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ANION TRANSPORT IN RELATION TO PROTEOLYTIC DISSECTION OF BAND 3 PROTEIN

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Summary

Sulfate efflux was measured in inside-out vesicles obtained from human red cells. Inhibition was observed in vesicles derived from cells pretreated with DIDS (4,4′-diisothiocyano-2,2′-stilbene disulfonate) or after addition of dipyridamole to the vesicles, both agents being specific and potent inhibitors of anion transport in cells. Trypsinization of the cytoplasmic side of the membrane in order to release a 40000 dalton fragment from band 3 (the purported anion transport protein) had no effect on sulfate efflux. Further degradation of band 3 to a 17000 dalton segment, by trypsinization of inside-out vesicles derived from cells that had been pretreated with chymotrypsin, also showed little reduction in transport activity. Furthermore, such vesicles derived from DIDS pretreated cells were inhibited by over 90%. In DIDS-treated cells, the agent is highly localized in band 3. In trypsinized inside-out vesicles, it is largely found in a 55000 fragment and in trypsinized vesicles derived from cells pretreated with chymotrypsin it is largely located in the 17000 fragment. The data suggest that both the anion transport and inhibitor binding sites are located in a 17000 transmembrane segment of band 3.

Introduction

On the basis of chemical labelling and reconstitution studies, it has been suggested that a 95000 dalton polypeptide of the human red cell, known as band 3 [1] is involved in anion transport. A number of anionic chemical probes that inhibit anion permeability bind relatively selectively to this protein [2—4] despite the fact that their chemical structures and reactivities are considerably different. A second group of inhibitors, including fluorodinitrobenzene [5] and

Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

pyridoxal phosphate [6] bind to other membrane components in addition to band 3. However, pretreatment of the cells with the more specific inhibitor DIDS (4,4′-diisothiocyano-2,2′-stilbene-disulfonate) results in a selective reduction of the amount of these labels located in band 3 [5,6].

Other evidence confirming the role of band 3 in anion transport has been obtained from the reconstitution of protein extracts from red cells in lipid bilayer vesicles [7,8]. Extracts enriched in band 3 [7] or containing relatively pure band 3 [8] caused an increase in sulfate transport. Prior treatment of the cells with DIDS [7] or pyridoxal phosphate [8], abolished this effect. Finally, red cell membrane vesicles selectively enriched in band 3 by detergent and alkaline extractions of other proteins are also capable of transporting anions [9]. The transport capacity is largely preserved under conditions whereby band 3 is enriched to the extent of about 95% of the remaining protein.

Digestion of the external surface of red cells with low concentrations of proteolytic enzymes results in the cleavage of band 3 into 65000 and 35000 dalton fragments with very little or no effect on its transport properties [10,11]. The 65000 dalton segment contains most of the DIDS binding sites [10], and it also seems to contain the anion transport activity because protein extracts enriched in this segment when reconstituted into phospholipid vesicles have an enhanced DIDS-sensitive sulfate transport [7]. After digestion of the cytoplasmic side of the membrane by trypsin in resealed ghosts the anion transport inhibitors are found in about equal amounts in fragments of band 3 of 48000 and 58000 daltons [11,12] and considerable anion transport activity is retained. The exact amount of retained activity is difficult to calculate because internal proteolysis of ghosts results in their fragmentation [12,13] which changes the surface-to-volume ratio in the resulting vesicles [11]; this makes the comparison between digested and control membranes difficult. After trypsin digestion of the cytoplasmic side of inside-out vesicles, transport of pyruvate is retained [14].

In the present study we have evaluated the effects of proteolytic fragmentation of band 3 from both the outside and the inside of the membrane on sulfate transport capacity and on the distribution of the covalently bound specific anion transport inhibitor, DIDS. The preparation chosen for the study was inside-out (IO) vesicles introduced by Steck and his collaborators [15,16]. Such vesicles can be prepared from cells that have been pre-exposed to proteolytic enzymes (so that the proteins on the outer face of the membrane have been hydrolysed). They offer the advantages that soluble protein fragments produced by proteolysis of the cytoplasmic face of the membrane can be washed away, and that the effects of the proteolysis on anion transport can be directly compared with untreated controls without the changes in surface to volume ratio that occur in proteolysed ghosts.

