Lepke et al., 1976; Ship et al., 1977; Jennings & Passow, 1979), binding to a specific site located in the 17,000-dalton transmembrane segment (Grinstein et al., 1978). It can therefore be used to quantitate the amount of band 3 peptide or of DIDS-containing fragments during fractionation procedures. Leaky ghosts were prepared by the procedure of Dodge, Mitchell and Hanahan (1963), using sodium phosphate buffer (5P8: 5 mM sodium phosphate, pH 8). Inside-out vesicles (IOV) were prepared by the procedure of Steck (1974).

Proteolysis of cells (25% hematocrit in PBS) was carried out for 16 hr with 0.4 mg/ml chymotrypsin at room temperature. Proteolysis of ghosts was carried out in 5P8 at a final protein concentration of 1–3 mg/ml. Unless otherwise indicated, the chymotrypsin concentration was 0.2 mg/ml, the time was 1.5 hr, and the temperature, 37 °C. Inside-out vesicles were suspended in phosphate buffer, 0.5P8 (0.5 mM sodium phosphate, pH 8) and the conditions of proteolysis were similar to those used for ghosts. Any departures from these procedures are noted in the text of legends.

After proteolysis of ghosts or vesicles, the extrinsic peptide segments were largely removed by "stripping" with 10 mM sodium hydroxide containing 0.1 mM EDTA (Steck et al., 1976). The "stripped" vesicles containing intrinsic peptides were further purified by partial extraction with low concentrations of Triton X-100 (0.5% in 10 mM Tris HCl, pH 7.4) (Wolosin, Ginsburg & Cabantchik, 1977). The residual vesicles are enriched in segments from band 3. The Triton X-100 soluble fraction was passed through a Cellex column. Elution with buffer (10 mM Tris HCl, 0.5% Triton X-100) containing a sodium chloride gradient of 0 to 0.6 M sodium chloride allowed some separation of peptide fragments.

The peptides present after various procedures were separated and visualized by SDS-acrylamide gel electrophoresis. For unproteolysed samples, the procedure of Weber and Osborn (1969) was used with 5.6% acrylamide. For assessing proteolytic products of lower mol wt, a urea-SDS system was found to be superior. It was effective with fragments as small as 2,000 daltons, not seen in the usual SDS procedures. The urea system was adapted from Swank and Munkres (1971). The samples were delipidated by extraction with chloroform-methanol, and then dissolved in solubilizer containing 1% SDS and 8 M urea. The gels, either slabs or discs, were 14.25% acrylamide and 6.25 M urea. Staining was with Coomassie blue. Scanning of the stained bands was carried out using a Beckman GS2 scanner on a Beckman Acta C11 spectrophotometer. Standards used for mol wt determinations included myoglobin (17,800), cytochrome C (12,600), and β-insulin (3600). For $^3H_2$-DIDS localization in gels, the procedure of Shami et al. (1978) was used. Proteins were determined by a modification of the method of Lowry et al. (1951).

Amino acid analyses were performed on a Durrum 500 amino acid analyzer as described previously (Ramjeesingh, Gaarn & Rothstein, 1980).

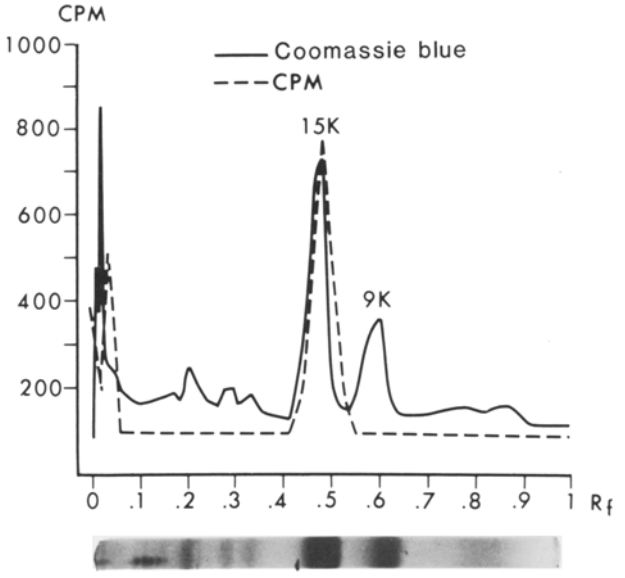

Fig. 1. Distribution of $^3H_2$DIDS and of peptides from vesicles derived from chymotrypsinized alkali-stripped ghosts after urea-SDS gel electrophoresis

