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The Sulfhydryl Groups of the 35,000-dalton C-terminal Segment of Band 3 Are Located in a 9000-dalton Fragment Produced by Chymotrypsin Treatment of Red Cell Ghosts

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Abstract

Five sulfhydryl groups of band 3, the anion-transport protein of the red blood cell membrane, can be labeled by *N*-ethylmaleimide (NEM). Two of these are located in a 35,000-dalton, C-terminal segment produced by chymotrypsin treatment of cells. Extensive treatment of unsealed ghosts with chymotrypsin results in the disappearance of the 35,000-dalton segment, but its two NEM-binding sites are preserved in a 9000-dalton peptide. The latter must therefore be a proteolytic product of the larger segment. Labeling of sulfhydryl groups of band 3 by an impermeant analog of NEM occurs in inside-out, but not in right-side-out vesicles derived from red cell ghosts, supporting the conclusion that NEM-reactive sulfhydryl groups, including those in the 35,000- and 9000-dalton segments, are exposed at the cytoplasmic face of the membrane. These findings support the conclusion that the 35,000-dalton segment crosses the bilayer, and suggest that the 9000-dalton segment may be a membrane-crossing portion of the 35,000-dalton segment.

Key Words: Anion transport; band 3; sulfhydryl membrane protein; red cell; chymotrypsin.

Introduction

Band 3 is an abundant intrinsic protein of the red blood cell membrane that has been implicated in anion transport activity (Cabantchik and Rothstein, 1974a; Passow *et al.*, 1975; Cabantchik *et al.*, 1978). Because it is a transmembrane protein partially exposed on each surface of the membrane, it is susceptible to the action of proteolytic enzymes. With chymotrypsin, cleavage can occur both at the outside face of the membrane (in intact cells)

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and at the inside face (in inside-out vesicles), splitting band 3 into three domains. Two of these, a 17,000- and a 35,000-dalton segment, the latter containing the C-terminus of band 3, are retained in the membranes, but a third, of 42,000 daltons, containing the N-terminus, is not (Cabantchik and Rothstein, 1974b; Steck *et al.*, 1976; Grinstein *et al.*, 1978).

When unsealed red cell ghosts are subjected to extensive treatment with chymotrypsin, the 17,000- and 35,000-dalton peptides are not found. The predominant peptides remaining in the membranes are 15,000 and 9000 daltons (Ramjeesingh *et al.*, 1980b). The former contains the covalent binding site for DIDS (4,4′-diisothiocyano-2,2′-stilbene disulfonic acid) (Ramjeesingh *et al.*, 1980a), a specific inhibitor of anion transport that is highly localized in band 3 (Cabantchik and Rothstein, 1974a; Lepke *et al.*, 1976). It is derived from the 17,000-dalton transmembrane segment of band 3 by an additional cleavage at its cytoplasmic side (Ramjeesingh and Rothstein, 1981). The latter (9000-dalton peptide) seems also to be derived from band 3 based on its equal abundance compared to the 15,000-dalton segment (Ramjeesingh *et al.*, 1980b). Its proposed derivation from the 35,000-dalton segment is consistent with the parallelism between its appearance and the disappearance of the putative parent segment during proteolysis of ghosts (DuPre and Rothstein, 1981).

In the present study, direct evidence is presented supporting the conclusion that the 9000-dalton peptide is derived from the 35,000-dalton segment. The larger peptide contains two sulfhydryl groups that can be labeled by *N*-ethylmaleimide (NEM) and that are unusual inasmuch as they are cryptic to NEM in ghosts unless a reducing agent is present (Rao, 1979). In the present study, two sulfhydryl groups with the same unique property are demonstrated to be present in the 9000-dalton peptide.

