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INHIBITION OF ANION TRANSPORT ASSOCIATED WITH CHYMOTRYPTIC CLEAVAGES OF RED BLOOD CELL BAND 3 PROTEIN

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Right-side-out vesicles derived from red blood cells treated with chymotrypsin retain specific anion transport function (defined as transport sensitive to the specific inhibitor, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS)), even though the transport protein, band 3, is cleaved into two segments of 60 and 35 kdaltons. In contrast, vesicles derived from alkali-stripped ghosts treated with relatively high concentrations of chymotrypsin retain almost no specific anion function. The loss of function appears to be related to additional cleavages of band 3 protein that occur in treated ghosts, the 60-kdalton segment being reduced first to a 17- and then to a 15-kdalton segment and the 35-kdalton segment being reduced to a 9-kdalton segment plus a carbohydrate containing fragment. The chymotryptic cleavages of band 3 protein of ghosts are preferentially inhibited by high ionic strength, the production of the 9-kdalton segment being somewhat slower than that of the 15-kdalton segment. Vesicles derived from ghosts treated with chymotrypsin at different ionic strengths show a graded reduction in specific anion transport activity, but it was not possible to determine, definitively, which of the additional cleavages was inhibitory. In the light of these data and other information, the functional role of the segments of band 3 is discussed.

Introduction

Band 3, the anion transport protein of the red blood cell [1,2], spans the bilayer and is, therefore, susceptible to proteolytic cleavage at the inner and outer faces of the membrane by a number of enzymes, including pronase [3–5], papain [5,6], trypsin [7,8], and chymotrypsin [4,5,7]. Certain of the cleavages are inhibitory to anion transport, whereas others are not. Treatment of the outside face of the membrane in intact cells with pronase [5,9,10] or with papain [5,6] can result in substantial inhibition of anion transport, whereas treatment with chymotrypsin or trypsin has no inhibitory effect [4,5]. The treatment of the cytoplasmic face with trypsin, in released ghosts [8], or chymotrypsin, using inside-out vesicles [11], also results in little or no loss of transport activity.

The cleavage sites in band 3 are clearly defined in the case of chymotrypsin. Exposure of the outside face of the membrane to the enzyme results in a single cleavage, producing membrane-bound segments of 60 and 35-kdaltons [3–5,7], the latter containing the carbohydrate of band 3 and its C-terminus [12–14]. Exposure of the inner face of the membrane results in an additional cleavage of the 60-kdalton segment. About 40-kdaltons of peptide from the N-terminal end of band 3 becomes soluble and a 17-kdalton transmembrane segment remains in the membrane [7]. After either, or both cleavages, transport activity is retained [11].

Recently it has been demonstrated that treatment of 'leaky' ghosts with chymotrypsin can result in

Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

additional cleavages of band 3. The residual membrane-bound peptides are 15 and 9 kdaltons, the former derived from the 17-kdalton transmembrane segment and the latter from the 35-kdalton, C-terminal segment [15]. Preliminary, qualitative observations indicate that some specific anion transport activity is retained by vesicles containing the 15- and 9-kdalton segments as the predominant peptides [16]. In the present study, however, vesicles in which band 3 was 'completely' cleaved to 15- and 9-kdalton segments were found to be devoid of specific anion transport activity. Under conditions of controlled proteolysis, correlations were attempted between the particular cleavages in band 3 and the loss of transport activity in an attempt to determine the functional role of its various segments.

Materials and Methods

Recently outdated human red blood cells were used for all experiments. Cells were separated from plasma by centrifugation and washed three times with 4 volumes of phosphate-buffered saline (150 mM NaCl, 5 mM sodium phosphate, pH 8) at 4°C. 80 ml of washed cells were resuspended in phosphate-buffered saline at 25% hematocrit. One-half of the cell suspension was incubated with 10 μM DIDS (4,4'-diisothiocyano-2,2'stilbenedisulfonic acid) for 1 h at 37°C. Under these conditions, essentially all of the specific DIDS-sensitive anion transport sites are blocked irreversibly by a covalent reaction of the probe [1,17]. Excess DIDS was removed by washing cells once with phosphate-buffered saline containing 0.5% bovine serum albumin (BSA, Sigma Chemical, St. Louis, MO) and twice with phosphate-buffered saline. Washed cells were treated with 1.5 mg/ml of chymotrypsin (Sigma Chemical) for 1.5 h at 37°C to produce external cleavage of band 3 into membrane-bound segments of 60 and 35 kdaltons [4]. Proteolysis was stopped with 100 μg/ml of the inhibitor phenylmethylsulfonyl fluoride (PMSF), and the chymotrypsin was removed by washing twice with phosphate-buffered saline containing 0.5% bovine serum albumin and twice with saline alone.