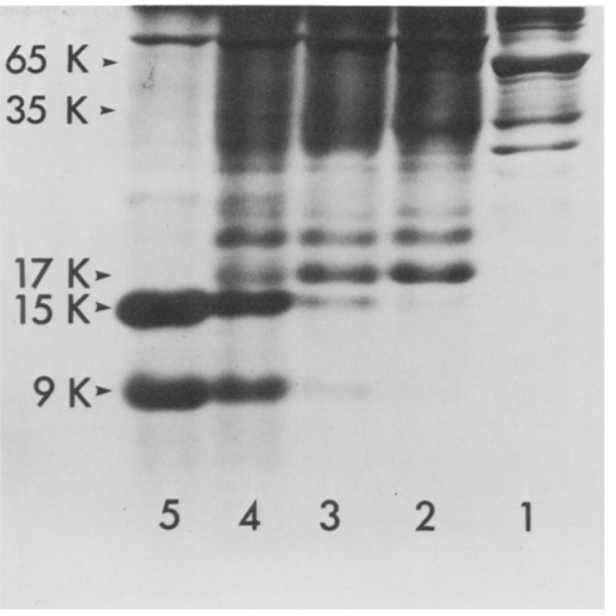

Fig. 2. Urea-SDS slab gel showing the production of the 17,000-dalton fragment and its conversion to the 15,000-dalton fragment at low and intermediate concentrations of chymotrypsin. (*1*): Alkali-stripped ghosts prepared from chymotrypsinized red blood cells. (*2*): Ghosts as in sample *1* Incubated with chymotrypsin (1.5 mg protein per ml with 5 μg enzyme per ml in 5P8). (*3*): 50 μg enzyme per ml. (*4*): 250 μg enzyme per ml. (*5*): 1.5 mg enzyme per ml

Results

The conclusion of Jennings and Passow (1979) that the 35,000-dalton C-terminal portion of band 3 (produced by chymotrypsin treatment of intact cells) is not further cleaved even with prolonged periods of treatment of the cells with high enzyme concentrations, was confirmed. The problem of visualization of the peptide segment evident from the literature (Cabantchik & Rothstein, 1974*b*; Steck et al., 1976; Jennings & Passow, 1979; Rao, 1979) was improved by use of the urea-SDS gel system (described in Methods) rather than the more commonly used procedures of SDS-acrylamide gel electrophoresis.

When leaky ghosts were treated with chymotrypsin, no 35,000-dalton peptide was found in the membrane fractions. The predominant peptides evident in the urea-SDS gels were of 15,000 and 9,000 daltons, with smaller amounts of higher mol wt material (Fig. 1). This is in contrast to previous reports (Steck et al., 1976; Grinstein et al., 1978), in which a single predominant peptide of 17,000 daltons was reported. The 9,000-dalton peptide was not evident in the previous studies either because it did not stain well or because it leached out of the gels in the usual SDS system (it can be seen as a faint band in heavily loaded gels). On the other hand, the considerably lower porosity of urea-SDS gels minimizes the losses of low mol wt peptide, and staining of the 9,000-dalton peptide is satisfactory.

The 15,000-dalton segment seems to be derived from the 17,000-dalton transmembrane segment produced by cleavages at the two sides of the membrane (Steck et al., 1976; Grinstein et al., 1978). Firstly, the 17,000-dalton segment contains a single DIDS-binding site of band 3 (Grinstein et al., 1978) which is retained in the 15,000-dalton segment (Fig. 1). Secondly, with careful control of the conditions, the sequence of proteolysis can be observed. Exposure of ghosts to low concentrations of chymotrypsin results in production only of the 17,000-dalton segment, intermediate concentrations a mixture of 17,000 and 15,000, and high concentrations only the 15,000-dalton segment (Fig. 2). Thirdly, the reported difference in molecular weight is real. It is not only based on careful calibration of the gel electrophoretic system, but also on amino acid analytical data as well. The 17,000-dalton segment contains 154 residues (Steck et al., 1978), whereas the 15,000-dalton segment contains only 137 residues (Ramjeesingh et al., 1980), according approximately for the difference in mol wt (Table 1).

