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THE LOCATIONS OF THE THREE CYSTEINE RESIDUES IN THE PRIMARY STRUCTURE OF THE INTRINSIC SEGMENTS OF BAND 3 PROTEIN, AND IMPLICATIONS CONCERNING THE ARRANGEMENT OF BAND 3 PROTEIN IN THE BILAYER

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The intrinsic domains of band 3 protein contain three cysteine residues, one in a 17 kDa middle segment and two in a 35 kDa C-terminal segment. The latter are retained in an 8 kDa fragment produced by chymotrypsin treatment of ghosts. Cleavage of cysteine residues by 2-nitro-5-thiocyanobenzoic acid (NTCB) allows localization of this amino acid in the primary structure of the 8, 17, 35 and 52 (17 plus 35) kDa segments of band 3 protein. The mapping of these residues taken with other information concerning accessibility of various sites at the two sides of the membrane leads to the conclusion that band 3 protein crosses the membrane at least five times, or ten times in a dimer structure. The implications of this conclusion in terms of band 3 protein structure and function are briefly discussed.

Introduction

Band 3 protein, the anion transport protein of the red blood cell, can be subdivided into three domains based on proteolytic cleavages at the two sides of the membrane [1–3]. The N-terminal segment of 42 kDa, produced by cleavage at the cytoplasmic side of the membrane is soluble. Its removal does not diminish the capacity for transport [4,5]. The other segments, 17 and 35 kDa, produced by a second cleavage at the outside of the membrane, are membrane-bound [1–3]. Each has been demonstrated to traverse the bilayer [1,6]. Both are apparently required for transport function [7,8].

Structural information related to the arrangements of the intrinsic segments of band 3 proteins in the membrane has been largely derived from use of proteolytic enzymes and of covalently reacting inhibitory probes applied to either the outside or cytoplasmic faces of the membrane, and by application of chemical cleaving agents directly to peptide segments. For example, the 17 kDa segment can be further cleaved by chymotrypsin treatment of ghosts to 15 kDa segments [9]. Both the 15 and 17 kDa segments contain two methionine residues cleavable by cyanogen bromide (CNBr). From the cleavage patterns, it has been possible to locate the binding site for 4,4′-diisothiocyano-2,2′-stilbene sulfonic acid (DIDS), a specific inhibitor of anion transport, within the primary structure of the peptide segments [10,11]. Furthermore, because DIDS can only interact with its binding site from the outside of the membrane, it can be inferred that this domain of band 3 protein is folded in a particular way within the bilayer [10].

Less is known about the arrangement of the 35 kDa segment. It contains the C-terminus of band 3

Abbreviations: CNBr, cyanogen bromide; DIDS, 4,4′-diisothiocyano-2,2′-stilbene sulfonic acid; NTCB, 2-nitro-5-thiocyanobenzoic acid; PMSF, phenylmethylsulfonyl fluoride.

protein [1]; the attachment site of the carbohydrate of band 3 protein, exposed at the outer face of the membrane [1]; and two sulfhydryl groups, reactive with *N*-ethylmaleimide, exposed at the cytoplasmic face of the membrane [6]. When 'leaky' ghosts are treated with high concentrations of chymotrypsin at low ionic strength the 35 kDa segment undergoes further cleavages [9]. Two membrane bound fragments have been identified, one of undetermined molecular weight containing the carbohydrate of band 3 protein [8], and one of about 8 kDa * containing the two sulfhydryl groups (cysteine residues) of the parent 35 kDa segment [12]. The 15 and 17 kDa segments also contains one cysteine residue [1,9], but it is cryptic to reaction with *N*-ethylmaleimide [12].

The present paper reports the use of the agent, 2-nitro-5-thiocyanobenzoic acid (NTCB) to cleave the cysteine residues of the 15 and 35 kDa segments, and of the 8 kDa fragment derived from chymotrypsin treatment of the latter. The results allow conclusions to be made concerning (a) the location of the three sulfhydryl groups in the primary structure of the intrinsic portions of band 3 protein; (b) the location of the 8 kDa fragment within its 35 kDa parent segment; (c) the approximate location of the sugar attachment site; and (d) the arrangement of the 35 kDa peptide within the bilayer.

Materials and Methods

Trypsin (TPCK treated) was purchased from Sigma. Non-radioactive DIDS and tritiated dihydro-DIDS ($^3H_2$DIDS) were synthesized from their diamino analogs as previously described [13]. 2-Nitro-5-thiocyanobenzoic acid (NTCB) was purchased from Eastman Kodak. [^{14}C]NTCB was prepared by the method of Degani and Patchornick using K^{14}CN [14]. Molecular weight standards for gel electrophoresis were obtained from British Drug House (44247 2L).

Recently outdated human red blood cells were used for all experiments. Cells were separated from plasma by centrifugation and washed three times with 4 vol. of phosphate-buffered saline (PBS: 150 mM NaCl, 5 mM sodium phosphate, pH 8) at 4°C. Some cells at 25% haematocrit were labelled with 10 μM $^3H_2$DIDS for 1 h at 37°C. Excess $^3H_2$DIDS was removed by washing the cells once with phosphate buffered saline containing 0.5% bovine serum albumin (Sigma Chemical) and twice with phosphate-buffered saline. For membrane preparation, labelled or unlabelled cells were lysed against 30 vol. 5 mM phosphate, pH 8 (5P8) and washed three times with the same solution until white.