It has been suggested that the sulfhydryl groups of the 35,000-dalton segment are exposed to the cytoplasmic compartment based on the finding that they are inaccessible to an impermeant maleimide derivative (glutathione-maleimide) in intact membranes, but are reactive in unsealed ghosts (Rao, 1979). In the present study, labeling with the impermeant derivative of NEM has been carried out using right-side-out and inside-out vesicles derived from red cell ghosts. The findings indicate that the two sulfhydryl groups of the 9000-dalton peptide are exposed only on the cytoplasmic side of the membrane. It is concluded that the 9000-dalton peptide may be a membrane-crossing portion of the 35,000-dalton segment.

Materials and Methods

N-ethyl[2,3-^{14}C]maleimide was obtained from Amersham Corporation. Ampoules of 50 μCi activity (S.A. 2–10 mCi/mmol) were stored at -20°C.

Dilutions were made from a stock solution of 100 mM *N*-ethylmaleimide in 50% ethanol and 0.005 N HCl. Each ampoule was used within 5 days of opening and dilution with unlabeled *N*-ethylmaleimide. L-[glycine-2-^{3}H]glutathione (1500 Ci/mmole) was obtained from New England Nuclear. It was used to synthesize [^{3}H]glutathione-maleimide. Bismaleimidomethyl ether was prepared according to the method of Tawney *et al.* (1961), and its monofunctional adduct, glutathione-maleimide, was synthesized according to Abbott and Schachter (1976).

Recently expired blood bank cells were washed three times in phosphate buffered saline (PBS) (150 mM NaCl, 5 mM sodium phosphate, pH 8). The cells were then washed once with 12 mM Tris-Cl, 150 mM NaCl, pH 7.4, and reacted at 50% hematocrit in the same buffer with 10 mM [^{14}C]*N*-ethylmaleimide (S.A. 0.625 Ci/mmol) for 1 hr at 37°C according to the procedure of Rao (1979). After reaction, the cells were washed three times with PBS and a fraction treated at 50% hematocrit in PBS with chymotrypsin (1.5 mg/ml) for 1.5 hr at 37°C. This treatment results in the cleavage of band 3 into two segments of 60,000 and 35,000 daltons (Cabantchik and Rothstein, 1974b; Steck *et al.*, 1976). The protease-treated cells were washed twice with PBS containing 0.5% albumin and twice with PBS. Ghosts from chymotrypsin-treated, NEM-labeled cells or untreated, NEM-labeled cells were prepared by the procedure of Steck and Kant (1974) using sodium phosphate buffer (5P8: 5 mM sodium phosphate, pH 8). Samples of ghosts from normal and proteolysed cells were dissolved in 2% sodium dodecyl sulfate in preparation for polyacrylamide gel electrophoresis. Other samples of ghosts were stripped of extrinsic peptides with 10 mM sodium hydroxide containing 0.1 mM EDTA and further proteolysed to give the 15,000-dalton and 9000-dalton peptides as described by Ramjeesingh *et al.* (1980b) using 2.5 mg of chymotrypsin per milliliter of solution with a membrane protein concentration of 2 mg/ml buffer, for 1.5 hr at 37°C (DuPre and Rothstein, 1981). Ghosts prepared as described above are unsealed, so that the proteolytic enzyme has access to band 3 exposed at both the outside and inside faces of the membrane.

In some experiments, ghosts, rather than cells, were treated with NEM. The ghosts, prepared as above from cells, or from chymotrypsin-treated cells, were reduced according to the procedure of Rao with 5% β-mercaptoethanol for 1 hr at 37°C. The ghosts were then washed three times in 5 mM sodium phosphate (pH 8) that had been deoxygenated by bubbling with nitrogen for 1 hr before use. Reduced or unreduced ghosts were then reacted with 2 mM [^{14}C]NEM for 1 hr at 37°C at a concentration of 2 mg of membrane protein per milliliter of 5 mM sodium phosphate, pH 7.0. At the end of the reaction, membranes were washed three times in 5 mM sodium phosphate, pH 8.0. Further proteolysis of the labeled ghosts to give the 15,000- and 9000-dalton peptides were done as described above.