Materials and Methods

Acetylthiocholine chloride, 5,5′-dithiobis-(2-nitrobenzoic acid), phenylmethylsulfonylfluoride, trypsin, α-chymotrypsin and soybean trypsin inhibitor were all purchased from Sigma Chemical Company. DIDS and tritiated dihy-

dro-DIDS ([^{3}H]H_2-DIDS) were synthesized from their diamino analogs as previously described [2].

Vesicles from human erythrocyte membranes were prepared from recently outdated blood essentially as described by Steck [15]. Sodium phosphate, 0.5 mM pH 9, was used for the vesiculation step. Sealed vesicles were separated from the top of a 4% Dextran T-40 density barrier after centrifugation for 45 min at 50000 × *g* and washed with 0.5 mM sodium phosphate, pH 9. The sidedness of the vesicle preparations was determined by measuring acetylcholinesterase activity in the presence and absence of Triton X-100 as described by Steck and Kant [16]. Such determinations were carried out only with the non-digested vesicles since proteolytic enzymes affect the cholinesterase activity of red cells [17].

Chemical and enzymic modification of the membranes. Chemical modification of intact erythrocytes with DIDS was carried out as follows: washed red cells (25% hematocrit) were incubated at 37°C for 30 min in either phosphate-buffered saline (150 mM NaCl, 5 mM sodium phosphate, pH 8) or HEPES-buffered saline (150 mM NaCl, 10 mM HEPES, pH 7.4) containing 20 μM DIDS. The cells were then washed once with HEPES saline containing 0.5% albumin and two times with phosphate-buffered saline, before enzymatic treatment or hemolysis. A similar protocol was followed for labelling the cells with [^{3}H]H_2-DIDS. The binding of this compound to band 3 protein at 37°C in relation to its effects on anion transport are very similar to those of DIDS [18,19], but the reduced form has a much higher specific activity.

Washed cells (25% hematocrit) were digested with 0.4 mg/ml chymotrypsin in phosphate-buffered saline for 16 h at room temperature. Proteolysis was stopped by adding 30 μg/ml of phenylmethylsulfonylfluoride and cooling the sample on ice. The cells were then washed three times with 5 volumes of phosphate-buffered saline before the vesiculation procedure. Digestion of inside-out vesicles was carried out by resuspending the washed vesicle pellet to a final concentration of 1—3 mg protein/ml in a medium containing 5 mM Na_2SO_4, 0.1 mM $MgSO_4$, 1.0 mM Tris · HCl, pH 7.4 (solution A), and 50 μg/ml trypsin. The mixture was incubated for 30 min at 37°C. The reaction was then stopped with one volume of an ice-cold solution of soybean trypsin inhibitor (100 μg/ml) in solution A. The preparation was centrifuged at 45000 × *g* for 15 min and samples of the pellet and supernatant were taken for radioactivity and protein analysis. Finally, the pellet was washed once more in solution A. Where indicated, extrinsic proteins were removed by incubating the vesicles for 5 min in an ice-cold medium containing 0.01 M NaOH and 0.1 mM EDTA [15,20] after which they were immediately centrifuged and washed with solution A.