Hemoglobin-free leaky red blood cell ghosts were prepared according to Steck and Kant [18] by lysing cells in low ionic strength phosphate buffer (5 mM sodium phosphate, pH 8) at 4°C. Ghosts were extracted for 2 min with 10 mM NaOH containing 0.1 mM EDTA at 4°C to remove extrinsic proteins [7] that are unrelated to anion transport [11]. The 'stripped' ghosts were immediately washed twice with the lysing buffer at 4°C. Aliquots containing 4 mg of protein in 4 ml of suspension were subjected to a second treatment with chymotrypsin for 1.5 h at 37°C. Proteolysis was stopped by adding 100 μg/ml of PMSF and cooling the samples on ice. Each suspension was then quickly frozen and thawed three times to allow release of any enzyme trapped in vesicles, and then washed twice with 10 volumes of phosphate buffer containing 0.5% bovine serum albumin and twice with buffer alone. Virtually complete cleavage of band 3 was found to occur with 1.5 mg of chymotrypsin per ml under these conditions, so this concentration was chosen as a standard treatment.

For experiments where the ionic strength was varied during proteolysis, the 'stripped' ghosts were divided into aliquots containing 4 mg protein each (usually 400–500 μl) and kept on ice. Ionic strength was varied by adding small volumes of ice-cold concentrated NaCl solution (300 mM NaCl dissolved in 5 mM sodium phosphate, pH 8) to give the final concentration desired. Chymotrypsin was added and the final volume was brought to 4 ml with ice-cold phosphate buffer. Samples were then transferred to a 37°C water-bath for 1.5 h. Final protein concentration was 1 mg/ml, and chymotrypsin concentration was 1.5 mg/ml unless otherwise indicated. Proteolysis was stopped and samples were washed as described above.

Proteolysed ghosts were transformed into right-side-out vesicles using the method of Steck and Kant [18]. Sealed vesicles, separated from unsealed membrane fragments by flotation on the top of a Dextran T-40 density barrier (density = 1.03) after centrifugation for 45 min at 50 000 $\times g$, were washed with phosphate buffer by centrifuging and resuspending [11].

For sulfate efflux measurements, sealed vesicles were resuspended to a final concentration of 1–2 mg protein/ml in a medium containing 10 mM Na_2SO_4, 0.1 mM $MgSO_4$, and 1 mM Tris-H_2SO_4, pH 7.4 (sulfate medium). The vesicle suspension was equilibrated overnight at 4°C with approx. 5 μCi/ml $^{35}SO_4^{2-}$ (New England Nuclear, Boston, MA). After

equilibration, the extravesicular isotope was removed by filtering 250 μl of the suspension through a 3 ml column of Dowex AG 1X4 (20–50 mesh, chloride form, Bio-Rad Laboratories, Richmond, CA) that had been washed and equilibrated with sulfate medium. Vesicles were eluted from the column with 2 ml of sulfate medium (room temperature). Efflux determinations were started immediately by filtering 0.4-ml aliquots through 1.5 ml anion exchange columns. Vesicles were eluted from the column with 1.6 ml of ice-cold sulfate medium. The filtrate, which contained the vesicle suspension free of extravesicular isotope, was collected directly into scintillation vials and counted with 14 ml Aquasol (New England Nuclear). Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc.). The amount of $^{35}SO_4^{2-}$ 'trapped' in the vesicles at the start of each experiment was estimated by extrapolation to zero time.

The method used, involving separation of a membrane fraction by flotation on top of a dextran solution of defined density, provides a vesicle population that is 'sealed' to dextran. The vesicles float because they have an internal aqueous compartment of lower density, whereas unsealed vesicles or membrane fragments will be of higher density and will pass into the dextran cushion under centrifugal force. In the present experiments, in which the membranes were subjected to varying degrees of proteolysis, peptide analysis was not an appropriate measurement for determining the amount of membrane in the various vesicle fractions. Quantification was therefore based on analysis of their organic phosphate content (primarily phospholipid phosphate [11]). Washed vesicles were hydrolysed by digestion with perchloric acid and the total phosphorus was determined by the method of Rouser et al. [19].

