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THE AMINO ACID CONJUGATE FORMED BY THE INTERACTION OF THE ANION TRANSPORT INHIBITOR 4,4′-DIISOTHIOCYANO-2,2′-STILBENEDISULFONIC ACID (DIDS) WITH BAND 3 PROTEIN FROM HUMAN RED BLOOD CELL MEMBRANES

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Summary

The specific anion transport inhibitor 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) and its reduced analog (H_2DIDS), when irreversibly bound to band 3 protein of the red blood cell membrane, form amino acid conjugates through interaction with the ϵ-amino group of a particular lysine residue. The specific residue is located in a transmembrane segment of band 3 protein and appears to be a close neighbor of the transport site.

Introduction

The disulfonic acid 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) and its reduced form, dihydro-DIDS (H_2DIDS), are among the most potent and specific inhibitors of anion transport across the red blood cell membrane [1—4]. The probes bind covalently largely to band 3 protein, an abundant intrinsic membrane protein [5,6], but the irreversible reaction is preceded by reversible binding and inhibition [7,8]. A kinetic analysis of the reversible inhibition indicates that DIDS and Cl^- compete for a common binding site, suggesting that the probe interacts in a 1 : 1 ratio with the transport site [9]. Consistent with this conclusion is the finding that at maximal inhibition the ratio of bound DIDS (or H_2DIDS) to the number of band 3 monomers is 1 : 1 [7,8],

Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; H_2DIDS, dihydro-DIDS; DADS, 4,4′-diaminostilbene-2,2′-disulfonic acid; H_2DADS, dihydro-DADS; SDS, sodium dodecyl sulfate.

and that each molecule of band 3 protein binds with one molecule of DIDS [10]. The covalent binding site has been located in a 2000 dalton fragment in the middle of a 15 000 dalton transmembrane segment of band 3 protein [11].

The nature of the particular ligands in band 3 protein with which DIDS reacts has not yet been determined specifically. The covalent reaction of the probe involves an isothiocyano group which is capable of reaction with lysine, arginine, histidine, tyrosine or cysteine residues, although the specificity is probably highest for lysine [12]. On the other hand, it has been demonstrated that the covalent reaction of H_2DIDS with the myelin proteins involves an arginine residue [13]. In order to determine the particular amino acid residue with which H_2DIDS and DIDS react covalently in band 3 protein, the protein was hydrolyzed to its constituent amino acids and the chromatographic behavior of the H_2DIDS- and DIDS-containing conjugates were compared to those of model compounds prepared separately. The H_2DIDS- and DIDS-amino acid conjugates derived from band 3 protein had properties similar to those of H_2DIDS- and DIDS-lysine.

Methods

DIDS and tritiated dihydro-DIDS ([$^3H_2$]DIDS) were synthesized from the diamino analog 4,4′-diaminostilbene-2,2′-disulfonic acid (DADS) as previously described [5]. The tritiation of this diamino analog was carried out by New England Nuclear (Boston, MA). Silica-gel precoated thin-layer chromatography glass plates (Sil G-25/UV 254) and precoated thin-layer chromatography plastic sheets (sil sheet 60) were obtained from Brinkman (Toronto, Canada). Anion-exchange resin AG1-X4 was obtained from Bio-Rad Laboratory (Richmond, CA). Polylysine and polyarginine were from Sigma Chemical (St. Louis, MO).

Recently expired blood bank cells were washed three times in phosphate-buffered saline (150 mM NaCl/5 mM sodium phosphate, pH 8). The cells at 25% haematocrit in phosphate-buffered saline were treated for 1 h at 37°C with 15 μM DIDS or 15 μM [$^3H_2$]DIDS (specific activity, 250 Ci/mol). This treatment allows almost complete interaction of all the band 3 protein inhibitory sites with the probe [8]. In one experiment, an intermolecular cross-linking of band 3 protein by H_2DIDS was produced by incubation of cells for 1 h at pH 9.5 [10]. The treated cells were washed with phosphate-buffered saline containing 0.5% bovine serum albumin to remove any unreacted probe. They were then hemolysed in 5 mM sodium phosphate (pH 8). The ghosts were recovered by centrifugation and washed in the same medium. Extrinsic peptides were removed by alkaline extraction [14].