$^{35}SO_4$ flux determinations. For efflux measurements a vesicle suspension (3—5 mg protein/ml) in solution A was incubated overnight at 5°C with 4—6 μCi $^{35}SO_4$/ml. After this loading period, extravesicular $^{35}SO_4$ was removed by filtering an aliquot of the vesicle suspension through a small (2 ml) anion exchange column (Dowex AG 1—X4, chloride form, 20—50 mesh). This procedure takes only 20—30 s so that losses of intravesicular sulfate are minimal. The filtered vesicles were resuspended in a solution containing 8 mM NaCl, 0.1 mM $MgSO_4$ and 1 mM Tris · HCl, pH 7.4, at room temperature, to a final concentration of 200—400 μg protein/ml. The resuspended vesicles were immediately

split into two fractions and dipyridamole (final concentration 50 μM) was added to one of them. Ten minutes were allowed for equilibration with the inhibitor. Then, 1-ml aliquots of the suspension were withdrawn at known time intervals and the amount of $^{35}SO_4$ remaining in the vesicles was determined by Millipore filtration. The filters were dissolved in 10 ml Aquasol and counted on a Packard Tricarb Liquid Scintillation Counter. At the end of the flux measurements a sample of the final vesicle suspension was employed for protein and phosphorus determinations.

Electrophoretic analysis was performed on gels containing 5.6% acrylamide and 1% SDS (sodium dodecyl sulfate), following the procedure of Fairbanks et al. [1]. Staining of gels for proteins (Coomassie Blue) and analysis of radioactivity profiles were done as previously described [2]. Total phosphorus was assayed by the method of Rouser, Siakotos and Fleischer [21] following digestion of the samples with perchloric acid. It was assumed throughout this work that most of the phosphorus is derived from phospholipids. Protein was determined by a modification of the method of Lowry et al. [22]. Unless otherwise indicated, all the values are given as the mean ±1 S.E.M. of four determinations.

Results

Analysis of protein composition. The protein profiles of normal and proteolyzed IO vesicles are shown in Fig. 1. In agreement with previous reports [10,

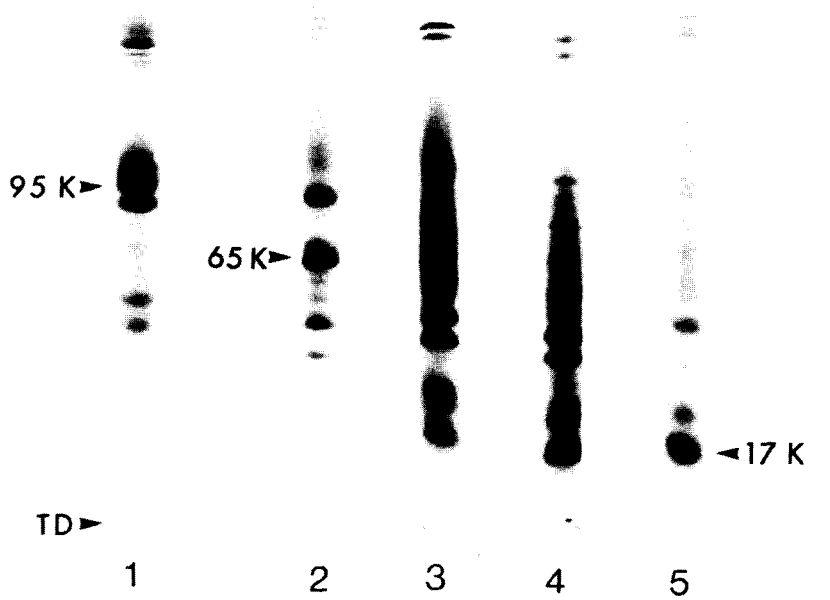

Fig. 1. Effects of proteolytic enzymes on the proteins of IO vesicles. Gel 1, control IO vesicles; Gel 2, IO vesicles, obtained from chymotrypsin-treated cells; Gel 3, trypsinized IO vesicles; Gel 4, vesicles obtained from chymotrypsin-treated cells were then trypsinized; Gel 5, same treatment as in Gel 4, followed by alkaline extraction.