Right-side-out vesicles from ghosts with intact band 3 and inside-out vesicles from ghosts derived from chymotrypsin-treated cells or untreated cells were prepared by the procedure of Steck (1974). The sidedness of the vesicle preparations was determined by measuring acetylcholinesterase activity in the presence and absence of Triton X-100 as described by Steck and Kant (1974). Right-side-out and inside-out vesicles were reduced by β-mercaptoethanol in a similar manner to that described for ghosts above. Right-side-out or inside-out vesicles, either reduced or unreduced, were incubated with 40 mM ^{3}H-glutathione-maleimide (S.A. 3.5 mCi/mmol) in 5 mM sodium phosphate, pH 7.0, for 1 hr at 37°C at a protein concentration of 2.5 mg protein/ml solution. At the end of the reaction period, the inside-out vesicles or right-side-out vesicles were washed three times with 5 mM sodium phosphate, pH 7.0. For further proteolysis with chymotrypsin (as described above) to produce the 15,000- and 9000-dalton peptides, the membranes were disrupted by freezing and thawing several times, allowing access of the enzyme to both sides of the membrane.

Proteins were determined by the method of Lowrey *et al.* (1951). SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) (12% acrylamide) and of Swank and Munkries (1971) (14.5% acrylamide, 10 M urea). After electrophoresis, gels were stained with Coomassie Blue and destained before slicing. Gel slices were solubilized in 0.6 ml of 30% hydrogen peroxide at 70°C overnight before counting in 6 mls of aquasol. The amount of *N*-ethylmaleimide and of glutathione-maleimide bound to band 3 was estimated by summing the radioactivity in slices corresponding to the 60,000-, 35,000-, and 9000-dalton bands in the acrylamide gels. In Table I, band 3 radioactivity is expressed as moles of reagent reacting per mole of band 3 by assuming a molecular weight of 100,000 and values of 25% and 50% respectively for the fraction of total

Table I. Numbers of NEM-Binding Sites in Band 3 and Its Proteolytic Fragments[a]

Peptide	Cells and reduced membranes	Membranes not reduced
Band 3	4.66 ± 0.28 (8)	3.2 ± 0.35 (3)
60K segment[b]	3.2 ± 0.4 (5)	2.95 (1)
35K segment[b]	1.96 ± 0.4 (5)	0.25 (1)
9K segment	1.85 ± 0.5 (9)	0.1 ± 0.18 (3)

[a]Number of determinations are in parentheses.

[b]Some ^{14}C-NEM (average of 7% of the total in five estimates) is located on the top of the gels as in Fig. 1b, due to formation of aggregates, largely of band 3 segments. The ratio of ^{14}C-NEM in the 60,000- and 35,000-dalton peaks is close to 3 to 2. Based on the assumption that the same ratio applies to the aggregated material, the ^{14}C-NEM located on the top of the gels was apportioned to the 60,000- and 35,000-dalton segments accordingly. If the ^{14}C-NEM of the aggregate was omitted from the calculation, the moles of NEM per band 3 segment would be a little lower, but the conclusions based on the data would be unchanged.

protein that constitutes band 3 in ghosts and alkali-extracted ghosts (Guidotti, 1972). The value of 50% (band 3/total membrane protein) is also assumed for inside-out and right-side-out vesicles. For the 60,000- and 35,000-dalton segments it is assumed that 1 mole of band 3 produces 1 mole of 60,000-dalton peptides and 1 mole of 35,000-dalton peptides. For the 9000-dalton peptide it is assumed that in the membranes from proteolysed ghosts the 15,000-dalton and 9000-dalton peptides account for 73 ± 3.5% of the total peptide. This average value was arrived at by scanning of six Coomassie Blue stained gels of proteolysed membranes on a Beckman Acta C11 spectrophotometer. It is consistent with a previously determined value (Ramjeesingh *et al.*, 1980b). Staining intensity is probably a reasonable measure of relative peptide content in this case, because the 15,000- and 9000-dalton peptides have been found to stain with about equal intensity. Thus the mole ratio of the two peptides, after chymotrypsin treatment of ghosts, was 1.19 ± 0.17 based on staining intensity and 1.07 ± 0.15 based on direct peptide analysis (Ramjeesing *et al.*, 1980b).