In some experiments, the amount of extraneous peptide was substantially reduced by treating alkaline-stripped ghosts (resuspended in 5 mM sodium phosphate, pH 8, with 1 mg protein/ml buffer) with 1.5 mg/ml chymotrypsin for 1.5 h at 37°C [15]. The resulting vesicles retain the DIDS of the ghosts, most of it in a transmembrane segment of 15 000 daltons derived from band 3 protein. For further purification, the vesicles were dissolved and the peptides separated by electrophoresis in an SDS/urea-polyacrylamide gel system [15, 16]. The 15 000 dalton DIDS-containing segment was excised from the gels for hydrolysis and analysis as described below.

Preparation of DIDS- and H_2DIDS-labelled conjugates of band 3 protein

Stripped ghosts or vesicles from chymotrypsin-treated ghosts derived from 20 ml cells treated with DIDS or H_2DIDS were delipidated with 10 vol. $CHCl_3$/CH_3OH (1 : 1, v/v). The protein pellet obtained by centrifugation after cooling on ice for 2 h was dissolved in double-distilled 6 M HCl and hydrolysed at 110°C for 14 h. After freeze-drying, the sample was dissolved in 1 ml water, adjusted to pH 3.5 with 6 N sodium hydroxide, and passed through an anion-exchange resin (AG1-X4) column (0.8 × 24 cm). Successive elutions with 25 ml water, pH 3.5, and 35 ml 2 M HCl separated the unmodified amino acids from the DIDS- or H_2DIDS-modified amino acids. At the pH used for dissolving the amino acids (pH 3.5), most of the carboxyl groups of the unmodified amino acids are protonated, whereas the sulfonic acid groups of the DIDS or H_2DIDS conjugates are not. Only the latter are retained on the ion-exchange column. The fractions collected were assayed for amino acids by the ninhydrin method [17], and counted for radioacitivity in the case of samples labelled with [$^3H_2$]DIDS.

Preparation of DIDS and H_2DIDS lysine and arginine conjugates

10 mg polylysine or polyarginine were dissolved in 10 ml water. 5 mg DIDS or H_2DIDS dissolved in 3 ml water were added dropwise to the poly-amino acids with constant stirring. After 1 h at room temperature, the final volume was adjusted to 15 ml with saturated NaCl. The labeled polyamino acid that precipitated out of solution was separated by centrifugation. The DIDS- or H_2DIDS-labeled polyamino acids were hydrolysed with 6 M HCl and separated from unlabeled residues by use of an anion-exchange resin as described above. Separation could also be achieved by gel filtration using a Sephadex G-15 column. The unlabeled amino acids are retarded, whereas the conjugates are eluted at a ratio of elution volume to void volume of 1.85. The conjugates were characterized by high voltage electrophoresis in 6% acetic acid, adjusted with formic acid to pH 1.9, and by thin-layer chromatography as described below. Spots were detected by fluorescamine staining viewed under an ultraviolet lamp [11].

Thin-layer chromatography of the DIDS and H_2DIDS conjugates

The DIDS-amino acid conjugates derived from band 3 protein and the DIDS-lysine or -arginine conjugates were run on analytical thin-layer chromatogrphy silica-gel plates by ascending chromatography using pyridine/acetic acid/water (20 : 2 : 80, v/v). Spots were visualized under an ultraviolet lamp and developed with iodine vapor.

The [$^3H_2$]DIDS-labeled amino acids and [$^3H_2$]DIDS-labeled standards were run on analytical thin-layer chromatography silica gel plastic sheets by ascending chromatography using *n*-butanol/formic acid/water (75 : 50 : 5, v/v). Spots were visualized under ultraviolet light or on Kodak XR-1 X-ray film by fluorography [18]. Radioactive profiles were also obtained by cutting the sheets into 3-mm strips. Each strip was incubated with 1 ml formic acid for 1 h before counting in 10 ml Aquasol in a Packard liquid scintillation counter.

