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PEPSIN CLEAVAGE OF BAND 3 PRODUCES ITS MEMBRANE-CROSSING DOMAINS

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After prolonged treatment of red-cell ghosts with pepsin followed by SDS-urea-acrylamide gel electrophoresis of the membrane peptide fraction, a heavily stained band representing peptides of about 4 kDa (with traces of higher molecular weights) was found. If the cells were first labelled with the disulfonic stilbene, DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid) or with *N*-ethylmaleimide, probes that react with specific sites in Band 3 the anion transport protein, both agents were largely located in the 4 kDA band. With less intensive pepsin treatment, Stained bands of about 17, 12 and 8 kDa were also visible, and DIDS labelling was associated with these higher molecular weight peptides. The 4 kDa band apparently contains at least five or six different peptides. A single peptide containing the DIDS-binding site was separated from others in the band by ion-exchange chromatography. The location of the DIDS-peptide in the primary structure of Band 3 was determined by matching the known location of DIDS and of a methionine residue cleavable by cyanogen bromide. It is concluded that two additional 4 kDA peptides are labelled with *N*-ethylmaleimide. Because the location of the *N*-ethylmaleimide-binding sites are known, these two peptides could also be mapped in the primary structure of Band 3. The findings are consistent with the suggestion that pepsin can digest those portions of Band 3 (and probably of other intrinsic peptides) that are exposed on either side of the membrane, leaving only those domains that cross the bilayer. For Band 3, the data are consistent with a structure containing five crossing strands per monomer, each crossing strand being about 4 kDa.

Introduction

Band 3, the anion transport protein of the red blood cell [1,2], can be split into three segments by proteolytic cleavages at the two sides of the cell membrane [3–5]. The C-terminal segment of about 35 kDa and an internal segment of about 17 kDa are membrane bound, whereas the *N*-terminal segment of about 42 kDa, located on the cytoplasmic side of the membrane, is soluble. The arrangement of the membrane-bound segments (17 and 35 kDa) has been the subject of considerable investigation using proteolytic and chemical cleavages combined with covalent agents that can react with the peptides from one side or the other of the bilayer. As a result of these studies, it has been proposed that Band 3 must cross the bilayer several times [6–8].

Intensive chymotrypsin treatment of ghosts, rather than intact membranes, results in additional cleavages. Two stainable Band 3 components remain in the membrane, a 15 kDa fragment derived from the 17 kDa segment [9] and and 8 kDa fragment derived from the 35 kDa segment [10].

Abbreviation: DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid.

Both the 17 [11] and 15 [9] kDa segments contain the binding site for the disulfonic stilbene, DIDS, a potent anion-transport inhibitor that competes with Cl^- for a binding site essential for transport [12]. It has been proposed that the 15 kDa peptide crosses the bilayer three times [6–8,13]. Both the parent 35 kDa [14,15] and the daughter 8 kDa segments [15] contain two cysteine residues that can bind *N*-ethylmaleimide. It has been proposed that the 8 kDa peptide crosses the bilayer twice [15].

Treatment of red-cell ghosts with thermolysin [7,16] or pepsin [16,17] results in additional cleavages of Band 3 and a somewhat different array of end-products. In testing the effects of more intensive pepsin treatment, it was found that further proteolysis of Band 3 could be attained. Neither the 35, 17, 15 nor 8 kDa peptide was found except in trace amounts. In fact, the only end-products found in substantial amounts were all of about 4 kDa. It proved possible to separate a DIDS-containing 4 kDa peptide from other 4 kDa components by ion-exchange chromatography at low pH. The former was found to contain one of the two methionine residues of the 17 or 15 kDa peptides, cleavable by cyanogen bromide [13,18]. Because the locations of the DIDS-binding site and the methionine residues are already known, it was also possible to map the location of this pepsin-produced DIDS-containing 4 kDa peptide (called DIDS-4K for convenience). The results suggest that it is a bilayer-crossing segment. Furthermore, additional information suggests that pepsin can cleave all of those peptide domains that are exposed at either side of the membrane. The residual membrane-bound 4 kDa peptides may, therefore, represent a series of bilayer-crossing domains, including those labelled with *N*-ethylmaleimide. These findings support the proposed structure for Band 3 involving multiple crossing of the bilayer.