Although the vesicles on the top of the dextran cushion can be presumed to be 'sealed' to dextran, they are not necessarily 'sealed' to SO_4^{2-}. If, for example, only a small fraction of the vesicles were participating in the SO_4^{2-} flux measurements, correlations of transport activity with degrees of proteolytic cleavage (which involves acrylamide gel electrophoresis of peptides from the whole vesicle population) might be misleading. In the literature this question is not often addressed. It was decided to use high molecular weight poly(ethylene glycol) or dextran as markers for extravesicular space in a packed pellet of vesicles, tritiated water to measure the total water space, and $^{35}SO_4^{2-}$ to measure vesicular sulfate space, allowing calculation of the size of the vesicular water compartment and the fraction occupied by sulfate. Similar procedures have been used to determine the fraction of vesicular space in which Rb^+ is trapped [20,21]. Briefly, for water space, vesicles were incubated in sulfate medium (see above) for 10 min at room temperature with 10 μCi ^3H_2O (Amersham, 5 mCi/ml) and with 1 μCi poly(ethylene glycol) (^{14}C-labelled PEG, New England Nuclear, 250 μCi/ml) per ml. Aliquots (800 to 900 μl) of the vesicle suspension containing approx. 80 μg of phosphorus per ml were centrifuged at 35 000 $\times g$ for 30 min at 4°C. The pellets were dissolved in 1 ml of 1% SDS and transferred to counting vials containing 11 ml of Aquasol.

For sulfate space determinations vesicles were incubated overnight at 4°C with $^{35}SO_4^{2-}$ as for the flux studies. [^{3}H]Dextran (Amersham, 1 mCi per ml) was added at 12.5 μCi per ml. Aliquots were pelleted and counted as described in the preceding paragraphs. The vesicular sulfate space was calculated using the dextran determinations to correct for extravesicular sulfate. An alternative procedure involved the separation of vesicular trapped sulfate by use of the anion-exchange column as outlined above for flux measurements, except that the temperature was 4°C (rather than room temperature) to minimize sulfate efflux during the separation procedure (less than 1 min). The results by the two procedures were essentially identical.

Electrophoretic analysis was performed on slab gels containing 15% acrylamide, 6.25 M urea, and 0.1% sodium dodecyl sulfate (SDS). This system was adapted from that of Swank and Munkres [22]. Aliquots of vesicles containing 100 μg protein each were dissolved in solubilizer containing 1% SDS and 8 M urea. Pyronin Y was used as the tracking dye. The gels were stained with Coomassie Blue and scanned with a Beckman GS2 scanner on a Beckman Acta CII spectrophotometer.

DIDS was synthesized in our laboratory by Dr. M. Ramjeesingh, as described previously [1]. Protein determinations were made by a modification of the method of Lowry et al. [23] or by the Bio-Rad protein assay system (Bio-Rad Laboratories).

Results

Of the total membrane material derived from either control or chymotrypsinized (1.5 mg for 1.5 h at 37°C) ghosts, 70 to 75% (based on phospholipid phosphorus determinations) floats on the top of a dextran cushion and can be considered to be 'sealed' to dextran, that is, to exclude dextran from the intravesicular space. Much of the remainder is found in a visible band within the dextran cushion, considered to be unsealed vesicles or membrane fragments. When the 'sealed' fraction is centrifuged to form a vesicle pellet, the extravesicular compartment (measured by PEG or dextran)'was 40 to 50% of the total, for membranes from both control and chymotrypsinized ghosts. Intravesicular water space based on ^3H_2O determinations was somewhat higher in vesicles from chymotrypsinized ghosts, 0.54 compared to 0.46 μl per μg of P (averages of two estimates, each in duplicate). These data suggest that the vesicles derived from chymotrypsinized ghosts are slightly but not substantially larger than those derived from control ghosts. This conclusion is borne out by electron microscopic examinations which indicate that the vesicles in each case are of relatively uniform size (estimated to average about 0.5 μm in diameter). The water space based on $^{35}SO_4^{2-}$ determinations were about 30% higher than those based on ^3H_2O, 0.77 and 0.61 μl per μg of P for vesicles derived from chymotrypsinized and control ghosts, respectively. No specific explanation for the larger water space calculated from $^{35}SO_4^{2-}$ can be given. It may however be due to the presence of a membrane binding component for SO_4^{2-} (which might be significant, given the high surface to volume ratio of the vesicles). The differential in SO_4^{2-}-space between the vesicles derived from control and from chymotrypsinized ghosts was not always as great as indicated above. In four different experiments the average ratio for proteolysed versus non-proteolysed ghosts were 1.04 ± 0.02 (S.E.).