Results

Conjugates resulting from hydrolysis of peptides from DIDS- or H_2DIDS-treated cells and DIDS- or H_2DIDS-treated polylysine or polyarginine can be separated from unmodified amino acid residues either by elution from anion-exchange columns or by gel filtration. In Fig. 1, data are presented using the anion-exchange system for H_2DIDS conjugates. The elution profiles in each case show two peptide peaks, one of which corresponds to the tritium peak of

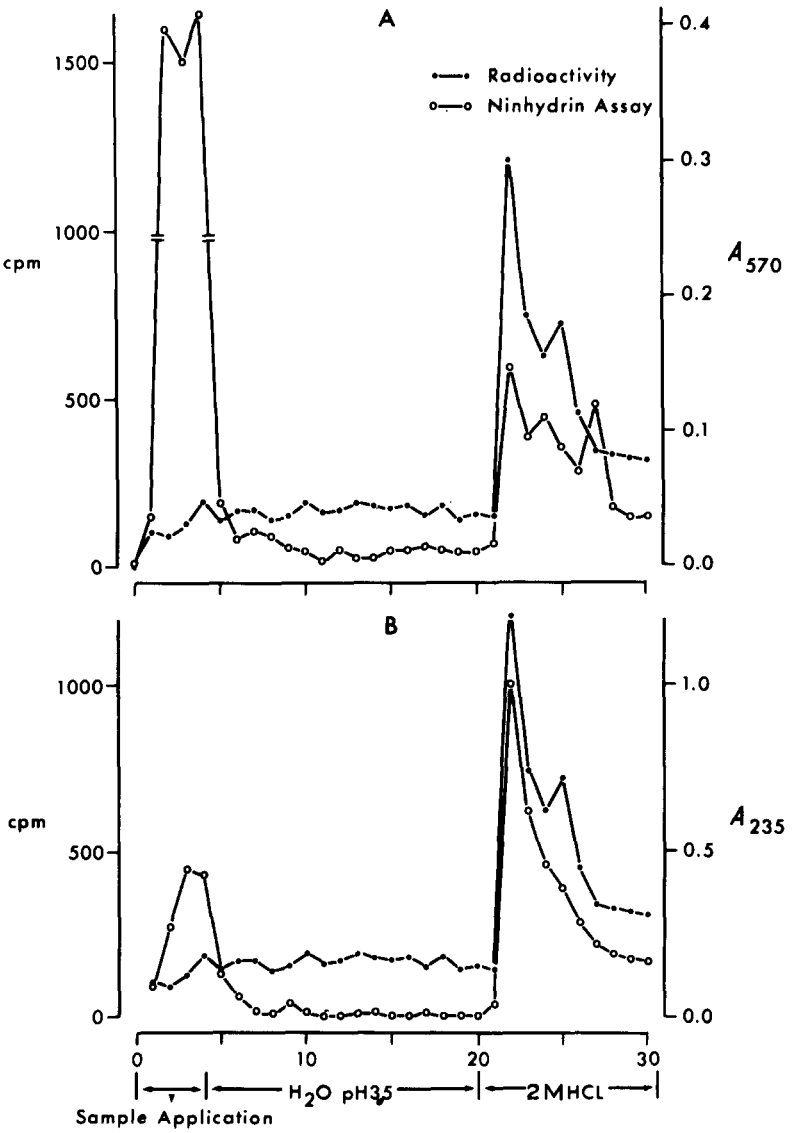

Fig. 1. Separation of [$^3H_2$]DIDS-conjugates from free amino acids on an anion-exchange resin (Bio-Rad AG1-X4) column. Resin was prepared by first washing with 2 M HCl, then extensively with water. Samples were adjusted to pH 3.5 before application to column. A, Hydrolysate from alkali-stripped chymotrypsinized ghosts; B, hydrolysate from polylysine.