11,20,23,26], the addition of chymotrypsin to the cells results in the disappearance of most of band 3 and the simultaneous appearance of a 60000 dalton polypeptide (gel 2). In a few previous studies [10,11,20], a 35000 dalton fragment has also been found to remain attached to the membrane after external proteolysis; however, its recovery is non-stoichiometric and rather low. In other studies no such fragment was detected in the gels [23—26]. Negative results were also obtained in this study. This could be explained by the prolonged exposure to chymotrypsin (16 h), leading to a more extensive proteolytic degradation of the 35000 dalton fragment and perhaps its detachment from the membrane. This segment is heavily glycosylated [27,28] so smaller fragments left in the membrane might be poorly stained.

Trypsinization of the cytoplasmic (external) surface of IO vesicles produced results consistent with those reported previously [20]. Most of the band 3 was cleaved (gel 3). Two main membrane-bound (integral) fragments were produced: a broad band with an average molecular weight of 55000 ± 5000, which has been identified by Steck et al. [20] as a major cleavage product of band 3, and a less intense 17000 dalton fragment which we interpret as a degradation product of the 55000 (see below). A 40000 dalton polypeptide was found in the supernatant after centrifugation of the trypsinized vesicles. It has been concluded that this fragment is derived from band 3 based on its staining intensity, its molecular weight, its capacity to bind glyceraldehyde-3-phosphate dehydrogenase and on cross-linking experiments with *o*-phenanthroline-$CuSO_4$ [20,29].

When IO vesicles derived from chymotrypsin digested cells were exposed to trypsin, the 60000 dalton polypeptide segment of band 3, resulting from external proteolysis, was transformed into a 17000 dalton fragment (gel 4), with the concomitant release of the 40000 dalton fragment to the medium. However, a considerable amount of protein is also found in the 50000—60000 dalton region of the gel. This could be attributed to incomplete digestion of the 60000 dalton fragment derived from band 3 or to the fragmentation of other high molecular weight proteins. Since proteolysis of the extrinsic protein bands 1 and 2 (M_r 200000 and 250000) is known to occur under these conditions, attempts were made to remove their degradation products by extracting the vesicles in an alkaline medium in the presence of EDTA [15,20]. Gel 5 shows the result of this extraction. The 17000 dalton fragment remains firmly attached to the membrane, whereas most of the other bands are substantially removed. Pretreatment of the cells with either DIDS or its dihydro derivative did not change the protein pattern of the vesicles nor the efficiency of the enzymes.

Measurement of sulfate fluxes. At the end of the equilibration period in solution A, the vesicles trapped 0.983 ± 0.013 nmol $SO_4/\mu g$ P_i. The amount of trapped SO_4 was the same when the vesicles had been treated with trypsin or were derived from cells treated with chymotrypsin, suggesting that neither the shape nor the size of the vesicles varied appreciably after a single proteolytic treatment from inside or outside. A small decrease in the trapped volume was observed in membranes treated with both enzymes (0.822 ± 0.071 nmol $SO_4/\mu g$ P_i).

To determine whether sulfate fluxes in IO vesicles takes place through the

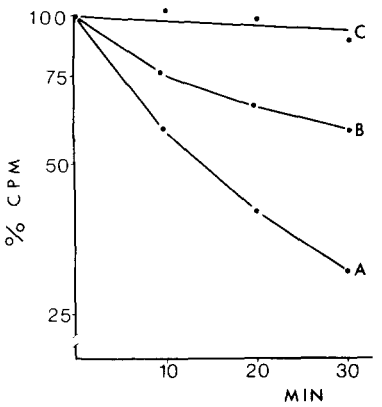

Fig. 2. Effect of inhibitors on sulfate efflux from IO vesicles. The penetrating inhibitor dipyridamole (50 μM) was added to the external solution 10 min before the first determination. DIDS (20 μM) was bound to intact red cells before vesiculation. Ordinate: percentage of the $^{35}SO_4$ remaining in the vesicles. Abscissa: time in minutes. A, control; B, dipyridamole; C, DIDS.

