Biochimica et Biophysica Acta, 599 (1980) 127—139

BBA 78733

THE LOCATION OF A DISULFONIC STILBENE BINDING SITE IN BAND 3, THE ANION TRANSPORT PROTEIN OF THE RED BLOOD CELL MEMBRANE

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(Received October 3rd, 1979)

Key words: Anion transport protein; Disulfonic stilbene; Band 3 protein; Binding site; (Erythrocyte membrane)

Summary

The binding site for 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid, a specific, potent, irreversible inhibitor of anion transport in red blood cells is located in a 15 000 dalton transmembrane segment of band 3, produced by chymotrypsin treatment of ghosts stripped of extrinsic proteins. The segment was cleaved into three fragments of 7000, 4000 and 4000 daltons by CNBr. The C-terminus of the segment is located in the 7000 dalton fragment; the N-terminus in one of the 4000 dalton fragments; and the binding site for 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid in the middle 4000 dalton fragment. The latter was cleaved by *N*-bromosuccinimide into two fragments of 2000 daltons. The binding site for 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid was located on the fragment containing the newly formed N-terminus. It is concluded that the binding site is located about 9000 daltons from the C-terminus (at the outside face of the membrane) and 6000 daltons from the N-terminus (at the cytoplasmic face). In view of the existing evidence that the binding site may be located near the outside face of the membrane, it is suggested that the 15 000 dalton segment is folded, so that it crosses the bilayer three times.

Introduction

The disulfonic stilbene, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), is a potent inhibitor of anion transport that binds by a covalent reac-

Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; APMB, 2-aminophenyl-6-methylbenzenethiazol-3,1′-disulfonic acid.

tion and is highly localized in an abundant intrinsic membrane protein known as band 3, of molecular weight approx. 95 000 [1,2]. Its inhibitory effect is proportional to the amount of binding to band 3, with virtually complete inhibition when the number of molecules bound is about equal to the number of molecules of band 3 present in the membrane [3,4]. Recent evidence [5] supports the view that each molecule of band 3 contains a specific, DIDS-binding, inhibitory site. In its interaction with the site, DIDS behaves as an affinity probe. That is, it first binds reversibly, primarily by interaction of its sulfonic acid groups with positively charged groups in the inhibitory site, followed by a covalent interaction with an adjacent site [3,4,6].

The functional nature of the DIDS-inhibitory site has been assessed by the use of a reduced form of DIDS, H_2DIDS, with which the rate of the covalent reaction is slow at low temperatures, so that the kinetics of its reversible inhibition can be determined [6]. The results suggest that H_2DIDS (and DIDS) compete with Cl^- for binding to a functional site, the transport site, to which Cl^- must bind in order for its transport to occur. On the basis of the kinetic behavior of transport [2,7] and on the basis of the transmembrane effect of inhibitory probes such as DIDS [8], the site appears to behave like a 'mobile carrier', being alternately accessible to the two sides of the membrane. DIDS would therefore seem to be an ideal probe to determine the location of this 'mobile' transport site in the primary structure of band 3.

By proteolytic cleavages at two sides of the membrane, DIDS has been located in a particular transmembrane segment reported to be 17 000 in molecular weight [9]. This segment is located about 42 000 daltons from the N-terminus of band 3 and about 35 000 daltons from the C-terminus [10]. The present paper describes chemical cleavages of the transmembrane segment which allow conclusions concerning the precise location of the covalently bound DIDS in the primary structure of band 3.

Methods

DIDS and tritiated dihydro DIDS ([^{3}H]H_2DIDS) were synthesized from their diamino analogs as previously described [1]. The tritiation of the H_2DIDS precursor was carried out by New England Nuclear, Inc. CNBr and *N*-bromosuccinimide were obtained from Aldrich Chemical Co. Silica gel 60 (particle size 0.063—0.2 nm, 70—230 mesh ASTM) was purchased from Brinkmann. The peptides, β-insulin, cytochrome *C*, and myoglobin were obtained from Sigma Chemical Co.

Molecular weights of various cleavage products were determined by migration in urea-SDS gels [11], using 10 M urea, 0.1% SDS and 14.5 acrylamide. The tendency for formation of aggregates that do not enter the gel could be minimized by adjusting the pH of the running buffer to 8.0. The peptides, polymyxin B, β-insulin, cytochrome *C* and myoglobin, were used as molecular weight standards (M_r 1200, 3818, 12 300 and 17 000, respectively). The calibration curve is linear except for a slight curvature at the highest molecular weight. Staining of gels for proteins (Coomassie blue), and analysis of the radioactivity profiles were performed as previously described [1]. Descending paper chroma-

tography was carried out on Whatmann No. 3 paper which was washed and dried before use. For concentrating protein solutions, an Amicon ultafiltration cell (Model 8MC) with PM10 filters was used. Peptides were determined by a modification of the method of Lowry et al. [12] and/or by the ninhydrin method [13]. Amino acid analyses were performed on a Durrum 500 amino acid analyser. Samples were hydrolyzed in vacuo in double-distilled constant boiling-point HCl at 110°C for 24 h. The following corrections were made for hydrolytic losses: threonine, 4.8%; serine, 12%; methionine, 14.3% and tyrosine, 9%. A correction of 3.9% was applied for incomplete release of valine. Cysteine was determined as cysteic acid after performic acid oxidation [14]. Tryptophan was measured spectrophotometrically [15]. Partial sequences of amino acids were determined in a Beckmann Model 890C automated amino acid sequencer [16]. The phenylthiohydantoin amino acid derivatives were identified by gas chromatography [17], using a Hewlett-Packard 5700 A gas chromatograph.