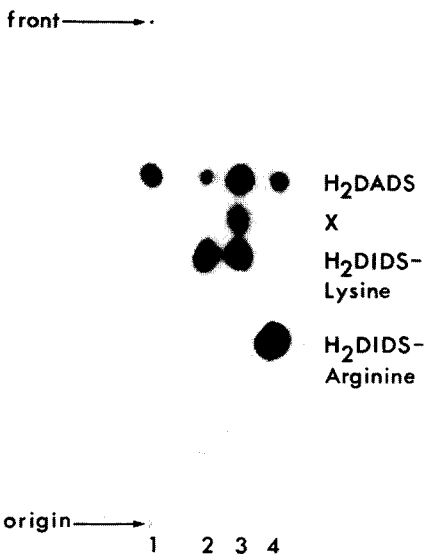

Fig. 2. Fluorography of [$^3H_2$]DIDS-conjugates from band 3 protein and of standards. A chromatogram was run on a silica gel glass plate in *n*-butanol/formic acid/water (75 : 50 : 5, v/v). 7% 2,5-diphenyloxazole in 25 ml diethyl ether was applied to the developed plate and Kodak XR-1 film was used for detection. 1, H_2DADS; 2, H_2DIDS-polylysine hydrolysate; 3, band 3 protein hydrolysate; and 4, H_2DIDS-polyarginine hydrolysate. X denotes an unidentified component.

[$^3H_2$]DIDS. The non-labeled peak (eluted by weak acid) contains the unmodified acids, whereas the labeled peak (eluted with strong acid) contains H_2DIDS conjugates. In the case of polylysine (Fig. 1B) and polyarginine (not shown), the labeled peaks are larger, indicating that the majority of the residues were conjugated with H_2DIDS. In the case of the hydrolysate from band 3 protein segments (Fig. 1A), the free amino acid peak is much larger than the H_2DIDS-labelled peak. The difference is expected because only one residue in the peptide segments should be labeled [7,8].

The material in the (labeled) peak eluted by strong acid was used for further characterization. After high voltage electrophoresis of hydrolysates from H_2DIDS-labeled polylysine and polyarginine, two peaks migrating towards the anode were observed in each case using fluorescamine. One, near the origin, corresponded in mobility to H_2DADS. The faster-moving spots are the H_2DIDS-lysine and -arginine conjugates. All of the spots contained [$^3H_2$]DIDS radioactivity.

After thin-layer chromatography on silica gel, the location of the disulfonic stilbenes were readily ascertained by fluorescence under an ultraviolet lamp. Radioactive spots in corresponding positions could be located by fluorography [18] (Fig. 2). None of the fluorescent or radioactive spots corresponded to the location of known unconjugated amino acid residues. A relatively intense spot of high R_F was found in hydrolysates of polylysine, polyarginine and membrane material. Its position corresponded in mobility to H_2DADS. It is the product that would be expected to result from exposure of H_2DIDS conjugates to the hydrolytic (acid) conditions. Fig. 3 shows the probable mechanism