same pathways as in the intact cell, the effects of inhibitors were measured. Since DIDS (unpublished data) and other stilbene derivatives [5] are ineffective when applied to the cytoplasmic surface of the membrane, pretreatment of the cells with DIDS before vesiculation was necessary. On the other hand, another anion transport inhibitor, dipyridamole [30] produced inhibition even when added to IO vesicles. The results of a typical experiment are illustrated in Fig. 2. When the cells were pretreated with DIDS, the flux was inhibited by more than 90%, suggesting that nonspecific leakage pathways are not contributing significantly to the measured flux. Addition of 50 μM dipyridamole to the vesicle suspension also caused a pronounced inhibition of the initial sulfate efflux. The observed increase in the inhibition with time suggests that penetration of dipyridamole through the membrane may precede the inhibition.

TABLE I

FLUXES IN IO VESICLES AFTER VARIOUS PROTEOLYTIC TREATMENTS AND ALKALINE EXTRACTION

Except for the last column, all the data are the mean ± S.E.M. of four determinations. The percentage of inhibition by DIDS is given as the range of 2—3 determinations. The chymotrypsin is added to intact cells prior to formation of IO vesicles and therefore represents proteolysis of the outside face of the membrane. The trypsin is added to the IO vesicles and therefore represents proteolysis of the cytoplasmic face of the membrane. The DIDS is also added to the cells prior to formation of IO vesicles (see Materials and Methods), whereas the dipyridamole is added to the vesicles during the flux measurements. K refers to the initial rate of SO_4 efflux, J is the flux of SO_4 per unit phosphate.

	K (h^{-1})	J (nmol/min per μg P_i)	Dipyridamole inhibition (%)	DIDS inhibition (%)
Control	2.23 ± 0.03	0.0365 ± 0.0009	50.4 ± 4.7	89.9—95.3
Chymotrypsin	2.08 ± 0.06	0.0358 ± 0.0012	58.7 ± 3.8	92.0—99.3
Trypsin	2.30 ± 0.11	0.0352 ± 0.0013	32.1 ± 3.7	85.6—92.7
Chymotrypsin + trypsin	2.15 ± 0.06	0.0293 ± 0.0018	47.8 ± 1.9	93.0—94.5
Chymotrypsin + trypsin + alkaline extraction	2.28 ± 0.06	0.0320 ± 0.0020	53.5 ± 8.4	—

The effects of proteolytic treatments on anion transport are shown in Table I. The transport is expressed as the initial flux per unit of phospholipid phosphate since considerable changes in the protein content of the vesicles are observed upon treatment with proteases. Neither prior treatment of the cells with chymotrypsin nor digestion of the IO vesicles with trypsin, modified the rate of sulfate efflux from the vesicles. Pretreatment of the cells with DIDS before either digestion resulted in a large (more than 90%) and reproducible inhibition of the efflux. Dipyridamole also consistently inhibited the efflux when added to the modified vesicles, although the magnitude of the effect was more variable.

Sulfate efflux was slightly decreased in the vesicles in which band 3 was reduced to a 17000 dalton segment after the combined enzyme treatment. This decrease was caused mainly by the small reduction (16%) in trapping of the ion since the rate constant of the efflux in these vesicles was 96% of that in the control vesicles (Table I). The reduced trapping can most simply be explained as a breakdown of the permeability barrier in a small fraction of the vesicles. Based on this assumption, the flux per intact vesicle would change by less than 5%. Even if the reduced trapping were due to a change in size of the vesicles, the concomitant change in the flux per vesicle would still be less than 20%. The inhibitory potency of DIDS and dipyridamole was not affected by the combined enzymatic digestion. indicating that both the transport and the inhibitor binding sites remain in the membrane after removal of all but a small segment of the 95000 dalton protein. Sulfate fluxes were also measured in vesicles that had been extracted with alkali after the combined proteolysis. These vesicles, in which the 17000 dalton fragment is the predominant remaining membrane protein component (gel 5, Fig. 1), transport sulfate at normal rates and this transport is inhibited by dipyridamole.