Erythrocytes from recently outdated blood bank were separated from plasma by centrifugation and washed three times in phosphate-buffered saline (150 mM NaCl, 5 mM sodium phosphate, pH 8). The cells were lightly labeled with [^{3}H]H_2DIDS (1 μM final concentration, 25% haematocrit, 37°C) for 30 min in phosphate-buffered saline buffer, spun down and then incubated with H_2DIDS and/or DIDS (15 μM final concentration, 25% haematocrit, 37°C) for 1 h in phosphate-buffered saline. The cells were washed twice with phosphate-buffered saline containing 0.5% albumin to remove any unreacted DIDS and twice with phosphate-buffered saline. Leaky ghosts were then prepared [18] by submitting the cells to a hypotonic medium: 5 mM sodium phosphate, pH 8 (5P8). The ghosts were frozen and thawed at room temperature, then stripped of extrinsic proteins by incubating for 2 min with ice-cold medium containing 10 mM NaOH and 0.1 mM EDTA [10], followed by two washings with 5P8, providing starting material for the cleavages.

The cleavage scheme involves enzymic proteolysis to produce the DIDS-containing 17 000 dalton segment; its cleavage by CNBr; the isolation of the DIDS-containing fragment; its cleavage by *N*-bromosuccinimide; with final separation of a 2000 dalton DIDS-labelled peptide.

Chymotrypsin cleavage of band 3. The alkali-stripped ghosts were resuspended to a final concentration of 1—3 mg protein/ml of 5P8 buffer containing 0.2 mg chymotrypsin/ml buffer. After incubation for 1.5 h at 37°C, proteolysis was stopped by adding 30 μg/ml of phenylmethylsulfonyl fluoride. The ghosts were then washed twice with 5P8 containing 0.5% albumin and twice with 5P8. The resulting vesicles, rich in the 15 000 dalton segment of band 3 [10], were resuspended to a final concentration of 1 mg protein/ml of 10 mM Tris-HCl buffer, pH 7.4, containing 0.2% Triton X-100. Stirring for 15 min at 4°C followed by centrifugation at 45 000 × *g* for 30 min gave the stripped, proteolyzed, Triton X-100-extracted vesicles as a pellet. The major peptide in these vesicles is the 17 000 dalton transmembrane segment of band 3. The vesicles were delipidated with a $CHCl_3/CH_3OH$ mixture (1 : 1) and run on urea-SDS gels. The band containing the 15 000 dalton segment was cut out of the gels, extracted with 1% SDS solution overnight, filtered and the filtrate concentrated in an Amicon microultrafiltration cell. The 15 000 dalton segment pre-

cipitated out of this solution on standing, with yields of 50—70% based on the amount of radioactivity ([^{3}H]H_2DIDS) in solution before and after precipitation. The yield can be optimized by adding $\frac{1}{3}$ vol. of CH_3OH to the solution. In our hands, this DIDS-containing transmembrane fragment reported to be 17 000 daltons [10]was found to be 15 000 daltons as determined by migration in urea-SDS gels. It will therefore be referred to as the 15 000 dalton segment in this paper. The difference in molecular weight may be due to differences in cleavage procedure (1.5 compared to 1 h, 37°C compared to 22°C, and cleavage of alkali-stripped rather than normal ghosts).

CNBr cleavage of the 15 000 dalton segment. The peptide (3 mg) was dissolved in 1 ml of 88% formic acid in a reaction tube. Water was added to reduce the formic acid concentration to 70%. 50—100 mg CNBr were added to the protein solution and the reaction tube closed under N_2 and wrapped with aluminum foil [19,20]. After selected periods of time up to 64 h at room temperature, the reaction mixture was evaporated to dryness under N_2 to give the products. With samples that were to be run on urea-SDS gels, 100 μl of 10% SDS were added before evaporation under N_2. This increases the solubility of the fragments in the urea-SDS gel solubilizer.