Methods

DIDS and tritiated dihydroDIDS ($^3H_2$DIDS) were synthesized from their diamino analogs as previously described [1]. The tritiation of the H_2DIDS precursor (H_2DADS) was carried out by New England Nuclear. *N*-Ethyl[2,3-^{14}C]maleimide was obtained from Amersham. Ampoules of 50 μCi activity (spec. act. 2–10 mCi/mmol) were stored at −20°C. Dilutions were made from a stock solution of 100 mM *N*-ethylmaleimide in 50% ethanol/0.005 M HCl. Cyanogen bromide and *N*-bromosuccinimide were obtained from Aldrich Chemical. Pepsin was obtained from Sigma. Cellex-D, having an exchange capacity of 0.80 mequiv./g, was obtained from Bio-Rad.

CNBr and *N*-bromosuccinimide cleavages were done as previously described [13]. Proteins were determined by the method of Lowry et al. [19]. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Swank and Monkries [20] (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 hydrogen peroxide and counted in 10 ml of aquasol. The amount of label in Band 3, 15 and 4 kDa bands was estimated by summing the radioactivity in slices to the corresponding band in the gel. Moles of reagent reacting for a mole of Band 3 was arrived at by assuming a molecular weight of 100000 for Band 3 and values of 25% and 50%, respectively, for the fraction of total protein that constitutes Band 3 in ghosts and alkali extracted ghosts [21]. For the 15 kDa peptide, it is assumed that in the membranes from proteolysed ghosts, the 15 and the 8 kDa peptides account for $73 \pm 3.5\%$ of the total peptide, and that the mole ratio between the two is essentially 1.0 [9]. for the pepsin-proteolysed ghosts, it is assumed that the 4 kDa peptides account for 90% of the stainable membrane protein. This value was arrived at by scanning four Coomassie blue-stained gels of the proteolysed membrane on a Beckman Acta C11 spectrophotometer. Molecular weights of the proteins and peptides were determined by migration in the urea-SDS gels using peptide molecular-weight standards from British Drug House (myoglobin, 16949; myoglobin I and II, 14404; myoglobin I, 8159; myoglobin II, 6214 and myoglobin III, 2512). Calibrations against peptides of known molecular weight in the 2–17 kDa range are reported to be linear by BDH (Bulletin on Molecular Weight Markers) and Swank and Munkries [20]. We have confirmed the linearity using many of the same standards. We have also reported a close correspondence in molecular weights determined by the

gel electrophoresis procedure and by amino-acid compositions for 15, 8, 4 and 2 kDa peptides derived from Band 3 by proteolytic or chemical cleavage [9,13]. Molecular-weight estimates based on this procedure must be considered to be approximate.

Recently expired blood bank cells were washed three times in phosphate-buffered saline (150 mM NaCl/5 mM sodium phosphate (pH 8)). The washed cells were labelled at 25% haematocrit with $^3H_2$DIDS by exposure to 12 μM of the probe for 30 min at 37°C [13]. The cells were then washed twice with phosphate-buffered saline/0.5% albumin to remove any unreacted DIDS and twice with ordinary phosphate-buffered saline. Leaky ghosts were prepared by the procedure of Dodge et al. [22] using sodium phosphate buffer (5 mM sodium phosphate, pH 8.0). Ghosts were labelled with *N*-ethylmaleimide as previously described [15]. Chymotrypsin treatment of ghosts was done as previously described [9] using 2.5 mg chymotrypsin per ml of the above mentioned pH 8.0 sodium phosphate buffer with a membrane protein concentration of 2 mg per ml buffer for 1.5 h at 37°C.

Pepsin treatment of ghosts

Ghosts labelled with DIDS or *N*-ethylmaleimide and with or without prior chymotrypsin treatment were washed with 40 vol. of ice-cold 0.1 M acetic acid. The acid-stripped pellet was resuspended to a protein concentration of 1 mg/ml of water which was then acidified to pH 3.0 with 2 M HCl, and pepsin was added at a concentration of 1.5 mg/ml. The exposure to acidity leads to vesiculation of the ghost membranes (observable by phase contrast microscopy). In order to ensure access of the pepsin to both sides of the membrane, the vesicle suspension was sonicated for 10 s and then incubated at 37°C for 0.5 h (Cell Disruptor Model W140, Heat Systems Ultrasonic). The membrane was then neutralized with 1 M ammonium bicarbonate to pH 7.0, spun down and washed twice with 10 mM Tris-acetate (pH 7.4), with two 5-s sonications between washes.