Gel-electrophoresis. Electrophoresis was carried out using SDS-urea-acrylamide gels (0.1%, 8 M, 15%) by the method described by Swank and Munkries [15], followed by staining using either Coomassie blue or silver [16]. In the former case about 50 μg of peptide was added to each gel and in the latter, 15 μg. For the radioactive profiles of gels, the gels were sliced into 0.5 mm slices, incubated at 80°C for 3 h in 0.8 ml of 30% hydrogen peroxide and counted with 10 ml of aquasol.

The urea-gel system can retain peptides as small as 1 kDa and can be used to approximate molecular weights by comparison with calibration curves using peptide standards of known size. Swank and Munkries [15] used nineteen different standards. We used a set of six standards provided by British Drug House (BDH 44247 2L) that covers the range 17000 to 1360. Fig. 5 of the paper illustrates the separation of the six peptide standards on the gel system. The relationship between mobility and log of molecular weight is linear. Although the molecular weight determinations must be considered approximate, their general validity is confirmed by independent determinations (based on amino-acid composition) for two of the fragments of band 3 used in this study (the 15 and 8 kDa peptides [9,10]), and also by the finding that the molecular weights of the cleavage segments produced from the 8 and 15 kDa segments add up approximately to the molecular weights of the parent peptides. By these criteria the estimates of molecular weights of fragments of band 3 protein

* In previous publications [8,9] the fragment was assigned a mass of 9 kDa based on approximate estimates of mobility in urea-SDS-acrylamide gel electrophoresis. Based on more precise calibrations of the gels used in the present study, the molecular weight of the segment is found to be closer to 8000. In this paper, it will be referred to as the 8 kDa fragment.

using the gel procedure might be deviant by about 15%.

Preparation of the 35 kDa segment. Intact red cells at 50% haematocrit were digested with chymotrypsin 0.1 mg/ml, at 25°C, overnight in phosphate-buffered saline. The enzyme was inhibited by addition of phenylmethylsulfonyl fluoride (PMSF, Sigma) to 100 μg/ml final concentration and the chymotrypsin was removed by washing twice with phosphate-buffered saline containing 0.5% bovine serum albumin and twice with phosphate-buffered saline alone. The cells were then lysed as above and peripheral membrane proteins were removed from the membrane by washing once with 10 volumes of 0.1 N Na(OH) at 0°C and twice with 5 mM phosphate, pH 8. Solubilization of the extracted membranes and purification of the 35 kDa peptide from the mixture were carried out using the method of Markowitz and Marchesi [17]. Purified fractions were characterized by gel electrophoresis and staining with silver. Only those fractions showing a single band were used for the cleavage reactions.

Preparation of the 52 kDa segment. The peptide was prepared essentially by the method of Markowitz and Marchesi [17]. The fractions were also run on urea-SDS gels and silver stained to check for purity, as above. Based on analysis of the N-terminal amino acid, the segment is about 95% homogeneous.

Preparation of the 15 and 8 kDa fragments. The fragments were prepared and purified by extraction from urea-SDS-acrylamide gels as previously described [10].

The NTCB cleavage reaction. NTCB cleavage of the cysteine peptide bond occurs in two stages (Fig. 1). The first stage is derivatization of the cysteine residue to form a thiocyanate intermediate. This intermediate upon subsequent exposure to higher pH, undergoes as internal cyclization reaction to cleave the N-peptide bond of the modified residue giving one peptide with a newly formed C-terminus and the other peptide with an N-terminus blocked by a 2-iminothiazolidinyl ring structure [14]. If radioactive NTCB is used, the incorporation of radioactivity occurs at the derivitization stage and upon cleavage, the radioactivity ends up on the peptide with the blocked N-terminus. Because a β-elimination reaction occurs in parallel, that competes effectively with the internal cyclization cleavage reaction (Fig. 1), cleavage is incomplete and a mixture of all possible intermediate peptides are usually present.

NTCB cleavage of the 52 and 35 kDa segments. Each peptide in column buffer was dialysed exhaustively against 0.01% SDS using Spectrapor dialysis tubing No. 2 (mol. wt. cut-off 12000). Subsequently, the solution was made up to 5% β-mercaptoethanol and incubated for 3 h at 37°C and then freeze-dried. Fresh Tris-acetate buffer, pH 8.5, (100 mM Tris/3 M urea/5 mM dithiothreitol/0.2% SDS) prepared with deoxygenated water by bubbling nitrogen for at least 1 h, was added to the freeze-dried protein sample to give a final protein concentration of 0.5 mg per ml. NTCB dissolved in methanol was added to give a 50 mM final concentration of NTCB and 10% or less of methanol. The pH was quickly readjusted to 8.5 with Na(OH) and derivitization was allowed to proceed at room temperature for 1 h. The protein solution was readjusted to pH 9.5 and incubated at 37°C overnight. The reaction mixture after exhaustive dialysis against 0.01% SDS using spectrapor dialysis tubing NO. 3 (mol. wt. cut off 3500), was freeze-dried, redissolved in urea-SDS gel solubilizer for gel electrophoresis.

NTCB cleavage of the 15 and 8 kDa segments. Each peptide was dissolved in 0.2% SDS to give a protein concentration of 1.0 mg/ml. An equal volume of buffer (200 mM Tris-acetate, pH 8.5/6 M urea/20 mM dithiothreitol/0.2% SDS) was added and sample incubated for 1.5 h at 37°C. NTCB dissolved in methanol was added to give a final concentration of 50 mM in NTCB and 10% or less of methanol. The pH was quickly readjusted to 8.5 with NaOH and incubated at room temperature for 0.5 hour. The sample was readjusted to pH 9.0 and incubated at 37°C overnight. The mixture, after exhaustive dialysis against 0.01% SDS using Spectrapor dialysis tubing No. 3, was freeze-dried, redissolved in urea-SDS gel solubilizer for gel electrophoresis.