Separation of the 4000 dalton DIDS-labeled fragment. A slurry of silica gel in 88% formic acid was poured into a glass column (36 × 0.9 cm) and washed successively with 20 ml of 88% formic acid containing 30% *n*-butanol, 20 ml of 88% formic acid containing 50% *n*-butanol and butanol containing 10% formic acid. The CNBr fragments from complete cleavage of the 15 000 dalton segment (64 h) were taken up in 1 ml of 88% formic acid and diluted with *n*-butanol until the first signs of cloudiness appeared. A few drops of formic acid gave a clear solution which was applied to the top of the column. The column was eluted successively with 40-ml each of 88% formic acid/butanol mixtures, 1 : 9, 1 : 1 and 7 : 3. The fractions collected were counted for radioactivity and assayed for peptides by the ninhydrin method. The eluate containing the labeled peptide was evaporated to dryness using N_2. Recovery of the tritiated H_2DIDS was between 40 and 45%. After urea-SDS-acrylamide gel electrophoresis of the labeled fraction, one stained band was found of 4000 daltons containing all of the H_2DIDS. For large-scale preparation of the purified labeled 4000 dalton fragment, the starting material can be stripped, proteolyzed, Triton X-100-extracted vesicles rather than the isolated 15 000 dalton segment.

N-Bromosuccinimide cleavage of the DIDS-labeled 4000 dalton fragment. The 4000 dalton DIDS-labeled fragment (1 mg) was dissolved in 400 μl of glacial acetic acid and 120 μl of water were added to give a 70% final acetic acid concentration. *N*-Bromosuccinimide (0.05 M freshly prepared) was added, 100 μl every 5 min, until a yellow color persisted. A slight excess of imidazole was added to destroy the unreacted *N*-bromosuccinimide. The solution was heated for 1 h at 100°C [21]. The mixture was chromatographed on a Whatmann No. 3 chromatography paper, with 88% formic acid/butanol (1 : 1) separating two main bands, R_F values 0.65 and 0.35, that could be visualized by fluorescamine [22]. About 70% of the radioactivity was located at the higher R_F, none at the lower R_F, and the remainder at the origin. The radioactive and unlabeled bands were eluted with 50% formic acid, and the protein solution freeze-dried.

Results

The 15 000 dalton segment of band 3 is produced by two chymotrypsin cleavages, one at the outside face of the membrane producing the C-terminus of the segment and the other at the cytoplasmic face, producing the N-terminus [9,10]. The segment is insoluble in water but is soluble in detergents such as SDS or in strong organic acids such as formic and trifluoroacetic acids. After 2 h with CNBr in formic acid, the predominant cleavage fragments were 11 000 and 4000 daltons (Fig. 1, gel 2 h); at later times (6 h) a fragment of 7000 daltons was also present with a reduction in the amount of the 11 000 dalton fragment (Fig. 1, gel 6 h). After 24 h only 7000 and 4000 dalton fragments were present, with more intense staining in the latter (Fig. 1, gel 64 h). The [^{3}H]-H_2DIDS originally present in the 15 000 dalton segment was found initially only in the 11 000 fragment (except for aggregated material containing 20—30% of the H_2DIDS that did not enter the gels). As the cleavage proceeded, the 11 000 dalton fragment disappeared and in parallel, the [^{3}H]H_2DIDS shifted to the 4000 dalton region (Fig. 2). At no time was [^{3}H]H_2DIDS found in the 7000 dalton fragment. These findings indicate that two sequential cleavages result from the CNBr treatment, the first producing an 11 000 plus 4000, and the second, cleaving the 11 000 into a 7000 plus a second 4000 dalton fragment. The presence of two distinct 4000 dalton segments, one containing DIDS, was confirmed by their physical separation on silica gel (discussed later in the paper).

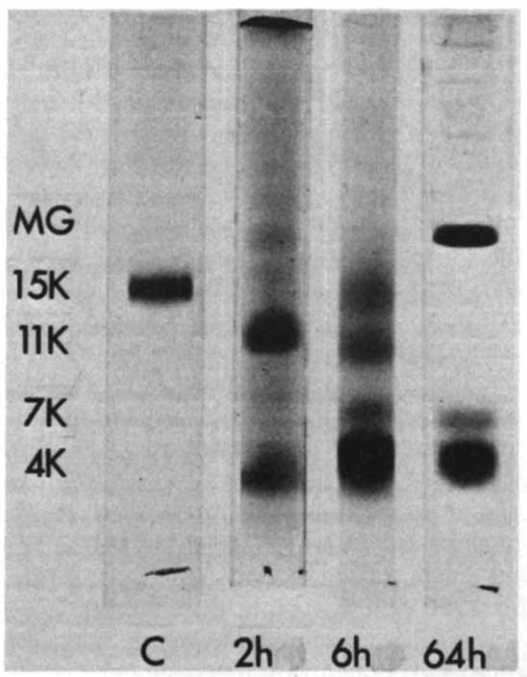

Fig. 1. CNBr celavage products from the 15 000 dalton segment assessed by urea-SDS-acrylamide gel electrophoresis. The times of cleavage and molecular weights are indicted. MG(myoglobin) is a reference peptide of M_r 17 200. The amount of peptide applied to the gels from the 6 h cleavage was larger than for the other samples (150 μg compared to 50 μg), accounting for the higher staining density.