The procedure outlined above differs from the previously reported pepsin treatment [16], in use of HCl rather than acetic acid, higher pepsin concentrations, longer times of exposure and the use of sonication.

Separation of DIDS-4K on Cellex-D column

Cellex D washed according to the manufacturer's recommendation was suspended in 10 mM Tris-acetate (pH 7.4)/0.2% triton X-100 and poured into a 10 ml plastic column. The pepsin-treated $^3H_2$DIDS-labelled ghosts prepared from 20 ml of red blood cells were dissolved in 20 ml of 10 mM Tris/0.2%Triton X-100 buffer and applied to the column. The column was first washed with 20 ml of this buffer and then with an acetic acid/acetate pH gradient generated with 0.2 M acetic acid/0.1% Triton X-100 and 0.2 M sodium acetate/0.1% Triton X-100. After the pH gradient, a further wash with 20 ml of 0.2 M acetic acid/0.1% Triton X-100 was done. Fractions collected were assayed for protein and counted for radioactivity.

Other procedures

For electron microscopy, ghost were pepsin treated as described above. After the acid had been neutralized with ammonium bicarbonate, the pellet was spun down and washed twice. The washed pellet was examined by negative staining and by fixation and sectioning.

Right-side-out vesicles were prepared from ghosts by the method of Steck and Kant [23].

For end-group analysis of the 4 kDa peptide fraction, ghosts were treated with pepsin as described above. The washed pellet of membrane vesicles was delipidated by extraction with 20 ml chloroform/methanol (1 : 1). The procedure removes virtually all of the phospholipid (less than 0.1% remains) as measured by phosphate analysis [24]. The extract contained no peptides that were stainable after urea-SDS-acrylamide gel electrophoresis. The 4 kDa band contained in the pellet was separated by urea-SDS-acrylamide gel electrophoresis as described above. The urea used in the procedure was deionized using a column of mixed-bed Dowex resins. The 4 kDa band was cut out of the gel and the end-groups were determined in a Beckman model 890C automated amino acid sequencer. The phenylthiohydantoinamino acid derivatives were identified by gas chromatography using a Hewlett Packard 5700A gas chromatograph.

Results

As previously reported [9,10], after chymotrypsin treatment of ghosts, peptides of 15 and 8 kDa are the end-products of Band 3 proteolysis, as illustrated by the stained bands after separation of the peptides by SDS-urea acrylamide electrophoresis of the dissolved membranes. The 15 kDa segment contains a single binding site for the anion-transport inhibitor, DIDS [13], and the 8 kDa segment contains two binding sites for the sulfhydryl reagent, *N*-ethylmaleimide [15]. In contrast, after proteolysis for 30 min with pepsin, a predominant band at about 4 kDa was seen with small amounts of higher molecular weight peptides (Fig. 1). Most of the DIDS and *N*-ethylmaleimide is located in the 4 kDa band (Fig. 2c and d). After shorter periods of pepsin exposure, stained bands of about 8, 12 and 17 kDa were evident (Fig. 1) and larger amounts of DIDS was associated with them (Fig. 2a and b). These results suggest that during pepsin treatment a series of cleavages occurs in Band 3, with the ultimate product(s) being 4 kDa, and with intermediate products being 17, 12 and 8 kDa. This finding, and the fact that some of the more heavily stained intermediate products have molecular weights that are approximately multiples of 4 kDa, indicate that the 4 kDa band must contain not only a DIDS-labelled peptide, but others as well, including *N*-ethylmaleimide-labelled peptides. This supposition is supported by quantification of the ratio of DIDS to peptide. In intact Band 3 [25,26] or in the 17 [11] or 15 [9] kDa peptides, one DIDS is present per peptide. In the 4 kDa band, on the other hand, the ratio of DIDS (by radioactivity) to peptide is closer to 1 : 6 (average of three estimates). Because of difficulties in determining the peptide contents in gels, the values in this case are based on the amount of peptide applied to the gels and the proportion of staining found in the 4 kDa band (see Methods). The numbers obtained must, therefore, be considered only approximate. A more quantitative procedure is described below.