Results

The findings of Rao (1979) on the binding of NEM to band 3 were confirmed. As in her experiments, cells were treated with [^{14}C]NEM and then osmotically lysed. The ghosts were "stripped" of their extrinsic proteins by alkali extraction and their intrinsic proteins separated by SDS-polyacrylamide gel electrophoresis. A large peak of activity was found in the band 3 region of 95,000 daltons (Fig. 1A). In labeled cells treated with chymotrypsin prior to preparation of ghosts, the band 3 peak was absent but most of the [^{14}C]NEM was found in peaks of 60,000 and 35,000 daltons (Fig. 1B), corresponding to the products of a single cleavage of band 3 at the outside face of the membrane designated as P_1 in Fig. 2, top line (Cabantchik and Rothstein, 1974b; Steck *et al.*, 1976). Some of the [^{14}C]NEM did not enter the gel (the peak at the far left of the histogram). This is due to aggregation of the 60,000- and 35,000-dalton peptides which occurs to variable degrees in different preparations. The 60,000-dalton segment contains the binding site of the anion-transport inhibitor, DIDS (Cabantchik and Rothstein, 1974b); the 35,000-dalton segment contains the carbohydrate attachment site (Steck *et al.*, 1976 and the review of Gabantchik *et al.*, 1978).

If ghosts rather than cells were reacted with [^{14}C]NEM in the presence of a reducing agent (β-mercaptoethanol), the results were the same as those found for NEM-treated cells. If, on the other hand, the reducing agent was omitted during the NEM-treatment of the ghosts, the labeling in band 3 was substantially reduced in amount, due to the loss of labeling in the 35,000-

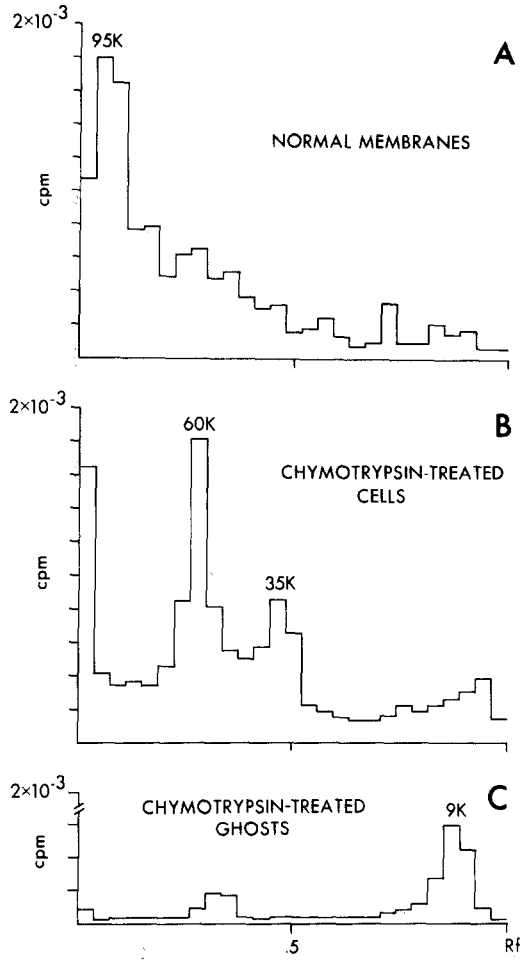

Fig. 1. Histograms of the distribution of [^{14}C]NEM radioactivity in peptides separated by electrophoresis [12% gel, Laemmeli, (1970)]: (A) membranes from cells treated with [^{14}C]NEM; (B) membranes from cells first labeled with [^{14}C]NEM followed by treatment with chymotrypsin; (C) chymotrypsin-treated ghosts prepared from cells labeled with [^{14}C]NEM. Each sample used for acrylamide gel electrophoresis contained 0.5 nmol of band 3 or of its daughter products. The histograms can therefore be directly compared for relative recovery of [^{14}C]NEM.