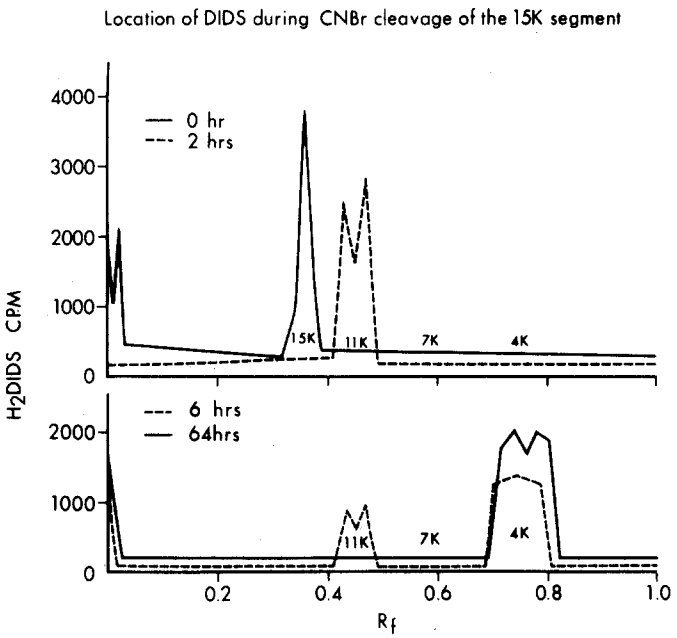

Fig. 2. Distribution of tritiated H_2DIDS in urea-SDS acrylamide gels after CNBr cleavage for different periods of time. The cleavage times and location of various fragments are indicated. The counts for each sample were normalized to the same total applied to the gels.

The finding of two cleavages is also consistent with the fact that CNBr, under acid conditions, cleaves primarily at methionine, and that the 15 000 dalton segment contains two methionyl residues ([23] and Table I).

The proposed location of the fragments and of the DIDS covalent binding site in the primary sequence are shown in Fig. 3. The first cleavage is designated as A and the second as B. The arrangement is based on the following considerations: (1) CNBr cleavage produces a homoserine lactone ring at each newly formed C-terminus. No lactone was found in the 7000 dalton segment (B to C_t, Fig. 3). This fragment must therefore contain the original C-terminal end of the 15 000 dalton peptide. The 4000 dalton fragments, on the other hand, both have homoserine lactones, so they must be toward the N-terminal end of the peptide. (2) The 4000 dalton segment containing DIDS must be the middle segment, based on the following observations. After the first cleavage at A to produce 11 000 and 4000 dalton fragments, the DIDS was localized in the former. The unlabeled 4000 dalton fragment must therefore be located at the N-terminal end of the 15 000 dalton segment (A to N_t, Fig. 3). After the second cleavage at B to produce a 7000 dalton plus a second 4000 dalton fragment, the DIDS was then found in the 4000 dalton region, but not the 7000 dalton C-terminal fragment. Thus, the DIDS-containing segment must be between the two cleavage points (A to B). This conclusion has been verified by another procedure. The DIDS-containing 4000 dalton segment was isolated (see below) and several amino acid residues were determined by sequencing from the N-terminus. They were Gly-Asp-?-Pro-Leu-Pro. The first six amino acids at

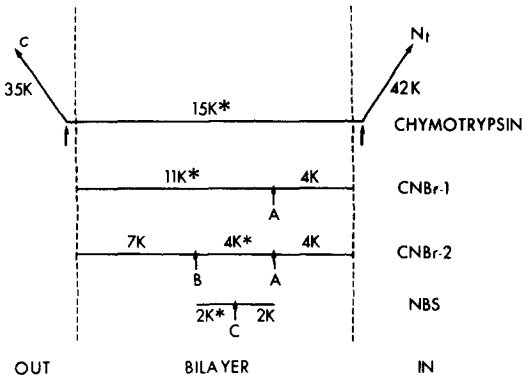

Fig. 3. Proposed arrangements of cleavage segments in band 3. The proteolytic cleavages at the outer and inner faces of the membrane are indicated on the top line. At A, the first cleavage with CNBr; at B, the second cleavage with CNBr and at C, the cleavage of the 4000 dalton DIDS fragment with *N*-bromosuccinimide. H_2DIDS location denoted by *.

the N-terminus of the 15 000 dalton segment, as determined by the same procedure could only be partially ascertained (?-Val-?-Gly-Ala-Val). Nevertheless, it is clear that the two sequences do not correspond. Thus the 4000 dalton fragment does not contain the N-terminal end of the 15 000 dalton segment. It must therefore be the interior fragment.