In previous studies, DIDS-containing peptide segments were found to be separable from non-labelled peptides at low pH under conditions where

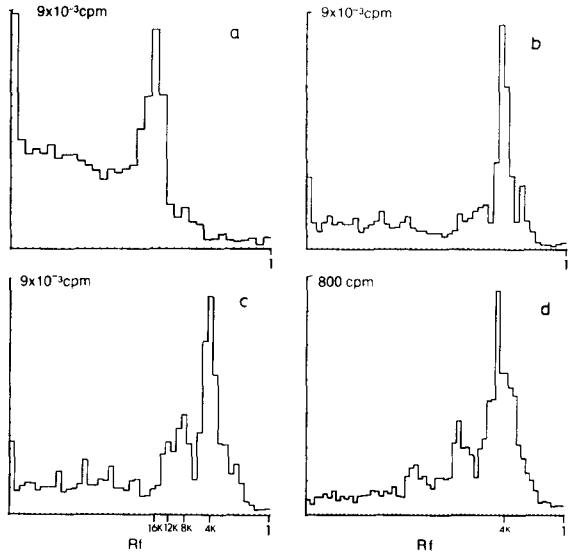

Fig. 2. The [$^3H_2$]DIDS and *N*-ethyl[^{14}C]maleimide-distribution in membrane peptides after pepsin treatment. The peptides were separated by SDS-urea acrylamide gel electrophoresis. Exposure of membranes to pepsin was for (a) 5, (b) 10 and (c) 30 min in the case of DIDS, and (d) 30 min in the case of *N*-ethylmaleimide (NEM). Molecular weights are indicated in kilodaltons.

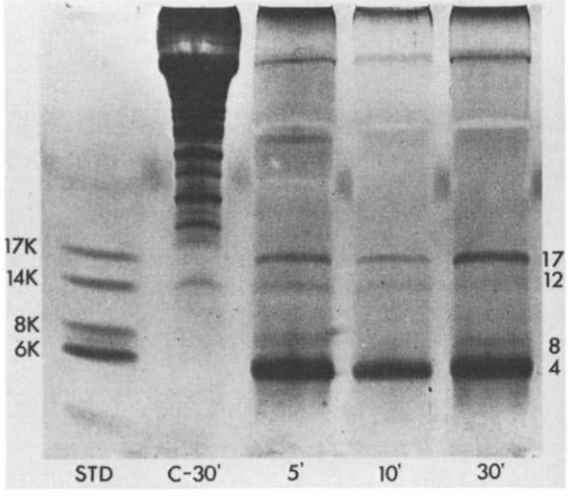

Fig. 1. SDS-urea-acrylamide gel electrophoretic peptide patterns. The left gel illustrates to location of molecular-weight standards (listed in Methods). The second gel represents the peptide pattern of unproteolysed membranes (60 μg peptide applied). The right band gels, labelled 5, 10 and 30, represent the peptide patterns of membranes treated with pepsin for the indicated times (min) (35 μg peptide applied to each gel). The heavily stained bands are about 4 kDa.

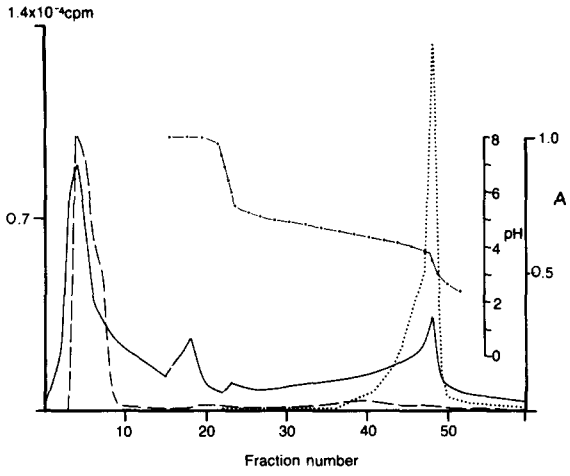

Fig. 3. Separation of [$^3H_2$]DIDS and *N*-ethyl[^{14}C[maleimide-labelled peptides on a Cellex D column using a pH gradient. Peptide content of the fractions was determined by the procedure of Lowry et al. [19]. (———) Peptide (A); (— — —) *N*-ethylmaleimide (cpm); (· · · · · ·) DIDS (cpm) and (-·-·-·-) pH.