The 9,000-dalton segment is generated only when unsealed ghosts are treated with chymotrypsin. As noted above, none is found after exposure of cells even to large concentrations of the enzyme for long periods of time (4 mg/ml for up to 14 hr), nor is it produced by a second cleavage at the cytoplasmic

Table 1. Amino acids compositions of segments of band 3.

Amino acid	9,000-dalton fragment	15,000-dalton fragment	17,000-dalton fragment
ASX	7.6±0.4	10.6±0.4	9.3±0.1
Thr	3.3±0.1	9.4±0.3	8.5±0.5
Ser	1.9±0.1	10.8±0.3	10.9±0.4
Glx	10.4±0.1	11.1±1.2	14.7±0.6
Pro	3.4±0.1	4.2±0.1	7.5±0.6
Gly	11.7±0.4	24.0±0.6	13.8±0.4
Ala	11.6±0.5	12.5±0.3	10.9±0.8
Val	6.6±0.1	11.3±0.4	11.2±0.6
Met	1.4±0.2	2.1±0.2	2.0±0.4
Ile	3.1±0.2	7.7±0.1	10.4±0.1
Leu	6.1±0.2	15.1±1.9	19.3±2.3
Tyr	0.9±0.1	2.3±0.4	5.5±1.2
Phe	2.8±0.1	8.4±0.6	11.5±0.7
His	2.6±0.1	1.8±0.1	2.1±0.2
Lys	5.3±0.1	7.2±0.3	5.3±0.2
Arg	4.0±0.1	4.5±0.1	6.5±0.2
Cys	2.1±0.1	1.1±0.2	1.4±0.3
Tryp	0	2.6±0.3	2.8±0.6
Sum	83.8	136.7	153.6
% hydrophobic	25	36	41
No. of samples	4	4	2

Hydrophobic residues are taken as Val, Met, Ile, Leu, Tyr, Phe and Trp.

The data for the 15,00-dalton fragment is from Ramjeesingh et al. (1980), and that for the 17,000-dalton segment from Steck et al. (1978)

Table 2. Mole ratio of 15,000 to 9,000 dalton segments in alkali and Triton X-100 stripped vesicles prepared from proteolysed ghosts

15 K (μg)	9 K (μg)		Ratio	Mole ratio (corrected for mol wt)
(*A*) Gel scanning				
46.7	21.1		2.2	1.32
77.1	45.5		1.7	1.02
77.9	45.3		1.7	1.02
55.4	24.2		2.3	1.39
92.7	46.0		2.0	1.20
		mean	1.98	1.19±0.17
(*B*) Peptide analysis (Lowry)				
52.5	36.0		1.46	0.88
334.5	157.5		2.12	1.27
64.5	33.3		1.94	1.16
52.5	30.6		1.72	1.02
112.5	64.8		1.74	1.04
		mean	1.80	1.07±0.15

Each pair of values represents a preparation from a different batch of cells

face of inside-out vesicles formed from the chymotrypsinized cells. Thus it is not a product of a cleavage at the cytoplasmic face of the intact membrane.

Two lines of evidence suggest that the 9,000-dalton segment is derived from 35,000-dalton, C-terminal segment of band 3:

(a) The first is based on quantitative determination of the amounts of the 15,000-dalton and 9,000-dalton peptides that are present in the vesicles derived from proteolysed ghosts. The amount of 15,000-dalton peptide was determined by use of the covalent probe, $^3H_2$DIDS. Virtually all of the probe is retained in the "stripped" proteolysed vesicles (as would be expected of a transmembrane peptide). After urea-SDS gel electrophoresis, 60% (6 estimates) of the probe bound to the cells was recovered in the 15,000-dalton segment with almost all of the remainder located in an aggregate that did not enter the gels as in Fig. 1. The 9,000-dalton segment is equally abundant in the vesicles. On the basis of scanning of the stained urea-SDS acrylamide gels after electrophoresis, the average ratio of 15,000 to 9,000 dalton peptide for four preparations is 1.19±0.17 (Table 2*A*). Based on a peptide analysis (Lowry) of the two peptides extracted from the gels, the ratio is 1.07±0.15 (Table 2*B*), not significantly different from 1.0. The latter estimate is probably more accurate than staining and gel scanning. The finding of a 1:1 mole ratio of 15,000 and 9,000 dalton segments indicates that the latter must be derived from a protein whose abundance is at least equal to that of band 3. No other intrinsic membrane protein can qualify. Band 3 is present in about 1.2×10^{-6} copies per cell (Steck, 1974; Lepke et al., 1976; Ship et al., 1977). Of the other abundant species, glycophorin is present in half as many copies, and band 4.5, in 1/4 to 1/3 as many copies (Lin & Spudich, 1974; Steck, 1974; Batt, Abbott & Schachter, 1976). Furthermore, in the case of glycophorin the intrinsic fragment left after proteolysis is only about 3,500 daltons (Marchesi, Furthmayr & Tomita, 1976). The only portion of band 3 that might be the source of the 9,000-dalton segment is the 35,000 C-terminal portion. Of the other parts of band 3, the 15,000 transmembrane segment is still present in the membranes and the N-terminal cytoplasmic portion is soluble and is removed by the alkali "stripping" procedure (Steck et al., 1976).