The DIDS-containing 4000 dalton fragment (A to B) was separated from the other fragments on a silica gel column (Fig. 4). The unlabeled fragments were eluted from the column at low formic acid concentrations, whereas the DIDS-

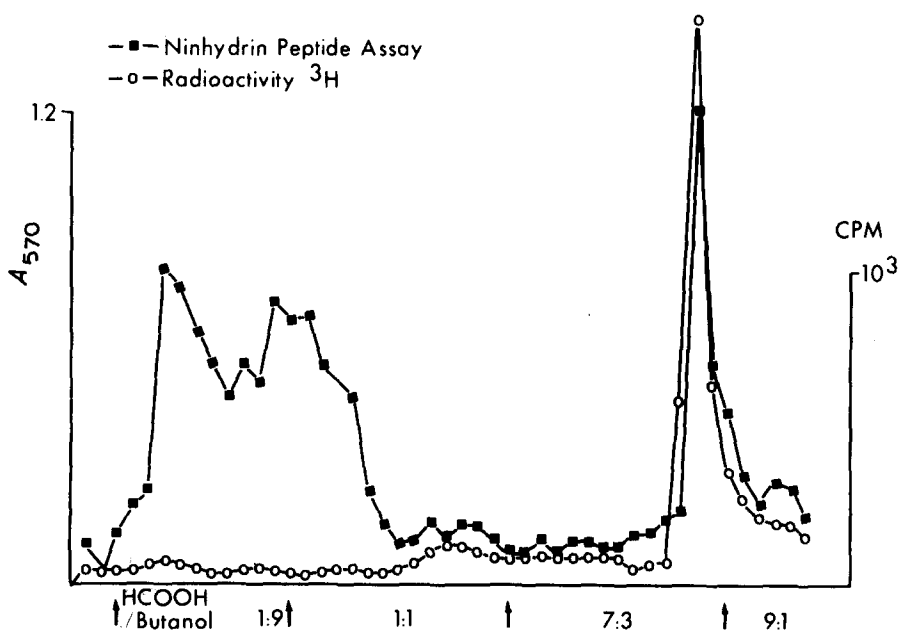

Fig. 4. The separation of the DIDS-containing peptide fragment by silica gel column chromatography.

labeled fragment came out only at high formic acid concentrations. The labeled fraction in the SDS-urea acrylamide gels contained a 4000 dalton peptide, and the unlabeled fraction contained 7000 and 4000 dalton fragments. The recovery of [^{3}H]H_2DIDS in the labeled fraction was about 40—45% of that present in the uncleaved 15 000 dalton segment. Presumably at low formic acid concentrations, the labeled fragment containing the two sulfonic acid groups of DIDS is sufficiently polar to bind to the silica gel. On the other hand, the unlabeled fragments are much less charged and do not bind as well to the silica gel. At high concentrations of formic acid, however, the sulfonic acids of the DIDS-labeled fragment would be protonated and the fragment less polar with its binding capacity to silica gel decreased. The importance of the sulfonic acid groups in the separation procedure is evident from the finding that when the unlabeled 15 000 dalton segment was used as starting material, the cleavage products could not be separated from each other by the silica gel column procedure.

The purified 4000 dalton DIDS fragment was subjected to cleavage with *N*-bromosuccinimide in acetic acid. A single cleavage resulted, producing two fragments. These were separated from each other by paper chromatography. One fragment, containing DIDS, also contained the lactone from the previous CNBr cleavage. It, therefore, must be on the C-terminal side of the *N*-bromosuccinimide cleavage point, as indicated in Fig. 3 (C to B). Its molecular weight was approx. 2000 as estimated from its position on urea-SDS acrylamide gels. The other, unlabeled fragment (C to A), was also approx. 2000 daltons.

Attempts were made to sequence the 2000 dalton, DIDS-containing fragment isolated by paper chromatography. In each of three such efforts, the sequencing stopped after cleaving the N-terminal amino acid (leucine, yield 64%). It seems possible that the sequencing stops because the DIDS conjugate is located next to the N-terminal leucine residue.

The amino acid compositions of the 15 000 dalton, the DIDS-labeled 4000 dalton, the DIDS-labeled 2000 dalton, and the unlabeled 2000 dalton peptides are given in Table I. The minimum number of residues (based on one cysteine per peptide) for the 15 000 dalton segment is 137, which is consistent with the molecular weight of 15 000 determined by urea-SDS gel electrophoresis. This value is somewhat lower than that reported previously by Steck et al. [10], but as discussed in Methods, this difference may be due to different conditions of proteolysis. The DIDS-labeled 4000 dalton fragment and its two 2000 dalton cleavage products also have minimal numbers of residues consistent with the molecular weights determined by the gel electrophoresis procedure (Table I). These correspondences and the fact that the sum of the weights of the fragments approximate to the weight of the parent segment indicate that the assigned values are reasonably correct.

The amino acid composition of the 15 000 dalton segment reported in Table I is similar to the published data [23] except for reduced numbers of the residues. The peptide contains numerous charged residues and the percentage of hydrophobic residues is not particularly high (36—40%), even though the segment behaves, in its solubility properties, as a hydrophobic peptide. The cleavage fragments have roughly the same proportion of charged and hydrophobic residues as the parent 15 000 dalton segment (Table I). Drickamer [24]

TABLE I

AMINO ACID COMPOSITIONS OF BAND 3

Data are expressed as residues/mol ± S.D. Hydrophobic residues were taken as Val, Met, Ile, Leu, Tyr, Phe and Trp.