the only negatively charged groups were the sulfonic acid residues of the DIDS [13]. A modified procedure using a anion-exchange resin (see Methods) was successful in the present study. Fig. 3 illustrates the elution of peptides using a pH gradient. A substantial amount of DIDS-free but *N*-ethylmaleimide-labelled peptide is eluted at the front, and a smaller amount of peptide containing almost all of the DIDS is retarded, eluting at low pH. As expected, acrylamide gel electrophoresis of each of the two fractions revealed a predominant 4 kDa band (by staining). In both the *N*-ethylmaleimide- and DIDS-labelled fractions the probes were largely located in the 4 kDa region. The ratio of DIDS to peptide (assuming a molecular weight of 4000) indicated that a single 4 kDa peptide fragment containing one DIDS binding site had been separated. The larger peptide peak (not retarded by the resin) contained 5.56 ± 0.73 (five estimates) as much peptide as the DIDS-containing (retarded) peak. Thus pepsin hydrolysis apparently results in the production of a number of 4 kDa fragments, one of which is labelled with DIDS. The two *N*-ethylmaleimide-binding sites are preserved during pepsin treatment, so two of the 4 kDa peptides may be labelled with *N*-ethylmaleimide. In this regard, it should be noted that the *N*-ethylmaleimide-labelled 4 kDa peak is somewhat broader than the DIDS-labelled 4 kDa peak (Fig. 2, right panels). This finding suggests that one *N*-ethylmaleimide-labelled peptide may be somewhat larger than 4 kDa and the other, somewhat smaller. The possibility of one peptide containing two *N*-ethylmaleimide-binding sites is, therefore, less probable.

Estimates of the number of peptides in the 4 kDa band were also attempted by determining the number and amounts of N-terminal amino acids in the fraction by standard amino acid analysis (see Methods). This approach was not particularly successful. Only small amounts of amino acids were released (amounting to yields of only a few percent), insufficient for quantitation. In three separate analyses, 3–5 amino acid residues were definitely identified, including valine, proline, phenylalanine, isoleucine and alanine. These tentative data support the conclusion that the 4 kDa fraction contains several peptides, and are con-

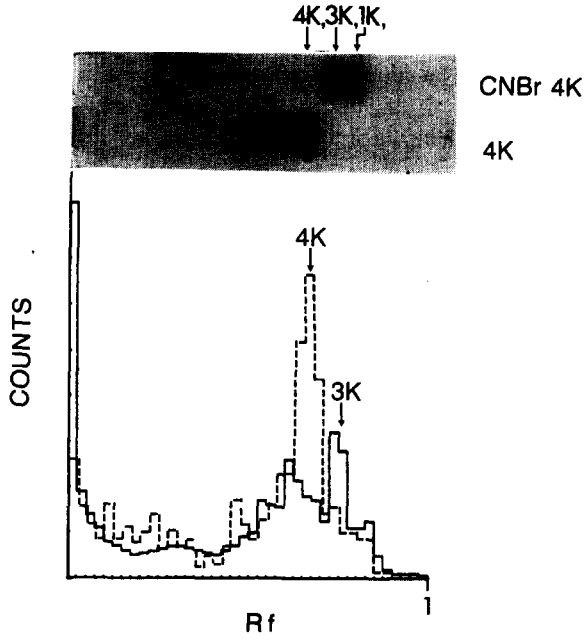

Fig. 4. Cyanogen bromide cleavage of the [$^3H_2$]DIDS-labelled 4 kDa peptide produced by pepsin treatment of membranes. At the top are stained gels of the 4 kDa peptide and its CNBr cleavage products (separated by SDS-urea acrylamide gel electrophoresis). The diagram represents the [$^3H_2$]DIDS location in the d kDa peptide (- - - - -) and its CNBr cleavage products (———). The size of the peptide fragments is indicated in kilodaltons.

sistent with the estimates noted above, using the ratio of total peptide to DIDS-labelled peptide.