Concanavalin A labelling of polyacrylamide gels. Coomassie blue stained gels containing the 35 kDa segment or NTCB treated 35 kDa segment, were soaked for 3 h at room temperature in phosphate-buffered saline buffer containing haemoglobin, 1 mg/ml. The gel was then placed in fresh phos-

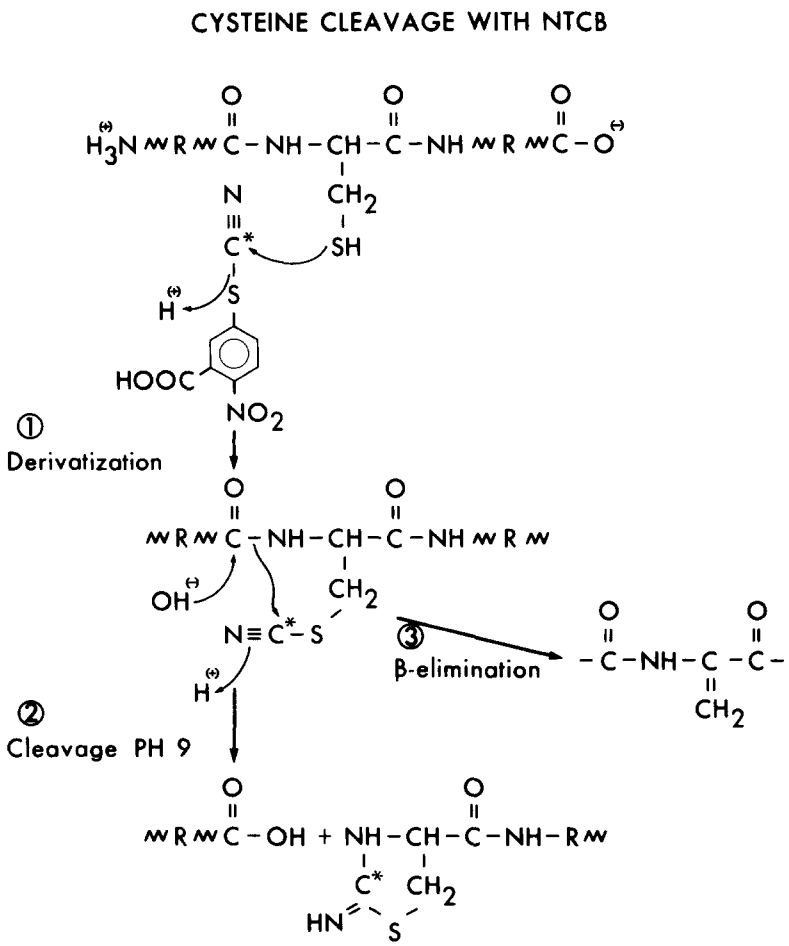

Fig. 1. Diagram of reactions of NTCB at cysteine residues of peptides.

phate-buffered saline-haemoglobin buffer containing 5 μg/ml of ^{125}I-labelled concanavalin A (100 μg containing $2.3 \cdot 10^6$ cpm of ^{125}I). After incubating for 4 h at room temperature, the gel was rinsed once and placed in 4 litres of phosphate-buffered saline with gentle agitation overnight before slicing and counting for radioactivity.

Results

As an aid in the presentation of the results, the three domains of band 3 protein, based on chymotrypsin cleavages at the two sides of the membrane, are illustrated in the diagram of Fig. 2: (a) an N-terminal, cytoplasmic, soluble, 42 kDa segment, containing three sulfhydryl groups; (b) a middle, transmembrane 17 kDa segment, containing one sulfhydryl groups, the DIDS attachment site, and another cleavage site at C_3 (to produce a segment of 15 kDa); and (c) a C-terminal, transmembrane 35 kDa segment, containing two sulfhydryl groups and the carbohydrate attachment site.

One of the objectives of the study was to determine the locations of the three cysteine residues of the intrinsic portions of band 3 protein (the 17 and 35 kDa segments), using NTCB as a cleaving agent. Unfortunately the use of this agent involves certain complications that are outlined in the Methods. First, the NTCB cleavages are incomplete and second, labelled reagent is inserted into both the cleaved (at the new-N-terminus), and uncleaved cysteine residues [14], as illustrated in Fig. 1. For this reason, a segment with one cy-

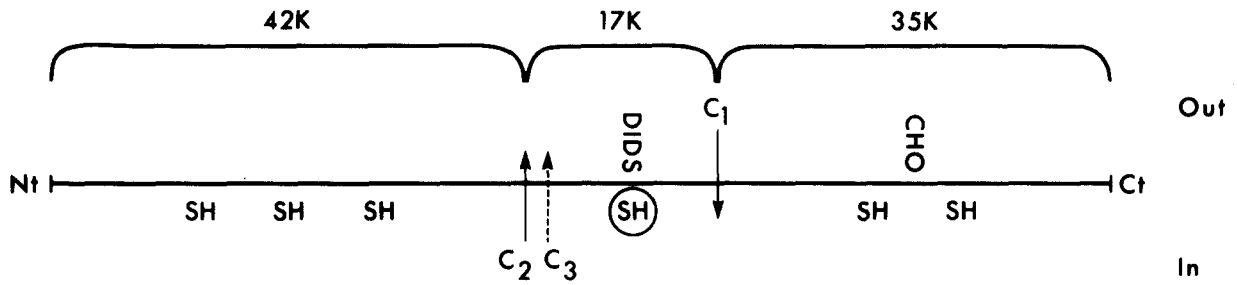

Fig. 2. Domains of band 3 protein based on chymotrypsin cleavage at the two sides of the membrane. The ouside and inside cleavage sites are disignated C_1 and C_2. An additional cleavage site (occurring in ghosts) is designated C_3. The N- and C-termini are designated N_t and C_t; the sulfhydryl groups by SH; the DIDS-binding site by DIDS; and the carbohydrate attachment site by CHO. The location of various sites with respect to the outside or inside faces of the membrane are indicated. The information is taken from Refs. 1, 2, 3, 5, 6, 9, 10 and 12.