Amino acid	15 000 dalton fragment	4000 dalton fragment (labeled)	2000 dalton fragment (labeled)	2000 dalton fragment (unlabeled)
Asx	10.6 ± 0.4	3.6 ± 0.4	1.8 ± 0.2	2.1
Thr	9.4 ± 0.3	2.3 ± 0.1	1.1 ± 0.1	1.3
Ser	10.8 ± 0.3	2.9 ± 0.8	1.3 ± 0.1	1.1
Glx	11.1 ± 1.2	3.2 ± 0.1	1.8 ± 0.5	1.5
Pro	4.2 ± 0.1	2.6 ± 0.1	1.2 ± 0.3	0.9
Gly	14.0 ± 0.6	5.2 ± 0.7	2.8 ± 0.3	2.1
Ala	12.5 ± 0.3	4.4 ± 0.4	1.8 ± 0.1	2.3
Val	11.3 ± 0.4	3.6 ± 0.2	2.1 ± 0.3	1.3
Met	2.1 ± 0.2	1	1	0
Ile	7.7 ± 0.1	1.9 ± 0.1	1.1 ± 0.3	1.1
Leu	15.1 ± 1.9	3.8 ± 0.5	2.3 ± 0.1	2.5
Tyr	2.3 ± 0.4	?	?	?
Phe	8.4 ± 0.6	1.1 ± 0.1	1.1 ± 0.1	0
His	1.8 ± 0.1	0.8 ± 0.2	0	1
Lys	7.2 ± 0.3	2.9 ± 0.3	1.7 ± 0.5	0.9
Arg	4.5 ± 0.1	1.8 ± 0.1	1.4 ± 0.6	0
Cys	1.1 ± 0.2	0	0	0
Trp	2.6 ± 0.3	?	?	?
Sum	136.7	41.1	22.5	18.1
% hydrophobic	36	30	34	27
No. of samples	4	4	4	1

has reported a composition for an 11 000 dalton CNBr cleavage fragment which is probably the same as our 11 000 dalton fragment (C_t to A, Fig. 3). By appropriate subtraction, compositions can be calculated for the 7000 dalton C-terminal and 4000 dalton N-terminal ends of the 15 000 dalton segments. Although such calculations are subject to errors, it is quite clear that these two segments also have similar percentages of hydrophobic residues, 41 and 46%, respectively [25].

Discussion

As pointed out in Introduction, it has been concluded on the basis of DIDS binding studies [2—5] and an analysis of the DIDS-inhibition kinetics [6], that each band 3 monomer contains one DIDS binding site that behaves kinetically as if it were the Cl^- binding site essential for anion transport [2,6,25]. This site is located in the 15 000 dalton transmembrane segment produced by proteolytic cleavages at the outside and inside faces of the membrane [10]. A second DIDS binding site located in the 35 000 dalton C-terminal portion of band 3 is only evident after incubation of DIDS-treated cells at high pH for longer periods of time [5]. Its possible role in transport has not been defined. The chemical information in the present paper supports the view that, within the limits of errors of the procedures, a unique DIDS binding site is located at

a particular residue in the sequence of the 15 000 dalton segment. For example, after complete CNBr cleavage, the only labeled peptide is the 4000 dalton fragment (except for a small amount of high molecular weight aggregate that does not enter the gels, shown as counts at the far left of Fig. 3). The recovery of count in the 4000 dalton region of the gels is approx. 70% of that in the 15 000 dalton segment used for the cleavage. The recovery of purified labeled 4000 dalton fragment from the silica gel column was over 40%. After its cleavage with *N*-bromosuccinimide, and separation of products by paper chromatography, 70% of the count was recovered in one 2000 dalton fragment with 30% remaining at the origin, probably due to aggregation. No significant count was found in a second 2000 dalton fragment. On sequencing, the labeled fragment behaves in a homogeneous manner. A single terminal amino acid (leucine) is cleaved (yield 64%), with no detectable release of any other residues on the second cleavage cycle, suggesting that essentially all of the DIDS-conjugated residues are located at the second position, resulting in a blockage of further cleavage.

In using covalent affinity probes to mark functional sites, it is important to note that their inhibitory effects result from a reversible association of their negatively charged groups with positively charged ligands of the membrane sites (in the case of DIDS, presumably with the transport site), and not on the covalent reaction [2—4,6]. Thus, the covalent reaction of the isothiocyano group must occur with a ligand that is a close neighbor of the presumed transport site. The distance between the transport site and the covalent site may, however, be somewhat greater than that calculated from the structure of DIDS. The probe binds by multiple interactions [2,26] and it may therefore induce structural rearrangements of the peptide, bringing into apposition, ligands that are normally a greater distance apart. Evidence that the DIDS interaction results in substantial conformational changes has been summerized elsewhere [2]. It must be concluded, therefore, that covalent reaction of DIDS may involve a ligand in band 3 that may be close to the transport site in the tertiary structure, but may be some distance away in the primary sequence. This problem of defining location is inherent in the use of any covalent affinity probe.