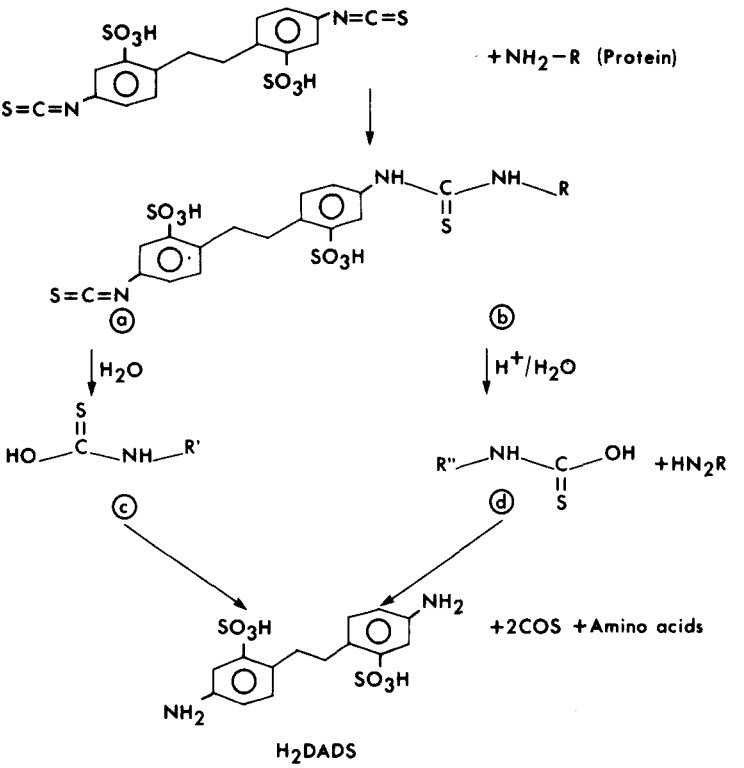

Fig. 3. H_2DIDS interaction with peptides and the probable mechanism of H_2DADS formation on hydrolysis.

of H_2DADS formation from H_2-DIDS-labeled peptides. The unreacted isothiocyano group (a) is easily hydrolyzed to a N-substituted thiocarbamic acid (c) which is unstable and breaks down to the amine and carbon oxysulfide [19]. This hydrolysis could easily occur during the protein preparation. The thiourea bonds (b) are very similar to peptide bonds of proteins and would be expected to undergo some cleavage with the 6 M HCl that is used for hydrolysis. The unstable thiocarbamic acid (d) is again the intermediate, with subsequent breakdown to the amine resulting in the formation of H_2DADS.

In addition to H_2DADS, the polylysine (Fig. 2, track 2) and polyarginine (Fig. 2, track 4) hydrolysates each give rise to a single intense fluorescent radioactive spot, taken to be H_2DIDS-lysine and H_2DIDS-arginine conjugates, respectively. The R_F for the former is 0.57 and for the latter, 0.46.

In the case of the hydrolysate from H_2DIDS-labeled membranes (Fig. 2, track 3), three spots were found, one matching the mobility of H_2DADS, one the mobility of H_2DIDS-lysine, and the other not matching any of the available reference substances (H_2DADS, H_2DIDS, H_2DIDS-lysine or H_2DIDS-arginine). It is unlikely to be H_2DIDS-cysteine, H_2DIDS-histidine, or H_2DIDS-tyrosine because such conjugates are unstable under the conditions used for hydrolysis of the peptides [12]. The reaction in each case would result in release of H_2DADS.