(b) Another kind of evidence indicating that the 9,000-dalton segment is derived from the 35,000-dalton C-terminal portion of band 3 is based on sequential proteolysis. Cells that were treated with chymotrypsin, carefully washed with inhibitor and albumin, then converted to ghosts. The 35,000-dalton segment present in these ghosts is evident in Fig. 3, gel 2. Exposure of the ghosts to a mild treatment with chy-

motrypsin resulted in a partial disappearance of the 35,000 and the appearance of some 9,000 dalton peptide (gel 4), whereas severe treatment resulted in almost complete disappearance of the larger segment and the appearance of substantial amounts of 9,000-dalton peptide (gel 5). The disappearance of the 35,000-dalton segment and the coincidental appearance of the 9,000-dalton fragment is perhaps more clearly evident in the densitometric scans illustrated in Fig. 4. The parallel appearance of the 15,000-dalton fragment and the coincidental disappearance of the 60,000-dalton fragment from which it is derived, is also demonstrated. The experiment suggests that in leaky ghosts, additional cleavages of the 35,000-dalton segment occur and that one of the cleavage products is the 9,000-dalton segment.

Although the predominant peptides in vesicles derived from proteolysed, stripped ghosts are the 15,000 and 9,000 dalton segments, other peptides are also present in lesser amounts (Fig. 1). Extraction with Triton X-100 results in some solubilization of the membranes, but even with 0.5% Triton for 1 hr, some vesicular material remains that forms a pellet on centrifugation. Examination of the extract and pellet by urea-SDS acrylamide gel electrophoresis reveals that the 15,000 and 9,000 dalton segments are partially dissolved but the other peptides are almost entirely dissolved (Fig. 5). Thus in the residual pellet of packed vesicles, the 15,000 and 9,000 dalton segments are highly enriched by removal of contaminating peptides. This procedure of negative purification is similar to that reported for production of vesicles highly enriched in intact band 3 (Wolosin et al., 1977). The procedure finally adopted is outlined in Methods. Recovery of 15,000 plus 9,000 dalton peptide in the vesicles after a single extraction, was 65% of the original amount present, and the two fragments accounted for over 80% of the total peptide. Higher purity can be obtained by a second Triton X-100 extraction with some additional loss. The molar ratio of 15,000 to 9,000-dalton segments in the extract and the pellet is about 1:1 in each case as determined by gel scanning, indicating that the two segments co-extract in Triton X-100.

On elution of the extract through a Cellex column, all of the $^3H_2$DIDS (marking the 15,000-dalton segment) elutes as a sharp peak, whereas peptides (determined by the procedure of Lowry et al., 1951), elute in all parts of the gradient (Fig. 6). Examination of the peptides in the $^3H_2$DIDS peak, by urea-SDS acrylamide gel electrophoresis, revealed the presence of not only the 15,000-dalton segment but also the 9,000-dalton segment in approximately a 1:1 mole ratio (Fig. 5), together with smaller amounts of other peptides. Attempts to separate the 15,000 and 9,000 dal-