Although the sulfate flux per unit of vesicle phosphate is not substantially altered as the stripping of membrane proteins by proteolysis and alkali treatment proceeds (Table I), the amount of protein per unit of phosphate is decreased by a factor of about 3 (Table II). The flux per unit of protein must, therefore, be increased by the same factor.

Location of DIDS-binding sites. [^{3}H]H_2-DIDS was allowed to react covalently with red cell membranes prior to vesiculation and proteolysis. In agreement with previous studies [2,18,19], most of the label is located in the 95000 dalton region of the gel in untreated membranes (Fig. 3A). Removal of the soluble 40000 dalton fragment from band 3 by trypsin treatment of the vesi-

TABLE II

RELATION OF THE AMOUNT OF DIDS-LABELLED PROTEIN COMPARED TO TOTAL PROTEIN AND PHOSPHOLIPID IN PROTEASE MODIFIED GHOSTS AND VESICLES

The results are the mean of two determinations.

	μg protein/μg P_i	cpm/μg protein	cpm/μg P_i
Chymotrypsin-treated ghosts	42.8	57.4	2453
+ Low ionic strength extraction	32.8	75.7	2376
+ Trypsin treatment	19.1	134.6	2577
+ Alkaline extraction	15.6	147.2	2296

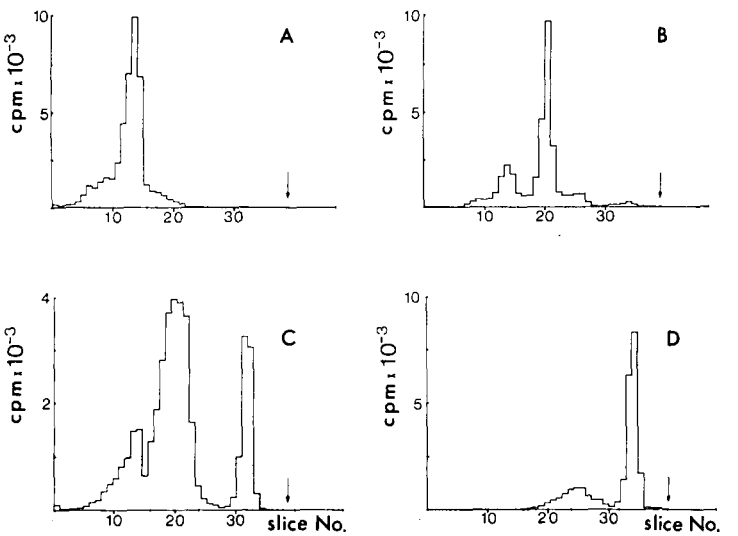

Fig. 3. Distribution of (^{3}H)H_2-DIDS in the proteins of proteolysed IO vesicles. Labelled vesicles were analysed by SDS polyacrylamide gel electrophoresis; the gels were then sliced and counted. Gel A, intact IO vesicles; Gel B, vesicles obtained from chymotrypsin-treated cells; Gel C, trypsinized IO vesicles: Gel D, IO vesicles from chymotrypsinized cells were then treated with trypsin. The arrows indicate the position of the tracking dye. Ordinates: thousands of cpm per slice. Abscissa: slice number.

cles did not decrease the amount of label bound to the membranes (Table II). In these vesicles the radioactivity was distributed in 3 zones: 14% was bound to undigested band 3 molecules (95000 dalton), 65% was distributed in the 50000—60000 dalton region, and the remaining 21% was found to coincide with the 17000 dalton Coomassie Blue-stained band (Fig. 3C), indicating that at least part of the 50 000—60 000 and 17 000 dalton polypeptides are derived from band 3. The mechanism by which the 17000 fragment is generated by trypsin alone is not certain. It could be due to the presence of unsealed vesicles or membrane fragments (even though attempts were made to separate sealed from unsealed vesicles (see Materials and Methods). Such unsealed vesicles would be accessible to trypsin on both faces of the membranes. Normally, in cells, trypsin does not digest band 3 on the outside face, but it does so at the low ionic strength [28] used in the vesicle studies.