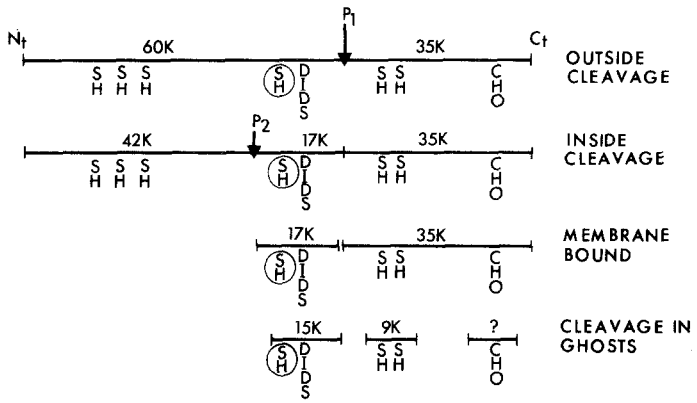

Fig. 2. A diagram to illustrate the chymotryptic cleavage sites and locations of sulfhydryl groups, carbohydrate attachment site, and DIDS-binding site of band 3. The size of the segments is given in kilodaltons and the N- and C-terminals are designated N_+ and C_+. Other details are given in the text.

dalton segment (Table I). This cryptic effect has been attributed to an internal sulfhydryl cross-linking (Rao, 1979).

If ghosts rather than cells are treated mildly with chymotrypsin, an additional cleavage occurs at the cytoplasmic side of the membrane (designated P_2 in Fig. 2) (Steck *et al.*, 1976). The 42,000-dalton, N-terminal segment becomes soluble and the membrane-bound, C-terminal segment of about 55,000 daltons (P_2 to C-terminus in Fig. 2, second line) contains the DIDS-binding site (Grinstein *et al.*, 1978). In membranes in which both sides of the membrane are exposed to chymotrypsin (the outside cleavage P_1 in cells, and the inside cleavage P_2 in inside-out vesicles made from those cells), a 17,000-dalton transmembrane segment (Fig. 2, third line) has been identified (Steck *et al.*, 1976) which contains the DIDS-binding site (Grinstein *et al.*, 1978). In such membranes the two membrane-bound segments derived from band 3 are 17,000 and 35,000 daltons, as illustrated in Fig. 2, third line.

If ghosts prepared from chymotrypsin-treated cells are then re-treated with relatively high concentrations of chymotrypsin, additional cleavages of band 3 are observed, resulting in the disappearance of the 60,000- and 35,000-dalton segments and the appearance of fragments of 15,000 and 9000 daltons (Ramjeesingh *et al.*, 1980b; DuPre and Rothstein, 1981). The same fragments are found if ghosts from unproteolysed cells are treated with chymotrypsin. The 15,000-dalton fragment retains the DIDS-binding site and must therefore be derived from the 17,000-dalton segment illustrated in Fig. 2. The 9000-dalton fragment is assumed to be derived from the 35,000-dalton segment. In the fragments derived from NEM-treated ghosts,

a large peak of labeling was found in the 9000-dalton peptide (Fig. 1C), and little or none in the 15,000-dalton segment (or elsewhere in the acrylamide gel).

A more quantitative analysis of the [^{14}C]NEM labeling pattern is presented in Table I, based on the amount of [^{14}C]NEM calculated from its specific activity and on the amount of peptide in each peak. Confirming the findings of Rao (1979), 5 mole of NEM bind to each band 3 monomer. Three of these are located in the 60,000-dalton segment, more specifically in a 42,000-dalton N-terminal cytoplasmic segment (P_2 to N-terminus in Fig. 2, second line) which is part of the 60,000-dalton segment (Rao, 1979)), and two that are cryptic in ghosts exposed to NEM in the absence of reducing agent (β-mercaptoethanol) are located in the 35,000-dalton segment. Each 9000-dalton peptide also binds 2 molecules of NEM, and as in the case of the