The DIDS-containing 4 kDa peptide fragment (DIDS-4K), on analysis, was found to contain one methionine residue. The parent 17 kDa segment has two, whose locations have been mapped by cleavage with cyanogen bromide [27]. The DIDS-4K peptide (taken from the ion-exchange column) was subjected to cyanogen bromide treatment. It was found to cleave into two fragments of about 3 and 1 kDa (Fig. 4). The larger one contains the DIDS label. During the cleavage a homoserine lactone ring is produced at the newly formed C-terminus. This structure was located on the larger fragment. This finding indicates that the larger, DIDS-containing fragment contains the original N-terminus of the parent DIDS-4K peptide.

The use of pepsin to treat membranes requires acid solutions of about pH 3 (using acetic or hydrochloric acid). The exposure to acidity leads to a vesiculation of the ghosts that can be observed by phase-contrast microscopy. Vesiculation also occurs when ghosts are extracted with alkali [28] or digested with trypsin [29] or chymotrypsin [9]; the ghost disappears and on centrifugation the membranes can be recovered as a pellet of vesicles. An electronmicroscopic examination of the pepsin-pellet after fixation and sectioning revealed apparently intact vesicles, in appearance and size, indistinguishable from those derived from ghosts by other procedures such as alkali extraction.

To test whether the bilayer retains its permeability characteristics under acid conditions, right-side-out vesicles were prepared and were subjected to pepsin digestion at low pH. Intact right-side-out vesicles have a relatively low density and float on top of a dextran gradient. After pepsin treatment at pH 3, only 10–20% of the vesicles floated, the remainder forming a pellet. Thus, the treatment does disturb the permeability properties of the membrane. That fraction of pepsin-treated vesicles that were intact (floated on the gradient) were solubilized in detergent and the peptides assessed by acrylamide gel electrophoresis. In addition to the 4 kDa peptides, considerable amounts of 8, 12 to 14 kDa and some larger peptides were found (not shown). Under similar conditions, with access of pepsin to both sides of the membrane, the predominant peptides are 4 kDa (Fig. 1). Because of the low yield of low-permeability vesicles and because some fraction is inside-out rather than right-side-out, it is not possible to attribute the effects of pepsin to a particular side of the membrane.

Discussion

Of the numerous proteolytic enzymes such as trypsin, chymotrypsin, papain, pronase and thermolysin that have been used to digest Band 3 in membranes [3–5,7,16,17,30–32], pepsin produces the most cleavages. Where the membrane-bound products of the other enzymes include 8, 15, 17, 35 and 55 kDa pepsin produces primarily 4 kDa products. At least 5–6 peptide fragments are produced based on the ratio of total peptide to DIDS (DIDS binds quantitatively to a single site in Band 3, so its amount can be used to denote a single 4 kDa peptide).

The pepsin proteolysis of Band 3 in the present experiments was more extensive than that reported by Tanner et al. [16]. They reported six end-products, including peptides of 35, 8, 16 and 17 kDa and larger peptides as well (estimated in gels containing urea, as in the present study). DIDS was localized (by its fluorescence) in the 17, 16 and to some degree in the 35 kDa bands. Our results with intermediate levels of pepsin proteolysis (Figs. 1 and 2), and with intact vesicles are consistent with those of Tanner et al. [16]. The more complete hydrolysis, to products of about 4 kDa, results from more intensive pepsin treatment combined with sonication of the membrane pellet that ensures access of the pepsin to both sides of the membrane.

It seems highly unusual that all of the end products of pepsin-digestion should have approximately the same molecular weight. A possible explanation is that the 4 kDa peptides represent the membrane-crossing domains of Band 3 and other intrinsic membrane proteins. The 4 kDa size is consistent with this hypothesis, for it has been calculated that a minimum of 3.2 kDa of peptide is required to cross the bilayer in α-helical form, the preferred structure of peptide in a hydrophobic domain [33]. Furthermore, the abundance of the predominant intrinsic membrane proteins also provides supporting information. for each cell

there are over a million copies of Band 3 [25,26,30,31] and 500000 of glycophorin [34]. The number of copies of Band 4.5, the putative sugar transporter, is not well determined, but estimates of around 300000 have been reported [35]. Band 3 and glycophorin are postulated to have five [36,37] and one [34] membrane-crossing domains, respectively. Little is known about the organization of Band 4.5, but it must cross the membrane at least once [38]. If the proposed five crossing peptides of Band 3 are present in the 4 kDa band (one of them labelled with DIDS), then the ratio of unlabelled to DIDS-labelled peptide would be 4:1. The actual experimental value was 5.5. Thus, five crossings of Band 3 can be accounted for, one for glycophorin and perhaps several for Band 4.5 as well, based on their lesser abundance in the membrane.