In vesicles generated from chymotrypsin-treated cells, about 95% of the radioactivity remains in the membrane, being distributed in two regions: a small fraction (15—23%) of the [^{3}H]H_2-DIDS remains in the 95000 dalton region whereas most of the label appears in the 60000 dalton region (Fig. 3B) confirming previous observations [10,11]. When IO vesicles from such chymotrypsinized cells were exposed to trypsin, less than 2% of the label was removed. Approximately 75% of the inhibitor was now found in the 17 000 dalton fragment (Fig. 3D), the rest being distributed in the 50000—60000 dalton area. This pattern was not modified by alkaline extraction of the membranes prior to electrophoresis, even though a number of stained bands are removed by this treatment (Fig. 1, gel 5). The failure of the digestion to convert all of the DIDS-labelled band 3 into the 17000 dalton peptide may be due to two factors. Firstly the chymotrypsin cleavage from the outside is at best 90% effec-

tive [10,11]. The uncleaved portion when proteolysed by trypsin on the inside face should give rise to a DIDS-labelled segment of about 50000 daltons. Inhibitor-labelled fragments of this size have been reported in resealed ghosts containing trypsin [11,12] and were also found (Fig. 3C) in trypsin-treated IO vesicles derived from normal cells (not treated with chymotrypsin). Secondly, up to 15—20% of the vesicles in the inside-out preparation are right side out (based on the determination of acetylcholinesterase activity) [15,16]. Such vesicles should be cleaved on the outside face by chymotrypsin but not by trypsin on the inside face. The DIDS in this case should be located in a segment of 60000 daltons [10].

Discussion

Considerable evidence has been reported supporting the conclusion that band 3, the 95000 dalton, intrinsic membrane protein, is directly involved in anion transport (see Introduction). Yet, in the present paper it is demonstrated that IO vesicles in which most (over 75%) of the 95 000 dalton protein has been reduced to a 17000 dalton fragment by proteolytic cleavages, show little loss of anion exchange capacity and display the same sensitivity to specific inhibitors as the intact cells (Table I). In parallel, the irreversible inhibitor, DIDS, which is highly localized in band 3, is largely found in the 17000 dalton fragment following proteolysis (Fig. 3D).

In order to discuss the present findings in terms of the localization of the anion transport activity, it is necessary to briefly summarize certain information concerning band 3. Its possible arrangement in the membrane is presented in Fig. 4, adapted largely from Steck et al. [20] and Jenkins and Tanner [32]. The primary chymotrypsin cleavage on the outer face of the membrane is designated P1. It produces segments of 60000 and 35000 daltons [10,11,20] with the C-terminus of the protein on the 35000 segment and the N-terminus on the 65000 segment [31,32]. Additional cleavages may occur in the 35000 segment either in the intact cell or after the proteins are dissolved in SDS [10], so that its recovery may be low. The primary inside cleavage with trypsin is marked P2. It produces segments of about 40000 and 50000 daltons [20]. The former

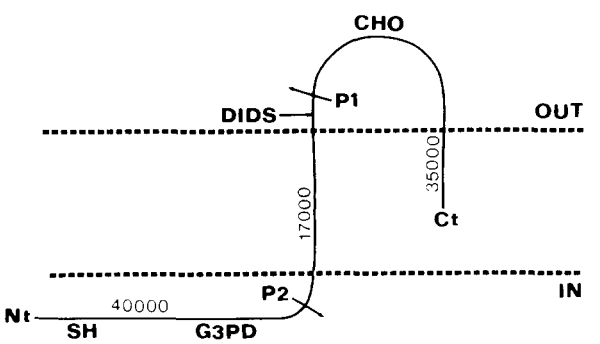

Fig. 4. Diagram of proposed arrangement of band 3 in the membrane. CHO, carbohydrates; Ct, C-terminal end; Nt, N-terminal end; P1, chymotrypsin cleavage; P2, trypsin cleavage; SH, sulfhydryl group; G3PD, glyceraldehyde-3-phosphate dehydrogenase binding site.