steine residue should give rise to uncleaved but labelled peptide, an unlabelled fragment containing the original N-terminus, and a labelled fragment containing the new N-terminus (and the original C-terminus). A peptide with two cysteine residues would be expected to give rise to five products plus uncleaved peptide, all labelled except the one containing the original N-terminus. Three of the segments would result from complete cleavage, whereas two of the segments would each contain one uncleaved cysteine residue. Despite these complications, the locations of the cysteine residues were determinable by comparing cleavage patterns of several of the intrinsic segments, including the 15 kDa peptide (C_3 to C_1, Fig. 2), the 35 kDa peptide (C_1 to C_t), the 52 kDa peptide (C_2 to C_t) and the 8 kDa peptide (resulting from intensive chymotrypsin treatment of the 35 kDa peptide).

A. NTCB cleavage of the 15 kDa, DIDS-containing segment

After NTCB treatment of the 15 kDa segment, the products were subjected to urea-SDS-acrylamide gel electrophoresis. Three stained bands were found at 15, 11 and 4 kDa (Fig. 3). The former represents the uncleaved peptide, and the smaller segments the products of a cleavage at the single cysteine residue. Most of the NTCB label is located in the 4 kDa region with little in the 11 kDa region, indicating that the smaller fragment contains the newly formed N-terminus (see Methods), and that the larger fragment contains the original N-terminus. A small amount of count is found in the 15 kDa band, representing incorporation of NTCB into the cysteine residue (without cleavage). The DIDS is located in two peaks, one at 15 kDa, representing uncleaved peptide, and the second at 11 kDa, representing the cleavage fragment containing the original N-terminus of the parent segment.

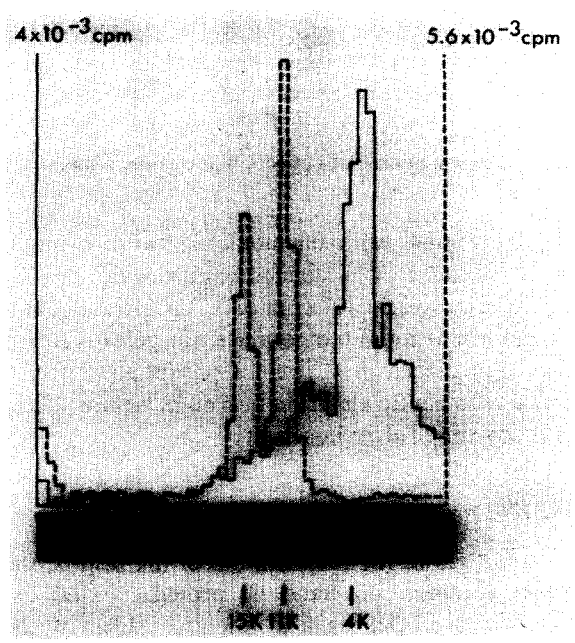

Fig. 3. NTCB cleavage of the 15 kDa segment. Solid line, [^{14}C]NTCB labelling. Broken line, DIDS labelling. Insert: Coomassie blue-stained peptides in SDS-urea gels. Details of labelling and cleavage procedures are given in Methods.

On the basis of the observations reported above, the cysteine residue is presumed to be located 4 kDa from the C-terminus of the 15 kDa segment. Its proposed location in relation to other previously identified sites, in the 15 (and 17 kDa) dalton segment is illustrated in Fig. 4.

B. NTCB cleavage of the 8 kDa segment

The 8 kDa segment contains two cysteine residues [12]. After cleavage by NTCB and separation of the products on urea-SDS-acrylamide gels, four distinct bands were found of approximately 7.5, 4.6, 2.9 and 1.7 kDa (Fig. 5). The profiles of radioactivity (not shown) indicate that all of the bands contain some labelled NTCB. Quantification of labelling was not, however, feasible because of the considerable overlap in the labelling pattern. Control studies without NTCB treatment indicate that the 7.5 kDa band represents uncleaved peptide, so the other three bands must represent cleavage products.

The expected number of cleavage products for a peptide with two cysteine residues is five (see above), rather than the three that were actually found. The apparent discrepancy can be explained as follows. It is proposed that the smallest fragment (1.7 kDa) lies between the two cysteine residues, and that the N- and C-terminal fragments are about the same size, 2.9 kDa (illustrated in Fig. 6, bottom section). This conclusion is based on the following considerations.

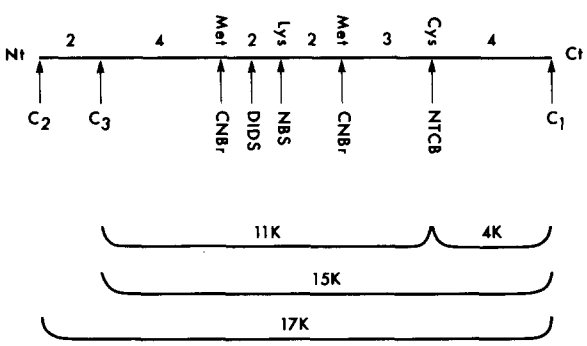

Fig. 4. Location of the cysteine residue and of other sites in the 17 kDa segment. The location of the other sites are based on Refs. 10 and 11. Chymotrypsin cleavage sites are indicated by C_1, C_2 and C_3; the N- and C-termini by N_t and C_t; the chemical cleavage sites by CNBr, *N*-bromosuccinimide (NBS) and NTCB; the DIDS-binding site by DIDS; and the methionine, lysine and cysteine residues by Met, Lys and Cys. The numbers indicate the sizes of the various segments in kDa.