Using the information developed in this paper, and the already known locations of the DIDS- and *N*-ethylmaleimide-binding sites and other markers in the primary structure of Band 3, it is possible to map the tentative locations of its five membrane-crossing domains. They presumably are located within the intrinsic (membrane bound) domains of Band 3. These have been identified as stainable

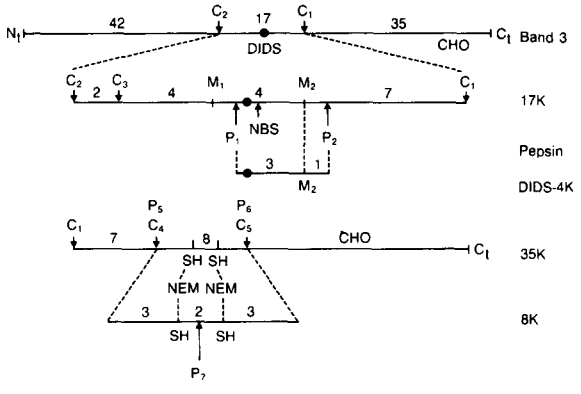

Fig. 5. Proposed maps of the locations of various sites in the primary structure of Band 3 and its 17, pepsin-DIDS-4, 35 and 8 kDa segments. The N- and C-termini are indicated by N_t and C_t. The chymotrypsin cleavage sites are indicated by C_1, C_2, etc., and the pepsin by P_1, P_2, etc.; M_1 and M_2 designate methionine residues cleavable by CNBr, and SH, cysteine residues reactive with *N*-ethylmaleimide (NEM), NBS indicates the cleavage site for *N*-bromosuccinimide (NBS); CHO, the carbohydrate attachment site; and DIDS (●) the DIDS-binding site. The numbers indicate the sizes of the peptide fragments in kilodaltons.

DIDS-labelled 15 and *N*-ethylmaleimide-labelled 8 kDa segments resulting from extensive chymotrypsin treatment of ghosts [9,10]. The locations of these two segments have already been determined. Band 3 can be divided into three segments of 42, 17 and 35 kDa based on mild chymotrypsin treatment at the outside and inside faces of the membrane [4,30,31]. The cleavages occur at C_1 and C_2 (Fig. 5, top line). The 42 kDa *N*-terminal segment (N_t to C_2) is soluble and will not be further considered. The 17 kDa segment (C_1 to C_2) contains the DIDS-binding site [11] (marked ○). With more extensive proteolysis by chymotrypsin (in ghosts), the 17 kDa segment is cleaved at C_3 (Fig. 5, second line), giving rise to the 15 kDa segment [18]. The 35 kDA C-terminal segment (C_1 to C_t) contains the carbohydrate attachment site and two sulfhydryl groups that can bin *N*-ethylmaleimide [14]. It is the parent segment from which the 8 kDa segment is derived by further cleavages with high concentrations of chymotrypsin (in ghosts) [9,10] (Fig. 5, bottom lines).

A detailed map of the 17 (and 15) kDa segments is presented in Fig. 5. If the two methionine residues (M_1 and M_2) are cleaved by cyanogen bromide, the 4 kDa peptide between them contains the DIDS-binding sites [13,27] *. This segment of peptide can be further cleaved by *N*-bromosuccinimide into two fragments of about 2 kDa each, the DIDS being located in the one between M_1 and the *N*-bromosuccinimide cleavage site, as indicated. Given these facts, it can be concluded that the pepsin-produced DIDS-4K must contain the methionine marked M_2 (Fig. 5, third line), and that the pepsin cleavages must occur at the points marked P_1 and P_2. This location of the pepsin produced DIDS-4K segment can account for its cyanogen bromide cleavage pattern. As noted, it is cleaved into fragments of 3 and 1 kDa (Fig. 4), with the DIDS and the newly formed C-terminus both located in the larger frag-

* One, rather than two, cyanogen bromide cleavages has recently been reported [39], at a location which appears to be the same as M_1 (Fig. 5, line 2). The second cleavage at M_2 requires more intensive cyanogen bromide treatment (higher concentrations and longer times).

ment. The alternative assumption that the pepsin-DIDS-4K contains the methionine marked M_1 is not a viable option, because the newly formed C-terminus could not then be on the same peptide fragment as the DIDS. It would be on the smaller of the two fragments.