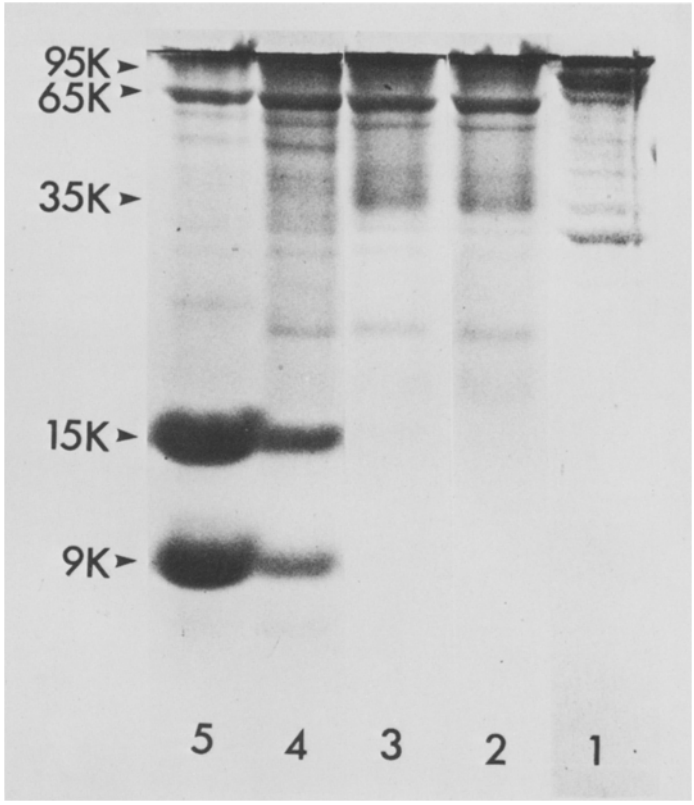

Fig. 3. Urea-SDS slab gel showing the proteolysis of 35,000 to 9,000 dalton peptide with increasing concentrations of chymotrypsin in alkali-stripped ghosts prepared from proteolysed red blood cells. (*1*): Nonproteolysed ghosts. (*2*) alkali-stripped ghosts prepared from chymotrypsinized red blood cells. (*3*): ghosts as in (*2*) (1 mg protein per ml) incubated with 200 μg chymotrypsin per ml in 5P8. (*4*): As in *2* but with 600 μg chymotrypsin per ml. (*5*): As in *3* but with 1.5 mg chymotrypsin per ml

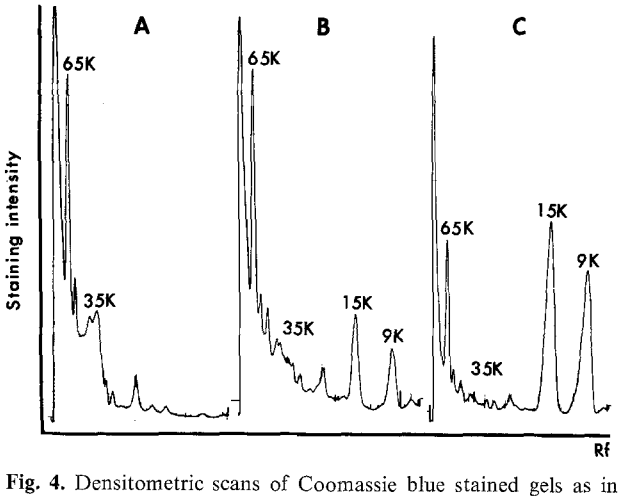

Fig. 4. Densitometric scans of Coomassie blue stained gels as in Fig. 3. Ghosts prepared from chymotrypsin-treated cells were exposed to a second treatment with chymotrypsin at the following concentrations: (*A*) 200 μg/ml; (*B*) 600 μg/ml; (*C*) 1.5 mg/ml