Quantification of hydrolysis products from [$^3H_2$]DIDS-treated membranes

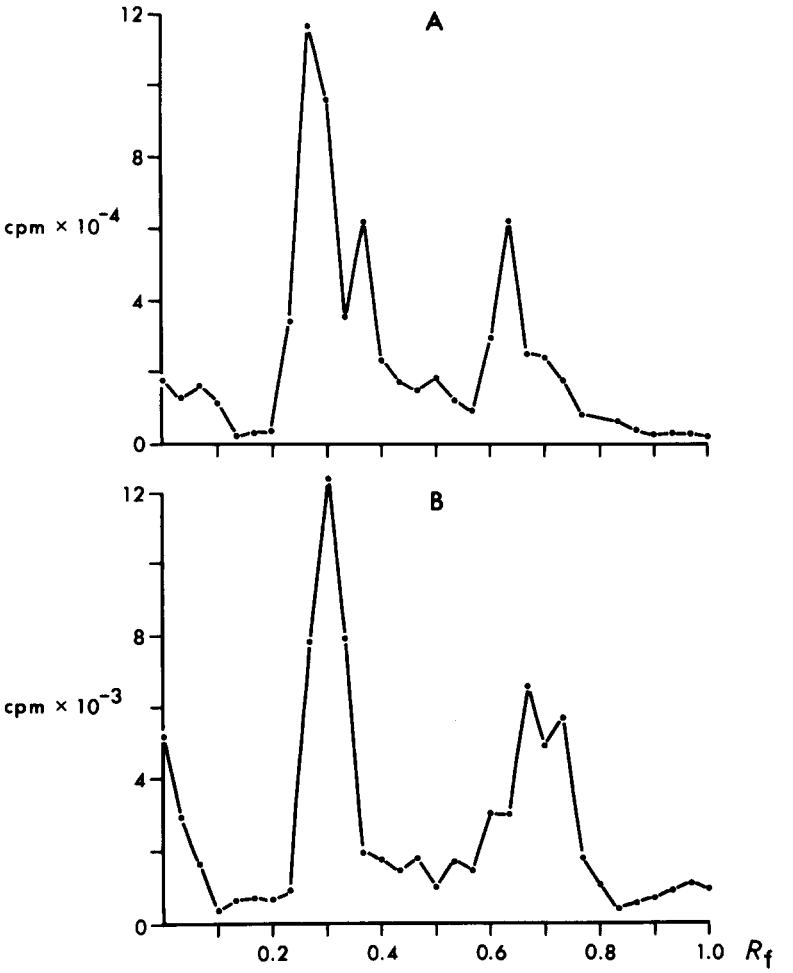

Fig. 4. Radioactive profiles from chromatograms of hydrolysed samples: A, [$^3H_2$]DIDS-labelled, alkali-extracted ghosts; B, [$^3H_2$]DIDS-labelled 15 000 dalton peptide. Chromatograms were developed in *n*-butanol/formic acid/water (75 : 50 : 5, v/v), then cut into 3-mm slices, incubated for 1 h in 1 ml formic acid, then counted in Aquasol.

was achieved by separation on precoated thin-layer chromatography plastic sheets so that successive 3-mm segments could be cut out and counted (Fig. 4A). Of the total amount of $^3H_2$DIDS, 20% was accounted for by H_2DADS, 40% by H_2DIDS-lysine, and 20% by the unidentified component. In attempting to identify the latter component, the possibility of a cross-linking reaction was considered, based on the fact that DIDS and H_2DIDS have two isothiocyano groups (Fig. 3). After reaction with band 3 protein at neutral pH, 85—90% is located in a 60 000 dalton segment produced by chymotrypsin cleavage of intact cells, with the remainder in a 35 000 dalton segment [20]. Under more alkaline conditions, an almost complete intermolecular DIDS cross-linking of the 60 000 and 35 000 dalton segments has been observed [10]. If such cross-linked band 3 protein is hydrolysed and the components separated on the thin-layer chromatography plates as above, instead of dis-

crete spots, there is a smear with a considerably greater proportion of radioactivity located between the H_2DIDS-lysine region and the H_2DADS region, indicating a large increase in unidentified material with an R_F greater than that of the H_2DIDS-lysine.

The H_2DIDS bound to the 60 000 dalton segment can, after further proteolysis, be recovered in a segment of 15 000 daltons [15]. This segment is relatively homogeneous by the criteria of end group analysis and cleavage behavior using chemical agents. After two such cleavages the H_2DIDS is almost completely recovered in a particular internal fragment of 2000 daltons [11]. Hydrolysate from the 15 000 dalton segment (separated by SDS/urea-polyacrylamide gel electrophoresis) yielded only two distinct peaks (Fig. 4B), one corresponding to H_2DADS and the second to H_2DIDS lysine. No peak corresponding to the unidentified component (Fig. 4A) is present, and the amount of radioactivity in the region is less than 10% of the total.

The increased amount of the unidentified H_2DIDS-conjugate under conditions producing a high yield of intermolecular cross-linking, and the substantially reduced amount when the purified 15 000 dalton segment of band 3 protein, presumably free of cross-linking, was used as a starting material suggests that the unidentified material is probably a conjugate between H_2DIDS, lysine and a second amino acid, by a cross-linking reaction.