The proposed location of the DIDS covalent binding site illustrated in Fig. 3 is about 9000 daltons from the outside chymotrypsin cleavage site (the C-terminus of the 15 000 dalton fragment) and 6000 daltons from the cytoplasmic cleavage site (N-terminus). Other inhibitory probes capable of covalent reaction have also been localized in band 3 based on chemical cleavages of the 65 000 dalton N-terminal segment produced by chymotrypsin treatment of cells [24, 27]. Several of the cleavages fall within the 15 000 dalton transmembrane segment which is located at the C-terminal end of the 65 000 dalton segment. With CNBr (under somewhat different conditions than in the present paper), a cleavage was reported which seems to correspond with our cleavage A (Fig. 3) but no cleavage at B was observed. With 2-nitro-5-thiocyanobenzoic acid and with hydroxylamine, cleavages are reported that are near to our cleavage B, although assignments of location are subject to some uncertainty because they are based on differences of molecular weight of larger fragments. Based on these cleavages, the location of 1-isothiocyano-4-benzenesulfonic acid, was reported to be within 7000 daltons of the external chymotrypsin cleavage point (approxi-

mately equivalent to C_t to B segment of Fig. 3). whereas with diazosulfinilic acid, only a small fraction seemed to be located in that segment. The major labeling is reported to be located between 7000 and 11 000 daltons, corresponding to the A to B segment in which DIDS is located.

In a topological sense, the DIDS covalent binding site behaves as though it is exposed at the outside surface. DIDS is a large non-penetrating probe [1], yet it reacts rapidly with its inhibitory site after addition to cells [6]. On the other hand, when added to the cytoplasmic surface (using inside-out vesicles), DIDS has no inhibitory effect [8]. The exposure of the DIDS binding site to the outside is also suggested by experiments in which DIDS covalently attached to the membrane can be cross-linked through its second isothiocyano group to added ferritin [28]. The interpretation, in this case, must be taken with some caution, however, because the ferritin cross-linking is most effective when the membrane is being stretched by exposure to hypotonic solutions. Another probe, 2-aminophenyl-6-methylbenenethiazol-3,7'-disulfonic acid (APMB), is inhibitory (reversibly) from either side of the membrane, but asymmetry in the location of the inhibitory site is evident from the finding that APMB linked to dextran by a short spacer group is effective from the outside but not from the inside. If, however, the probe is attached to the dextran by a long spacer group (40 Å), it is also inhibitory from the inside (Barzilay, M. and Cabantchik, Z.I., personal communication). This finding is consistent with the conclusion that the inhibitory site is toward the outside surface, but it must be treated cautiously because it has not been directly demonstrated that APMB inhibits by binding to the same site as DIDS.

Assuming that the DIDS covalent binding site is near to the outer surface of the membrane, its location in the primary structure at a point 9000 daltons from the C-terminus which is also located at the outside (because it is produced by a chymotrypsin cleavage at the outside face of the membrane) reinforces the suggestion that the 15 000 dalton segment may be folded within the bilayer, as proposed by Drickamer [24] and by Williams et al. [29], based on the use of other probes and cleavages. The simplest structure would be one in which the peptide crosses the membrane three times so that a loop and the C-terminus are exposed to the outside, and a second loop and N-terminus are exposed to the inside. The DIDS would be located on the loop exposed to the outside, as illustrated in Fig. 5. This arrangement would account for its location about one third of the distance from the N-terminus of the segment. Each of the three transmembrane segments would have over 4000 daltons of length, more than sufficient to cross the membrane [30].

A folded structure as in Fig. 5 could account for the finding that the 15 000 dalton segment remains associated with the bilayer except in the presence of detergents, even though it is not particularly hydrophobic in composition ([23] and Table I). The three transmembrane strands could form a somewhat cylindrical assembly in which the hydrophobic residues would be largely located on the outside in contact with the lipoidal region of the bilayer, and in which the hydrophilic residues would be largely internalized to provide an aqueous region through which anion transport could take place [2,25,31]. The sequence of polar and hydrophobic amino acid residues would presumably play a fundamental role in the stability of the assembly [30].

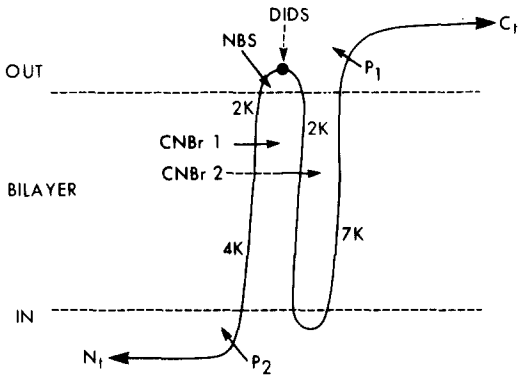

Fig. 5. Proposed arrangement of the 15 000 dalton transmembrane segment in the bilayer.