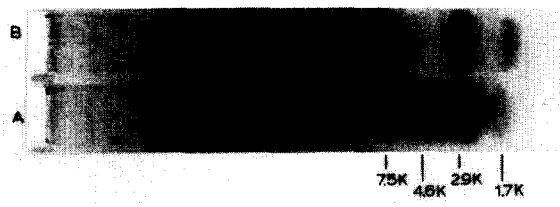

Fig. 5. NTCB-cleavage products from the 8 kDa segment separated by SDS-urea gel electrophoresis. (A) Cleavage products. (B) Molecular weight markers. They are from left to right 16949, 14404, 8159, 6214, 2512 and 1360. Staining is with Coomassie blue.

(a) Neither SH group can be very close to the N- or C-termini of the 8 kDa peptide, because in either case only two stainable NTCB-cleavage products would be found.

(b) The smallest fragment (1.700 kDa) must be a product of complete cleavage (both cysteine residues). The 2.9 kDa band appears to contain about twice as much peptide as the 1.7 kDa band based on its heavier staining and its somewhat broader profile (obvious from visual inspection and confirmed by densitometric scanning). It is presumed to contain the other two completely cleaved fragments, both being the same size. One of these, containing the original N-terminus, would be unlabelled, but the other containing a newly-formed N-terminus would be labelled. Thus, the absence of an unlabelled band (containing the original N-terminus) is explained.

(c) The 4.6 kDa band is also presumed to contain two peptide fragments, each with one uncleaved cysteine residue. Each would contain one of the 2.9 kDa fragments covalently linked to a 1.7 kDa fragment. This conclusion involves the corollary that the 1.7 kDa peptide, a presumed constituent of both 4.6 kDa segments, must be located between the two cysteine residues as illustrated in Fig. 6. If, for example, a 2.9 kDa peptide were located between the cysteine residues, then one cysteine would have to be located between two 2.9 kDa residues. If uncleaved by NTCB, a peptide of 5.6 kDa would be seen. No peptide of this size was found.

(d) The only fragment that must be common to both the 8.0 kDa peptide and the 35 kDa segment

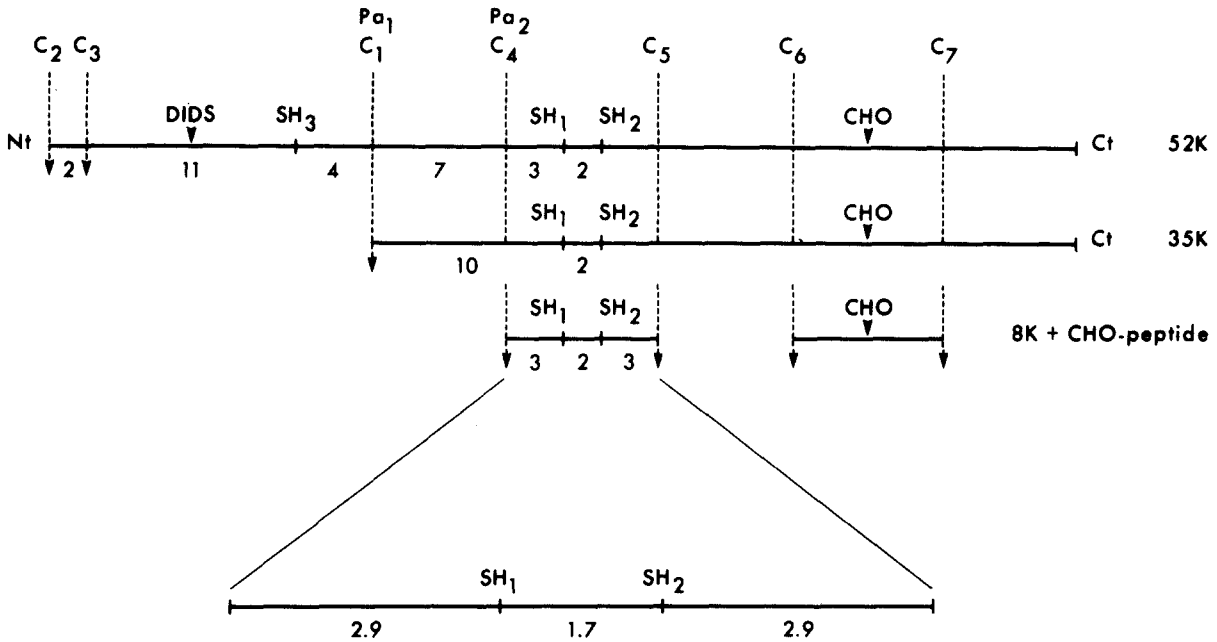

Fig. 6. Proposed locations of cysteine residues in various segments of band 3 protein produced by chymotrypsin treatment. The N- and C-termini are designated N_t and C_t; chymotrypsin and papain cleavage sites by C_1 to C_7, and Pa_1 and Pa_2; the DIDS-binding site by DIDS; the carbohydrate attachment site by CHO; and the cysteine residues by SH_1 to SH_3. The numbers indicate the sizes of the various segments in kDa.

from which it is derived, is the one located between the two cysteine residues, proposed to be the 1.7 kDa fragment. A peptide of this size was released by NTCB cleavage of the larger segment (see next section). It was the only matching fragment.