DIDS reacts with peptides in a manner similar to H_2DIDS. DIDS-conjugates from polylysine, polyarginine and from band 3 protein can be separated from unconjugated amino acids on anion-exchange columns in the same way as the H_2DIDS-conjugates. The solvent system used for separating H_2DIDS-conjugates did not allow good separation of the corresponding DIDS-conjugates on thin-layer chromatography. A different solvent system was, therefore, used for their separation (see Methods). DIDS appears to react with the same amino acid residues as does H_2DIDS. In the case of polylysine and polyarginine, hydrolysates gave rise to intense fluorescent spots taken to be DIDS-lysine and DIDS-arginine and to another less intense spot, in each case identified as DADS. In the case of band 3 protein hydrolysates, three fluorescent spots were found, one corresponding to DADS, a second to DIDS-lysine and a third not corresponding to any of the available standards. It is presumably the DIDS equivalent of the unidentified spot attributed to cross-linking in the case of H_2DIDS.

Discussion

On the basis of the present study it is concluded that the covalent reaction of DIDS and H_2DIDS with band 3 protein, occurring during interaction of the probes with intact red cells, involves formation of a conjugate with a lysine residue through its ϵ-amino group. This conclusion is based largely on the finding that DIDS- and H_2DIDS-conjugates derived from hydrolysis of band 3 protein match the behavior of DIDS- and H_2DIDS-lysine conjugates run in two two different chromatographic systems. Minor undefined DIDS- and H_2DIDS-containing components may involve lysine residues cross-linked to other components. The reaction with a free amino group of lysine is consistent with the expected specificity of isothiocyano-group reactions [12]. Because each band

3 monomer has only one DIDS- or H_2DIDS-binding site [7,8,10], located in a particular 2000 dalton segment [11], it can also be concluded that the probes appear to interact with one particular lysine residue of the 28 reported to be present in the protein [21]. This high degree of specificity results from two factors. Firstly, because band 3 protein is a transmembrane protein, many of its ligands are inaccessible to non-penetrating probes such as DIDS and H_2DIDS [2]. Secondly, these compounds behave as affinity probes [2]. They bind reversibly through ionic interactions of their two sulfonic acids with positively charged protein membrane ligands, as well as additional non-ionic interactions [1—4]. The covalent reaction occurs as a second step [7,8] between a DIDS or H_2DIDS molecule reversibly bound and an adjacent group, identified in this paper as an amino group of a lysine residue. Only one site on the externally exposed part of band 3 protein appears to possess the appropriate characteristics.

The reversible reaction of DIDS and H_2DIDS with band 3 protein results in inhibition of anion transport by competition with Cl^- for binding to the transport site [9]. The particular lysine residue with which the covalent reaction occurs must, therefore, be located in close proximity to the transport site. It is not clear, however, whether that lysine is also functionally important. A direct role is not likely, based on the finding that analogs of DIDS incapable of interaction with the lysine can nevertheless be potent inhibitors [2,3]. In a related study Passow et al. [22] have identified a lysine residue in band 3 with which fluorodinitrobenzene reacts, producing irreversible inhibition of anion transport. The binding of fluorodinitrobenzene prevents the interaction of DIDS, suggesting that the same lysine residue may be the site of covalent reaction for both probes. The authors conclude that fluorodinitrobenzene-lysine residue is not directly involved in transport, but that it is linked to the transport site in an allosteric relationship.

The proximity of the DIDS-lysine residue to the transport site does not necessarily indicate that they are neighboring ligands in the primary structure, but their proximity may be related to a particular tertiary arrangement [11, 22]. It has been proposed that the intrinsic (membrane-bound) portions of band 3 exist as an assembly of at least five membrane-crossing, interacting strands [23]. The DIDS interaction (reversible and irreversible) with band 3 protein may involve ligands from several elements of the assembly.

Acknowledgements

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