More than three segments of peptide may be involved in the intramembranous structure. Band 3, when extracted in Triton X-100 is a dimer [32] and recent evidence indicates that it is also a dimer in the membrane [33]. The dimer structure persists even after chymotrypsin cleavages to produce the 15 000 dalton transmembrane segment and another membrane bound segment of 35 000 daltons containing the C-terminus of band 3 [34]. The latter segment also crosses the bilayer [29,35] and it seems to be closely associated with the 15 000 dalton segment. Recently, a proteolytic fragment of 9000 daltons has been tentatively identified as a transmembrane fragment of the 35 000 dalton segment [25]. Thus four strands of peptide per monomer, or eight per dimer, may contribute to the intramembranous portion of band 3.

Acknowledgements

This work was supported by a grant from the Medical Research Council (Canada) MT 4665. We express our thanks to Mrs. Zinovia Duma for determining the amino acid compositions of the various peptide fragments and to Dr. T. Hoffmann for the determination of sequences.

References

1 Cabantchik, Z.I. and Rothstein, A. (1974) J. Membrane Biol. 15, 207—216
2 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239—302
3 Lepke, S., Fasold, H., Pring, M. and Passow, H. (1976) J. Membrane Biol. 29, 147—177
4 Ship, S., Shami, Y., Breuer, W. and Rothstein, A. (1977) J. Membrane Biol. 33, 311—324
5 Jennings, M.L. and Passow, H. (1979) Biochim. Biophys. Acta 554, 498—519
6 Shami, Y., Rothstein, A. and Knauf, P.A. (1978) Biochim. Biophys. Acta 508, 357—363
7 Gunn, R.B. and Frohlich, C. (1979) J. Gen. Physiol. 74, 351—374
8 Grinstein, S., McCulloch, L. and Rothstein, A. (1979) J. Gen. Physiol. 73, 493—514
9 Grinstein, S., Ship, S. and Rothstein, A. (1978) Biochim. Biophys. Acta 507, 294—304
10 Steck, T.L., Ramos, B. and Strapazon, E. (1976) Biochemistry 15, 1154—1161
11 Swank, R.T. and Monkries, K.D. (1971) Anal. Biochem. 39, 462—477
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Hirs, C.H.W., Moore, S. and Stein, W.H. (1956) J. Biol. Chem. 219, 623—641
14 Hirs, C.H.W. (1967) Methods Enzymol. 11, 59—61
15 Koziarz, J.J., Kohler, H. and Steck, T.L. (1978) Anal. Biochem. 86, 78—89
16 Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80—91

17 Pisano, J.J. and Bronzert, T.J. (1969) J. Biol. Chem. 244, 5597—5607
18 Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 31, 172—173
19 Gross, E. and Witkop, B. (1967) Biochemistry 6, 745—748
20 Gross, E. (1967) Methods Enzymol. 11, 238—255
21 Ramachandran, L.K. and Witkop, B. (1967) Methods Enzymol. 11, 283—299
22 Undefriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W. and Weigele, M. (1972) Science 178, 871—873
23 Steck, T., Koziarz, J.J., Singh, M.K., Reddy, G. and Koher, H. (1978) Biochemistry 17, 1216—1222
24 Drickamer, L.K. (1977) J. Biol. Chem. 252, 6909—6917
25 Rothstein, A., Ramjeesingh, M. and Grinstein, S. (1979) in Membrane Transport in Erythrocytes (Lassen, V., Ussing, H.H. and Wieth, J.O., eds.), Alfred Benzon Symposium No. 14, Munksgaard, Copenhagen, in the press
26 Barzilay, M., Ship, S. and Cabantachik, Z.I. (1979) Membrane Biochem. 2, 227—254
27 Drickamer, L.K. (1976) J. Biol. Chem. 251, 5115—5123
28 Rothstein, A. and Cabantchik, Z.I. (1974) in Comparative Biochemistry and Physiology of Transport (Bolis, L., Bloch, K., Luria, S.E. and Lynen, F., eds.), pp. 354—362, North-Holland, Amsterdam
29 Williams, D.G., Jenkins, R.E. and Tanner, M.J.A. (1979) Biochem. J. 81, 477—493
30 Guidotti, G. (1977) J. Supramol. Struct. 7, 489—497
31 Rothstein, A., Cabantchik, Z.I. and Knauf, P.A. (1976) Fed. Proc. 35, 3—10
32 Steck, T. (1974) J. Cell Biol. 62, 1—19
33 Nigg, E. and Cherry, R.J. (1979) Nature 277, 493—494
34 Reithmeier, R.A.F. (1979) J. Biol. Chem. 254, 3054—3060
35 Rao, A. (1979) J. Biol. Chem. 254, 3503—3511