C. NTCB cleavage of the 35 kDa segment

The behaviour of the 35 kDa segment during acrylamide gel electrophoresis is influenced by the amount and heterogeneity of its carbohydrate. It forms a broad, relatively poorly stained band, whose molecular weight cannot be assessed with any accuracy from its mobility [1–3]. In Fig. 7A, it has been located in the gels by exposure to the lectin, ^{125}I-labelled concanavalin A, which binds to its sugar residues. Its treatment with NTCB resulted in a low yield of cleavage products (probably due to the carbohydrate). Nevertheless, after treatment with NTCB, the concanavalin A binding component was definitely shifted to a somewhat lower molecular weight range but with considerable overlap with the untreated sample. Part of the overlap is presumed to be due to a fraction of the 35 kDa peptide that is uncleaved by NTCB. The molecular weight of the cleaved carbohydrate containing fragment cannot be determined precisely, but it is relatively large (20–30 kDa). No low molecular weight carbohydrate containing components were evident.

Because cleavage yields from NTCB treatment of the 35 kDa segment are relatively low, the cleavage products were difficult to assess on the gels by staining procedures. They could however be readily detected by the radioactivity of the incorporated [^{14}C]NTCB. Three labelled areas were observed in all cases, a relatively broad area in the high molecular weight region (about 25 000 to 35 000) and somewhat sharper peaks centered at 13 000 and 2000. The relative amounts of labelled peptides in the 13 and 2 kDa peaks varied considerably from sample to sample, but with an apparent reciprocal relationship, that is, when the 13 kDa peak was high, the 2 kDa peak was low and vice versa. Three examples are illustrated in Figs. 7B–D. The interpretation of these results is as

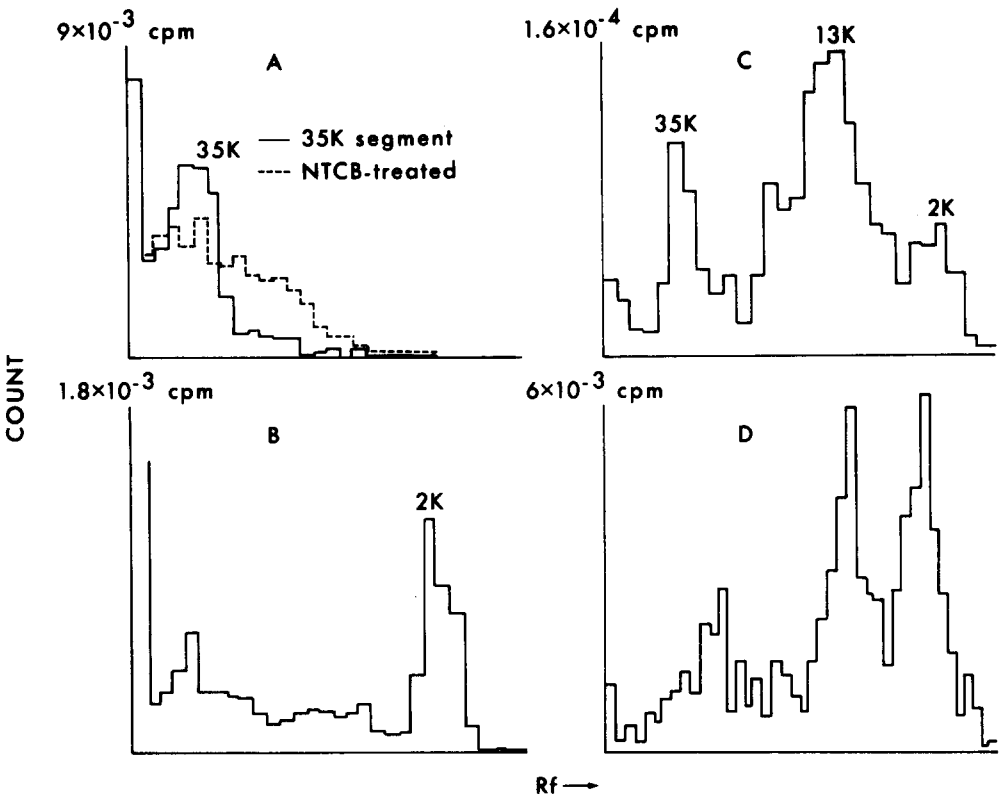

Fig. 7. NTCB-cleavage products from the 35 kDa segment separated by SDS-urea gel electrophoresis: (A) location of concanavalin-A binding components; (B to D) location of [^{14}C]-NTCB binding components in three different experiments. The numbers in (C) indicate the sizes of the various segments in kDa.

follows: (a) the broad higher molecular weight peak, in the same location in the gels as the concanavalin A binding components, is assumed to represent uncleaved (but labelled) 35 kDa peptide plus cleaved, labelled, glycopeptide fragments (SH_1 to C_t and SH_2 to C_t in Fig. 6); (b) as noted previously, it is assumed that the 2 kDa fragment is located between the two cysteine residues (the same fragment as released by NTCB cleavage of the 8 kDa segment); (c) the 13 kDa fragment is taken to result from the cleavage of a single cysteine (SH_2 in Fig. 6), representing the peptide between N_t to SH_2, with its radioactivity being that inserted into the uncleaved cysteine residue at SH_1; and (d) the reciprocal variability in labelling of the 13 and 2 kDa fragments is assumed to arise from variability in the NTCB cleavage versus incorporation reactions (Fig. 1). If incorporation at SH_1 predominates, then little labelled 2 kDa fragment (SH_1 and SH_2) will be released, but the amount of labelled 13 kDa fragment (N_t to SH_2) will be high (as in the top panel of Fig. 7B). If, on the other hand, the cleavage at SH_1 is relatively high, relatively larger amounts of the labelled 2 kDa fragment will appear and smaller amounts of labelled 13 kDa peptide will be seen (as in the lower part of Fig. 7). The 11 kDa product (N_t to SH_1) released by cleavage at SH_1 will not be labelled (its N-terminus is not produced by the cleavage reaction) and is threfore not detected.