dissolves out of the membrane (confirmed in the present study). It is located on the inner face of the membrane based on the presence of two markers, the S-S cross-linking site that is involved in formation of band 3 dimers [20] and the glyceraldehyde-3-phosphate dehydrogenase binding site [29]. These are marked as SH and G3PD in Fig. 4. This soluble fragment is not seen when ghosts are treated with trypsin presumably because it is trapped within vesicles that are formed as the hydrolysis of the membrane protein proceeds [12,13]. Furthermore, other cleavages of this segment by trypsin take place so that smaller fragments of 20000 daltons or less may be found rather than the intact 40000 dalton segment [20,28,24].

The 50000 dalton segment is membrane bound. In our hands, it appears as a broad band that contains most of the DIDS associated with band 3. Passow and his collaborators [11,12] were able to resolve the labelled band into a double peak (48000 and 58000 daltons). Tanner and Jenkins [32] also resolved the same segment into 2 peaks by staining techniques and demonstrated that the electrophoretic behaviour of the segment was due to carbohydrate heterogeneity rather than peptide heterogeneity.

When cleavages are made at both P1 and P2, the predominant remaining segment of band 3 by either staining or DIDS-labelling is the transmembrane 17000 dalton segment. Because the DIDS binding is approximately stoichiometric with band 3 [19] and because little DIDS-labelled material is solubilized by the proteolytic procedures, the portion of band 3 that contains the DIDS binding site, primarly the 17000 dalton fragment, must be almost fully preserved in the vesicles. This segment presumably contains the site of inhibition of anion transport by DIDS. Because the DIDS does not penetrate [2], the binding site must be located on ligands of the fragment that are exposed to the extracellular environment, presumably between the cleavage sites at P1 and the lipid penetrating portion.

Cleavage of band 3 at P1 and P2, with concomitant loss of the 40000 dalton fragment from the vesicles, and of any identifiable portions of the 35000 dalton fragment resulted in little loss of anion transport activity (Table I). This result is reasonably consistent with previous studies in which only one of the two cleavages was performed. Outside cleavage of band 3 with chymotrypsin can be accomplished without effect on the anion flux [10,11]. After inside cleavage carried out with trypsin in resealed ghosts much, but not all, of the anion transport activity is retained. The calculation of the degree of reduction in this case is complicated by the changes in surface to volume ratio due to fragmentation of the ghosts that accompany the trypsin treatment [12,13]. The changes in surface to volume ratios were avoided in the present study by measuring all fluxes in IO vesicles. The various enzyme treatments and extractions seem to produce little change in the size of the vesicles based on the observation that the amount of SO_4 that can be "trapped" in the vesicles after equilibration is not substantially altered. The largest change was a 16% reduction in trapped SO_4 in vessicles digested from both outside and inside. In contrast to the reported reductions in anion transport activity following trypsinization from within resealed ghosts [11,12], the same cleavage performed on IO vesicles either with or without chymotrypsin cleavage at the outer face of the membrane resulted in little loss of transport activity (Table I). A preliminary

report also indicates that tryptic cleavage of the same kind in IO vesicles has little effect on pyruvate transport [14].

The cleavage of 75% of band 3 to the 17000 dalton fragment without appreciable loss of anion transport activity suggests that this segment is responsible for the functional activity. The high degree of localization of DIDS in the segment supports this view. The DIDS binding site may also identify the anion-binding site of the transport system because it has recently been demonstrated that Cl^- binding by band 3 (detected by NMR techniques) is largely blocked by pretreatment of cells with DIDS [33], and that disulfonic stilbenes act as competitive inhibitors of Cl^- transport (Shami, Y., Knauf, P. and Rothstein, A., unpublished observations).

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