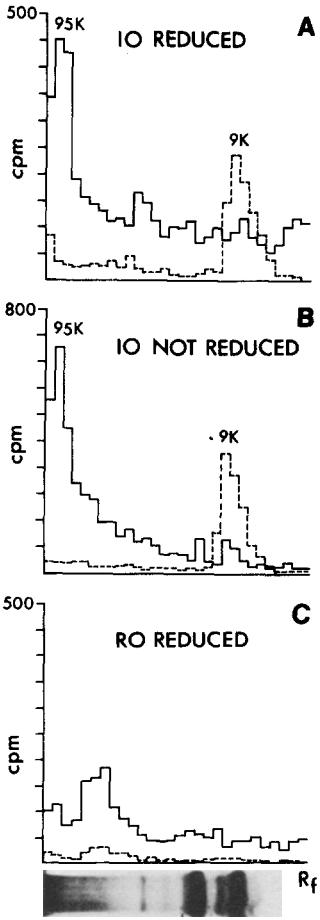

Fig. 3. Histograms of the distribution of [^{3}H] glutathione-maleimide in peptides separated by urea-SDS-polyacrylamide gel electrophoresis (Swank and Munkries, 1971): (A) inside-out vesicles labeled with glutathione-maleimide in the presence of reducing agent; (B) inside-out vesicles labeled with glutathione-maleimide with no reducing agent present; (C) right-side-out vesicles labeled with glutathione-maleimide in the presence of reducing agent. The samples used for acrylamide gel electrophoresis contained 0.125, 0.175, and 0.150 nmol of band 3 or of its daughter products for (A), (B), and (C) respectively. The solid lines in each case represent "normal" vesicles, and the dotted lines, chymotrypsin-treated, frozen and thawed vesicles. The gel pattern is for chymotrypsin-treated, frozen and thawed vesicles. The patterns were the same for right-side-out or inside-out preparations. The two heavily stained bands toward the right-hand side are the 15,000- and 9000-dalton peptides.

35,000-dalton segment, they are cryptic when prepared from ghosts that are treated with NEM in the absence of reducing agent.

Rao (1979) demonstrated that glutathione-maleimide, an impermeant agent (Abbott and Schachter, 1976), does not bind to band 3 in intact cells, but does so in unsealed ghosts. In the latter case, its distribution in band 3 is the same as that of NEM, with three sites in the 60,000-dalton segment and two in the 35,000-dalton segment. To gain further insight into the location of the binding sites in the membrane, the ^{3}H-labeled impermeant analog was applied to right-side-out and inside-out vesicles prepared from ghosts. In the case of the inside-out vesicles, band 3 and its daughter products were extensively labeled, in the same patterns and amounts as found for [^{14}C]NEM labeling of cells as illustrated in Fig. 1. In vesicles derived from "normal" ghosts, band 3 was labeled (Fig. 3A); in vesicles from ghosts derived from chymotrypsinized cells, the labeling was found in the 60,000- and 35,000-dalton segments (not shown); and in vesicles derived from chymotrypsinized ghosts, the label was found only in the 9000-dalton peptide (Fig. 3A). Inside-out vesicles, however, differed from cells or ghosts in one respect. The sites in the 35,000- or 9000-dalton peptides were labeled even in the absence of reducing agents (Fig. 3B). We have no simple explanation for this finding. In the case of right-side-out vesicles, in contrast, no significant labeling of band 3 or of any of its segments was found (Fig. 3C). A small peak of lower molecular weight than band 3 is found in the band 4.5 region. General labeling across the gel may represent sulfhydryl groups of minor peptides. Most of the labeling of the right-side-out vesicles is removed if they are treated with chymotrypsin (Fig. 3C).