D. NTCB cleavage of the 52 kDa segment

The proposed locations of the sulfhydryl groups illustrated in Fig. 6 were further tested by NTCB cleavages of the 52 kDa segment (C_2 to C_t in Fig. 2). The cleavages are more effective for this segment than for the 35 kDa segment, so that the

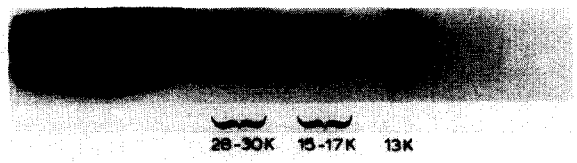

Fig. 8. NTCB cleavage products from the 52 kDa segment (C_2 to C_t in Fig. 2) separated by SDS-urea-gel electrophoresis. The sizes of the fragments are indicated in kDa. Staining is with silver.

products are visible as stained bands after gel-electrophoresis. The segment contains three cysteine residues, two located in the 35 kDa portion (C_1 to C_t) and one in the 17 kDa segment (C_2 to C_1). The latter should be located 13 kDa from the N-terminus of the 52 kDa peptide (see Fig. 4).

According to the scheme of Fig. 6, it would be predicted that after NTCB cleavage of the 52 kDa peptide, bands should be found at 2 kDa (SH_1 to SH_2); 13 kDa (N_t to SH_3); 16 kDa region (SH_3 to SH_1, and SH_3 to SH_2); 30 kDa region (N_t to SH_1, and N_t to SH_2); and broad peaks of higher molecular weight containing the sugar attachment site. These predictions are met. Stained bands of appropriate mobilities are illustrated in Fig. 8. The 2 kDa band is lightly stained and does not show up in the gel of Fig. 8. It can be seen, however, in gels overloaded with respect to the other peptides (not shown). The only band that does not fit the predicted pattern is a lightly stained one between the 28–30 kDa region and the heavily stained material toward the top of the gel. Also as predicted the DIDS is located in the 13 kDa peak (and in uncleaved 52 kDa peptide) and the carbohydrate label is in a broad zone in the higher molecular weight region at the top of the gel (not shown).

Discussion

Band 3 protein contains six cysteine residues [1], three in the cytoplasmic, soluble, N-terminal segment (C_2 to N_t in Fig. 2) [6] and three in the 52 kDa membrane-bound portion (C_2 to C_t). Of the latter, two are located in the C-terminal, 35 kDa glycopeptide segment. Both are reactive with *N*-ethylmaleimide and both are exposed on the cytoplasmic side of the membrane [6,12]. The third, located in the 17 kDa segment [1] does not react with *N*-ethylmaleimide [6]. Its crypticity may be due to its internal location within the bilayer.

The NTCB cleavage patterns described in the results indicate that the three cysteine of 52 kDa segment are located at approximately 13, 27 and 29 kDa from its N-terminus (Fig. 6). The latter two are located in the 35 kDa, C-terminal segment (C_1 to C_t) about 10 and 12 kDa from its N-terminus. The same two cysteines are also present in the 8 kDa fragment produced by further cleavages of the 35 kDa segment using intensive chymotrypsin treatment of ghosts, about 3 to 5 kDa from the N-terminus. By aligning the sulfhydryl groups of the 35 and 8 kDa peptides as in Fig. 6, it is possible to locate the N- and C-terminal ends of the smaller fragment at C_4 and C_5, 7 and 15 kDa from the N-terminus of the parent 35 kDa peptide. The carbohydrate attachment site of the 35 kDa segment must, therefore, be located towards its C-terminal end, between C_5 and C_t. After chymotrypsin treatment of ghosts carbohydrate assumed to be derived from band 3 protein is found in a smaller, membrane bound, glycopeptide [8]. The cleavage sites involved in its production are designated C_6 and C_7 in Fig. 6, but their exact location, and the size of the peptide fragment are not known at this time.

Certain of the identified peptide sites are known to be exposed, on one side or the other of the membrane. These include the DIDS binding site [5], the external chymotrypsin cleavage site (C_1) [18], and the carbohydrate attachment site on the outside [1], and the chymotrypsin cleavage sites C_2 and C_3 [11], and the two sulfhydryl groups, SH_1 and SH_2 at the inside [6,12]. The chymotrypsin cleavage sites C_4, C_5, C_6 and C_7 are produced in leaky ghosts and cannot be designated in terms of sidedness [9]. Other evidence, however, suggests that C_4 may be located at the outer surface of the membrane. It has been reported that papain treatment of intact cells results in one cleavage close to C_1 (Pa_1). There is, however, a second cleavage within the 35 kDa segment. As a result a carbohydrate containing segment of about 25 to 30 kDa is recovered [7]. After papain cleavage of cells, followed by formation of ghosts and treatment with chymotrypsin, the 8 kDa peptide is recovered intact (unpublished observations). These observations suggest that the second papain cleavage site

is close to C_4 (Pa_2). Because it occurs during papain treatment of intact cells, it can be inferred that C_4 (Pa_2) is exposed at the outer face of the membrane. It has previously been proposed [10,19,20] that the 17 kDa segment (C_1 to C_2) crosses the bilayer three times (illustrated in Fig. 9). The present observations can also be accommodated if it is assumed that the 35 kDa peptide crosses the bilayer at least twice. It is proposed that the 8 kDa fragment provides the two membrane crossing elements as illustrated in Fig. 9. The basis for this assumption is the following:

(a) The only membrane bound fragments remaining after intensive chymotrypsin treatment of ghosts are the 8 kDa fragment and a carbohydrate containing fragment of low but undetermined molecular weight [9] (designated CHO-peptide in Fig. 6). They presumably provide the membrane-crossing peptide domains.