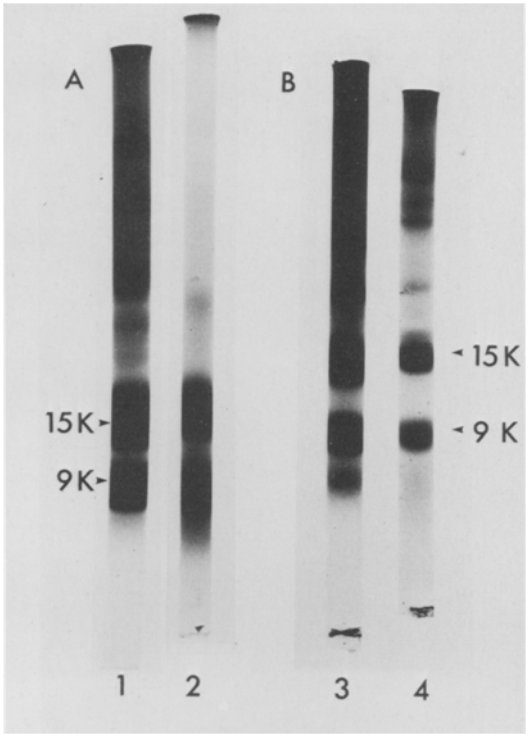

Fig. 5. Urea-SDS gels showing: (*1*) vesicles prepared by proteolysis with chymotrypsin of alkali-stripped ghosts; (*2*) pellet obtained by 0.5% Triton X-100 extraction of vesicles from *1*; (*3*) supernatant from *2*. The extract was dialysed in the presence of bio-beads overnight, freeze-dried, and then dissolved in solubilizer; (*4*) radioactive peak (*see* Fig. 5) from the *Cellex* D column

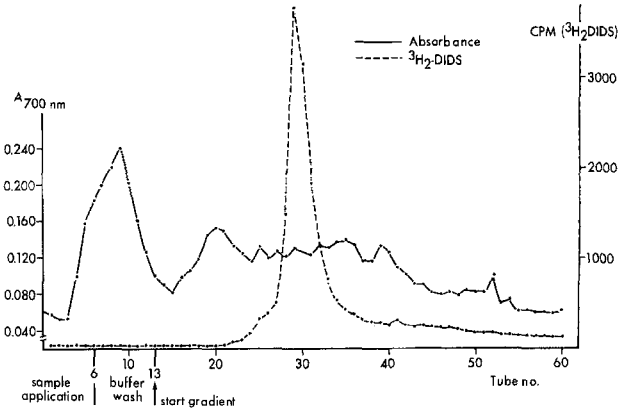

Fig. 6. Elution profile from a Cellex column of supernatant containing Triton X-100 solubilized proteins from vesicles derived from proteolysed, alkali-stripped ghosts

ton components from each other on Cellex columns by other eluting media, or by filtration techniques with sephadex columns were unsuccessful.

Under certain conditions the DIDS-binding site located in the 15,000-dalton transmembrane segment of band 3 can be cross-linked to the 35,000-dalton C-terminal segment (Jennings & Passow, 1979). If the cross-linked cells, using their procedure, were converted to leaky ghosts and retreated with chymotrypsin, there was no evidence of cross-linking of the 15,000 with the 9,000 dalton segments (which would result in the appearance of a 24,000-dalton peptide). Thus the cross-linking site of the 35,000-dalton segment is not located in the 9,000-dalton peptide.

The 15,000 and 9,000 dalton segments behave as intrinsic peptides, only extractable from the membranes with detergents or organic acids. They dissolve in SDS or in urea-SDS, but are only slowly solubilized in Triton X-100. Despite their hydrophobic behavior, their average composition is not particularly hydrophobic. The 15,000-dalton segment has numerous charged residues and only 36% of hydrophobic residues (Ramjeesingh et al., 1980). The 9,000-dalton segment is generally similar in composition (Table 1) with 25% of hydrophobic residues. Its minimal molecular weight based on amino acid composition is 9,200, about the same as the molecular weight based on gel electrophoresis. Band 3 contains about 6% of carbohydrate and is particularly rich in galactose and glucosamine, with little galactosamine. Most if not all of the carbohydrate is attached to the 35,000-dalton C-terminal portion of band 3 (Yu & Steck, 1975; Jenkins & Tanner, 1977*a*; Drickamer, 1978; Fukuda et al., 1978). Neither the 15,000 not the 9,000-dalton segments contained detectable amounts of glucosamine and thus do not seem to be major attachment sites for carbohydrate.

Discussion

The association of band 3 with the bilayer is not simple. A 40,000-dalton, N-terminal segment on the cytoplasmic side can be released as a soluble peptide by tryptic or chymotryptic digestion (Steck et al., 1976). An adjacent transmembrane segment of 17,000 daltons may be folded in the membrane so that it traverses three times (Drickamer, 1976, 1977; Williams, Jenkins & Tanner, 1979; Ramjeesingh et al., 1980). The remaining 35,000-dalton C-terminal segment has a portion exposed on the outside containing most, or all, of the carbohydrate of band 3 (Yu & Steck, 1975; Jenkins & Tanner, 1977*a*; Drickamer, 1978; Fukuda et al., 1978), but it also crosses the membrane at least once and perhaps more often, so that its cysteine residues (Rao, 1979; Rao & Reithmeier, 1979) and some sites of iodination by lactoperoxidase (Williams et al., 1979), are exposed on the cytoplasmic face. Thus the pattern of association of band 3 with the membrane involves a looping back and forth of the peptide through the bilayer. Some of the loops are closely associated. Thus under certain conditions, the 35,000-dalton segment can be cross-