Discussion

By amino acid analysis, band 3 is reported to contain six cysteine residues (Steck *et al.*, 1978). Five of these are reactive with NEM (Table I), confirming Rao's (1979) findings. Three are located in the 60,000-dalton N-terminal segment of band 3 (Table I), a product of chymotrypsin cleavage at the outer face of the membrane at P_1, as illustrated in Fig. 2. The 60,000-dalton segment can be further cleaved at the cytoplasmic face of the membrane (P_2 in Fig. 2) into a membrane-bound segment of 17,000 daltons, and a soluble N-terminal segment of 42,000 daltons (Steck *et al.*, 1976). Its three NEM-binding sites are found in the latter peptide (Rao, 1979). The 17,000-dalton transmembrane segment contains one cysteine residue (Steck *et al.*, 1978), but this particular residue does *not* react to an appreciable extent with NEM in either cells, ghosts, or inside-out vesicles. The other two NEM-reactive sites are located in the C-terminal 35,000-dalton segment. No

other peptide components account for any substantial fraction of the bound NEM.

In ghosts extensively treated with chymotrypsin, additional cleavages of band 3 occur (Ramjeesingh *et al.*, 1980b). The 42,000-dalton N-terminal segment, with its three NEM-binding sites, is solubilized from the membrane (Steck *et al.*, 1976). Thus the only substantial remaining sites of NEM binding must be derived from the 35,000-dalton segment. The 35,000-dalton segment itself disappears and the only NEM-labeled peptide is the 9000-dalton fragment (Fig. 1C). Both the 35,000-dalton (Steck *et al.*, 1978) and 9000-dalton peptides (Ramjeesingh *et al.*, 1980b) contain two cysteine residues and both bind 2 moles of NEM per mole of peptide (Table I). It seems evident, therefore, that the 9000-dalton segment is a proteolytic product of the 35,000-dalton segment. This conclusion is reinforced by the finding that the cysteine residues of the 9000-dalton segment are cryptic to NEM interaction unless the reaction of the agent with ghosts is carried out in the presence of a reducing agent (Table I), a unique property already reported for the binding sites in the 35,000-dalton segment, but not for the other NEM-reactive groups of band 3 (Rao, 1979, and Table I). These findings concerning the source of the 9000-dalton peptide are in agreement with previous evidence, of a less direct nature. First, during proteolysis, the rate of disappearance of the 35,000-dalton segment is approximately the same as the rate of appearance of the 9000-dalton segment (DuPre and Rothstein, 1981). Second, the 9000-dalton segment is present in a one-to-one mole ratio with the 15,000-dalton segment, a known band 3 daughter product (Ramjeesingh *et al.*, 1980b).

Rao (1979) has concluded that the two sulfhydryl groups of the 35,000-dalton segment are exposed on the cytoplasmic side of the membrane, based on the finding that in cells they can be labeled by the permeant agent NEM, but not by the impermeant glutathione-NEM analog (Abbott and Schachter, 1976), whereas in ghosts labeling occurs with both agents. These findings indicate that the sulfhydryl groups are not accessible to the impermeant agent from the outside of the intact membrane. They do not necessarily demonstrate that the groups are located on the cytoplasmic side of the membrane. They may be on the external side, but inaccessible to the impermeant maleimide unless the protein structure is altered by formation of ghosts or by exposure to low ionic strength. The results reported here, namely labeling of band 3 with the impermeant glutathione-maleimide in inside-out but not in right-side-out vesicles (Fig. 3), provides more definitive evidence for the cytoplasmic exposure of band 3 sulfhydryl groups, including the two located in the 35,000- and 9000-dalton peptides. Exposure of some portion of the 35,000-dalton peptide at the cytoplasmic side of the membrane has also been demonstrated by iodination procedures using lactoperoxidase (Williams

et al., 1979; Markowitz and Marchesi, 1981). Other sites on this peptide segment are known to be exposed at the outer surface, namely the site of proteolytic cleavage that forms its N-terminus (Cabantchik and Rothstein, 1974b; Steck *et al.,* 1976), and its carbohydrate attachment site (see review of Cabantchik *et al.,* 1978). These findings support the conclusion that the 35,000-dalton segment traverses the bilayer (Rao, 1979; Williams *et al.,* 1979; Markowitz and Marchesi, 1981).