(b) There must be one crossing between C_1, the external chymotrypsin cleavage site (the N-terminus of the 35 kDa peptide) and SH_1, known to be located on the cytoplasmic side [6,12]. The only available lipid-bound peptide is the N-terminal fragment (C_4 to SH_1) of the 8 kDa fragment. This fragment is 3 kDa long, about the correct length (as an α helix) to cross the bilayer.

(c) This conclusion is consistent with the proposed location of the second, external, papain cleavage site (Pa_2) close to C_4 (see earlier discussion), thereby designating C_4 (Pa_2) to SH_1 as a membrane-crossing peptide fragment.

(d) A second membrane crossing must occur between SH_2 which is cytoplasmic and the carbohydrate attachment site, which is external. The C-terminal part of the 8 kDa peptide (SH_2 to C_5) is 3 kDa, about the correct size to cross the bilayer. It is also possible that the CHO-peptide portion (C_5 to C_t) may provide the second crossing element, or may provide additional membrane crossings.

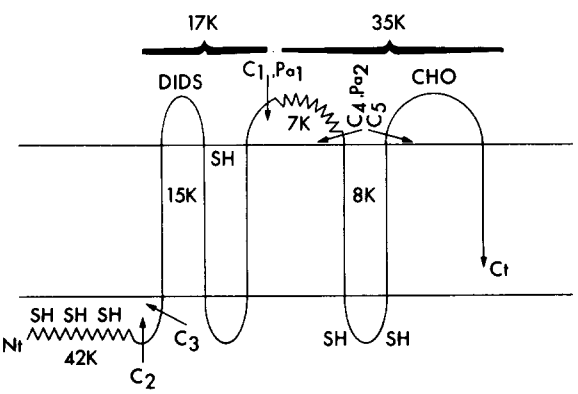

Fig. 9. Proposed arrangement of band 3 protein in the bilayer. A similar diagram is used in a brief review [24].

In the diagram of Fig. 9 each monomer of band 3 protein is presumed to cross the bilayer at least five times. Several observations suggest that the crossing strands exist as an assembly:

(a) The 15 kDa (C_3 to C_1) and 8 kDa peptides are not particularly hydrophobic in terms of amino acid composition (41 and 36%), nor are smaller cleavage fragments of the 15 kDa peptide [21]. Numbers of charged residues are present. Yet they behave like hydrophobic peptides, only extractable from the membrane by detergents or concentrated organic acids. These two apparently inconsistent observations have been explained by assuming that the crossing strands are present in an assembly in which the charged and hydrophilic residues are internalized to form an aqueous core (through which transport occurs) and in which the hydrophobic residues are arranged around the periphery associated with the fatty acid side chain of the phospholipids of the bilayer.

(b) The 17 and 35 kDa peptides [22], or the 15 and 8 kDa peptides [9] remaining after intensive chymotrypsin treatment, are associated with each other (are not separable) in non-ionic detergent.

(c) Part of the 35 kDa peptide is a close neighbor of part of the 17 kDa peptide, as indicated by a cross linking reaction of the anion transport inhibitor, DIDS [7].

(d) Several kinds of evidence suggest that both the 17 and 35 kDa segments are essential elements of the anion transport mechanism, and that they therefore must be closely associated in a functional structure [7,8,23,24].

In addition to intermolecular interactions between membrane-crossing elements within each monomer, a number of studies indicate that band 3 protein exists in the bilayer as a dimer structure [1–3], and that the dimer associations exist between the membrane bound segments [22]. For a dimer structure, an assembly of ten or more membrane crossing strands would be present.

Certain features of the assembly have been dis-

cussed elsewhere [25]. Thus each monomer has one DIDS-binding site [2,3,7]. In the dimer structure, the binding of bulky DIDS analog to one protomer of band 3 protein impedes the access to the second [26], and binding of DIDS reduces the covalent binding of eosin-isothiocyanate (which appears to bind the same site) by 50% [27]. These findings are consistent with data suggesting that the two DIDS sites of each dimer are 28 to 40 Å apart [26]. The DIDS sites are exposed to the outside medium [5], yet data on energy transfer suggest that they are located about 40 Å from the inner surface of the membrane or less than the thickness of the bilayer [28]. It has therefore been suggested that these sites lie within a cleft or pit in the band 3 protein structure.

From a functional point of view, DIDS appears to compete with Cl^- for binding to the anion transport sites [29]. Furthermore, each transport site of band 3 protein dimers appear to behave independantly [3]. It should also be noted that the aqueous interior of the band 3 protein assembly cannot be an open channel, but that it must have a diffusion barrier to account for the high electrical resistance of the membrane and for the fact that anion transport is predominantly a one for one, electroneutral exchange [2,3]. A number of models have been proposed to account for the kinetic behavior of transport in terms of band 3 protein structure. Each involves local, spontaneous conformational changes as a result of which the Cl^- binding site alternates (when occupied) between topological-out and topologically-in positions and vice versa [2,3,30–35]. Such a conformational change might involve a small shift of membrane-crossing elements with respect to each other.

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