linked by DIDS to the inhibitory site in the 17,000-dalton segment, suggesting that some portions of the two segments must be close neighbors. Because DIDS is a highly charged nonpenetrating probe (Lepke et al., 1976; Ship et al., 1977), the cross-linked site must be located near the outer surface of the bilayer. Further evidence of association is the finding that the 17,000 and 35,000 dalton segments after extraction with the non-ionic detergent Triton X-100, co-migrate in a centrifugal field (Reithmeier, 1979).

In the present paper it is demonstrated that the 17,000-dalton transmembrane segment can undergo further cleavage in ghosts to produce a 15,000-dalton segment that retains the DIDS binding site. The 35,000-dalton segment, resistant to further cleavage by chymotrypsin in the intact cell (Jennings & Passow, 1979) becomes sensitive in ghosts resulting in the production of a 9,000-dalton fragment. The increased sensitivity to proteolysis in the ghost may simply be due to the reduced ionic strength in the ghost preparation (Jenkins & Tanner, 1977*b*). Some of the additional cleavage sites in ghosts must be external because carbohydrate and DIDS cross-linking sites, both exposed at the external aspect of the 35,000-dalton segment, have been excised from the 9,000-dalton fragment. But the fragment retains the two sulfhydryl groups (Table 1) of the 35,000-dalton segment, both of which are exposed on the cytoplasmic side of the membrane (Rao, 1979; Rao & Reithmeier, 1979), so that it must cross the bilayer.

The 15,000 and 9,000 dalton peptides coextract in Triton X-100 in a 1:1 mole ratio and they also co-elute from a cellex column, suggesting that they are associated, as are the 35,000 and 17,000 dalton segments (Reithmeier, 1979; Jennings & Passow, 1979) from which they are derived.

The 15,000 and 9,000 dalton peptides span the bilayer and display considerable hydrophobic behavior, in that they are virtually the only peptides remaining in the membrane vesicles after Triton X-100 extraction (Fig. 5). Their amino acid composition is not, however, particularly hydrophobic in character. As reported previously (Ramjeesingh et al., 1980), the 15,000-dalton segment contains about 40% of hydrophobic residues and has many charged residues. The composition of the 9,000-dalton is somewhat similar (Table 1). It has only about 30% of hydrophobic residues. In order to rationalize their relatively non-hydrophobic composition with their relatively hydrophobic behavior, it can be assumed that they form a structure in which the hydrophobic residues are on an external face associated with the side chains of the phospholipids of the bilayer, and in which the hydrophilic residues are either internalized, or are exposed to the aqueous solution on the two sides of the membrane. As previously pointed out, such a structure could provide a protein pathway for the transport of anions through the membrane (Rothstein et al., 1976, 1980; Cabantchik et al., 1978).

Vesicles containing the 15,000 and 9,000 dalton segments as the predominant stainable peptides, are capable of anion transport activity that is inhibitable by specific inhibitors, DIDS and NAP-taurine (Rothstein et al., 1980). The inhibitors are localized in the 17,000 or 15,000 dalton segments (Grinstein et al., 1978; Knauf et al., 1978; Ramjeesingh et al., 1980). On the basis of a kinetic analysis, DIDS competes with Cl^- for the transport site (Shami et al., 1978) and NAP-taurine, when applied at the outer face of the membrane (Knauf et al., 1978), with the modifier site proposed by Dalmark (1976). These functional sites are therefore presumably located within the 15,000-dalton segment. This conclusion is subject, however, to the reservation that the covalent reaction site of the chemical probes is not the inhibiting portion of the molecule. The latter is attributed to the sulfonic acid moieties (Cabantchik et al., 1978). Thus the inhibitory site is close to but not identical with the site of covalent attachment and it might be at some distance in the primary structure of the peptide (Rothstein et al., 1980). At the present time, therefore, the functional roles of the 15,000 and 9,000 dalton segments cannot be strictly assigned. It has not yet been possible to separate these peptides in a functional state so that their separate contributions to anion transport can be evaluated.

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