After the 35,000-dalton peptide is cleaved by chymotrypsin treatment of ghosts, the 9000-dalton peptide is the only membrane-associated, daughter product identifiable by staining after SDS-urea acrylamide gel electrophoresis. Its association with the membrane appears to be largely due to hydrophobic interactions based on its detergent extractability (Ramjeesingh *et al.,* 1980b). Its insertion into the bilayer may protect it from further proteolysis, whereas exposed parts of the 35,000-dalton segment are presumably digested and solubilized. Although no other stained peptides are present in sufficient quantity to be considered as intrinsic parts of band 3 [the procedure can detect peptides as small as 1000 daltons (Swank and Munkries, 1971)], a glycopeptide has recently been detected by sugar labeling techniques that appears to contain the sugar attachment site of the 35,000-dalton peptide (Ramjeesingh, M., and Rothstein, A., unpublished observations). It forms a very broad, unstained band in the SDS-urea-acrylamide gel system, probably due to the amount and heterogeneity of its carbohydrate moiety. The size of the peptide portion has not yet been determined. Because the parent 35,000-dalton segment crosses the membrane, either or both the 9000-dalton and glycopeptide fragments must be the membrane-crossing portions. If other membrane-crossing peptides were present, they would have to be about 3000 daltons in size (Guidotti, 1977). Any such segments should be visible in the urea-SDS-acrylamide system used for separations. Unpublished studies (Ramjeesingh, M., and Rothstein, A.) based on cleavages of the 35,000- and 9000-dalton peptides at their cysteine residues indicate that the smaller peptide is located near the N-terminal end of its parent segment and that the carbohydrate-containing peptide is toward the C-terminal end, as illustrated in Fig. 2 (bottom line).

To accommodate this information and also the known exposure of the N-terminus and carbohydrate to the outside and of the two sulfhydryl groups to the inside, it is concluded that the 35,000 dalton-segment crosses the membrane at least two times, as illustrated in Fig. 4. At least one of the crossing strands would appear to be encompassed by the 9000-dalton peptide.

The 15,000-dalton segment is proposed to cross the bilayer three times, as illustrated in Fig. 4 (Ramjeesingh *et al.,* 1980a), so that the intact band 3 peptide appears to cross the membrane at least five times. Somewhat similar

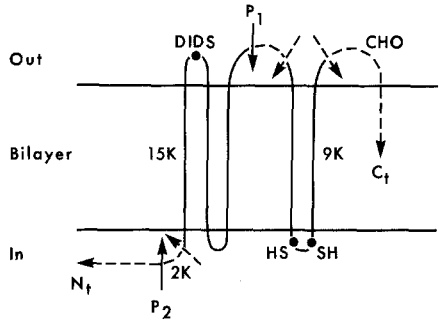

Fig. 4. A proposed arrangement of band 3 in the bilayer. P_1 and P_2 represent the external and internal chymotrypsin cleavage sites as illustrated in Fig. 2. The dotted arrows represent additional cleavage sites occurring after extensive proteolysis of unscaled ghosts. The 35,000-dalton segment is represented by the segment P_1 to C_+ and the 9000-dalton segment by the solid line segment containing the two SH (sulfhydryl) groups. Other details are given in the text.

multicrossing structures for band 3 have also been proposed by Drickamer (1980) and Tanner *et al.*, (1980). The crossing strands are probably closely associated with each other. Thus the 17,000-dalton and 35,000-dalton segments are sufficiently close neighbors to be cross-linked by the bifunctional agent DIDS (Jennings and Passow, 1979). These two segments are associated with each other after Triton-X-100 extraction (Reithmeier, 1979), as are their proteolytic products, the 15,000- and 9000-dalton segments (Ramjeesingh *et al.*, 1980b). These associated peptide fragments may therefore represent an assembly of membrane-crossing portions of band 3. This assembly may provide a protein pathway across the bilayer through which anion transport occurs (Rothstein and Ramjeesingh, 1980).

Acknowledgments

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