It has been proposed that the 17 (15) kDa segment crosses the bilayer three times [13,36], as illustrated in Fig. 6. In this scheme, the pepsin-DIDS-4K segment would be the crossing strand between the two pepsin cleavage sites, P_1 and P_2, at the inside and outside loops. Two additional pepsin-produced 4 kDa peptides would be derived from the segments between P_1 and C_3 (the N-terminus of the 15 kDa segment), and P_2 and C_1 (the C-terminus of the 15 kDa segment). These segments are about 5 and 6 kDa, so it must be assumed that pepsin cleavages occur at P_3 and P_4 which result in reduction of length to about 4 kDa in each case.

It has been proposed that the 8 kDa segment forms a loop through the membrane (Fig. 6) with its two sulfhydryl groups (*N*-ethylmaleimide-binding sites) exposed at the inside face of the membrane and with the two chymotrypsin cleavage sites (C_4 and C_5) at the outside face [15,37]. Its location in the primary structure of Band 3 and in its parent 35 kDa segment determined by cleavages at cysteine residues [37] is illustrated in Fig. 5 (bottom lines). It is suggested that pepsin also produces and 8 kDa segment by cleavages close to C_4 and C_5 (designated P_5 and P_6). An additional cleavage at P_7 on the inside loop between the two

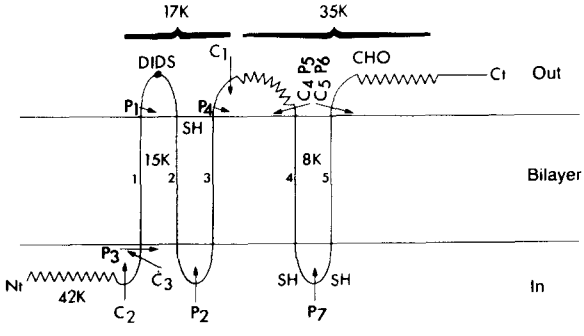

Fig. 6. A proposed arrangement of band 3 in the bilayer, adapted from Ref. 34. The symbols are as in Fig. 5. The numbers 1–5 represent the proposed 5 membrane-crossing peptide domains released by pepsin treatment.

sulfhydryl groups would produce two 4 kDa segments each containing one sulfhydryl group (*N*-ethylmaleimide-binding site). This proposed location is supported (but not proven) by two sets of data. First, as noted in results, the *N*-ethylmaleimide-labelled 4 kDa peak is distinctly broader than the DIDS-labelled peak. This finding can be explained if two *N*-ethylmaleimide-labelled were present, of somewhat different molecular weights (but approx. 4000). If one peptide were present containing both *N*-ethylmaleimide-binding sites, the *N*-ethylmaleimide-labelled peak would be expected to be about the same breadth as the DIDS-labelled peak. Second, if the pepsin cleavage site were not between the sulfhydryl groups, the products of cleavage would be distinctly unequal in size, about 3 and 5 kDa (Fig. 5, bottom line). Differences of this magnitude should be visible after separation by urea-SDS-acrylamide gel electrophoresis as two distinct peaks. No such double peak was found. If the assumptions are correct, the data support the view that two peptides of about 4 kDa are labelled by *N*-ethylmaleimide.

The analysis described above can account for five membrane-crossing peptides (labelled 1–5 in Fig. 6). It can also account for the appearance during pepsin proteolysis of intermediate peptides of about 17–15, 12 and 8 kDa (Figs. 1 and 2). The former could arise by cleavages at P_3 or P_4, and the smaller segments by additional cleavages at either P_1 or P_2, or release of the sulfhydryl-containing 8 kDa segment by cleavages at P_5 and P_6. It is suggested that pepsin digests and solubilizes all exposed portions of Band 3 and probably of other intrinsic peptides, leaving the bilayer, protected from proteolysis, all membrane-crossing peptides of about the same molecular weight (4 kDa) (labelled 1–5 in Fig. 6). Pepsin may therefore be a useful agent in examining the associations of other intrinsic proteins with bilayers.

Acknowledgement

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