The data reported above suggest that vesicles derived from chymotrypsinized ghosts do not differ substantially from those derived from normal ghosts will respect to the proportion that are sealed ('float' on a dextran gradient), size (no large differences in vesicular water space per unit of phospholipid-P), or the amount of trapped SO_4^{2-}. Furthermore, the vesicle population that is sealed to dextran appears to be sealed to SO_4^{2-} as well. It appears likely that most if not all of the vesicles are contributing to the measured sulfate fluxes, and that comparisons of fluxes and of degress of proteolysis of band 3 (as described below) involve the same populations of vesicles. Essentially similar conclusions have been reported previously for right-side-out vesicles prepared by the same procedure, with respect to the trapping of Rb^+ by essentially all of the vesicles collected from the top of the dextran cushion [20,21].

Vesicles derived from non-chymotrypsinized 'stripped' ghosts containing primarily the 60- and 35-kdalton segments of band 3 protein (resulting from prior chymotrypsin cleavage of the cells), displayed a sulfate efflux that was largely sensitive to DIDS (Fig. 1). In eight such experiments, 80–95% of that flux was inhibited by the agent. In contrast, vesicles

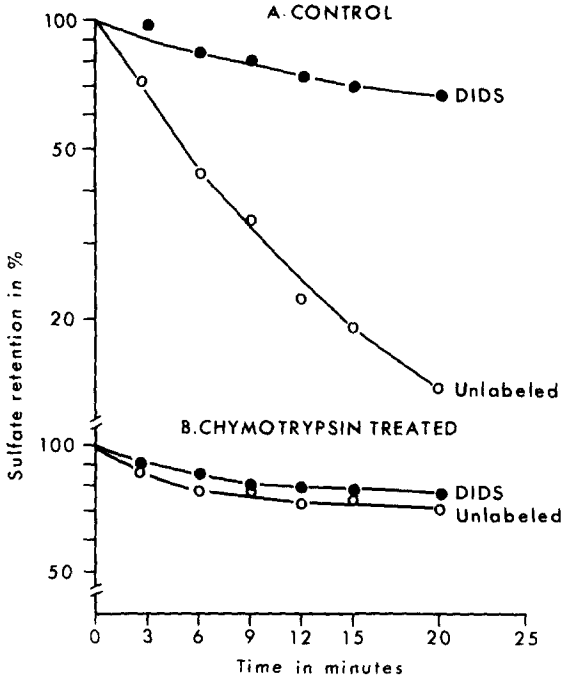

Fig. 1. Effect of DIDS on $^{35}SO_4^{2-}$ retention by vesicles derived from (A) control ghosts, and (B) chymotrypsinized ghosts. The DIDS was applied to cells prior to formation of ghosts. Its inhibitory effect persists because it binds irreversibly to band 3 protein [1]. Ghosts were made from cells treated with chymotrypsin to split band 3 protein into 60- and 35-kdalton segments [4].

derived from ghosts treated with chymotrypsin displayed a substantially decreased anion flux that was virutally insensitive to DIDS. At the end of the equilibration period in sulfate medium, the trapped volume of SO_4^{2-} (based on retention of labelled SO_4^{2-}) was the same for both sets of vesicles. The loss of specific DIDS-sensitive anion activity in vesicles from chymotrypsin-treated ghosts was therefore attributed to the further cleavages of band 3 protein. Examination of the cleaved peptides by urea-SDS-polyacrylamide gel electrophoresis indicates almost no detectable 60- or 35-kdalton segments. The predominant stained band 3 peptides are 15 and 9 kdaltons (see gel-scan in Fig. 2 for low ionic strength), as previously reported [15].

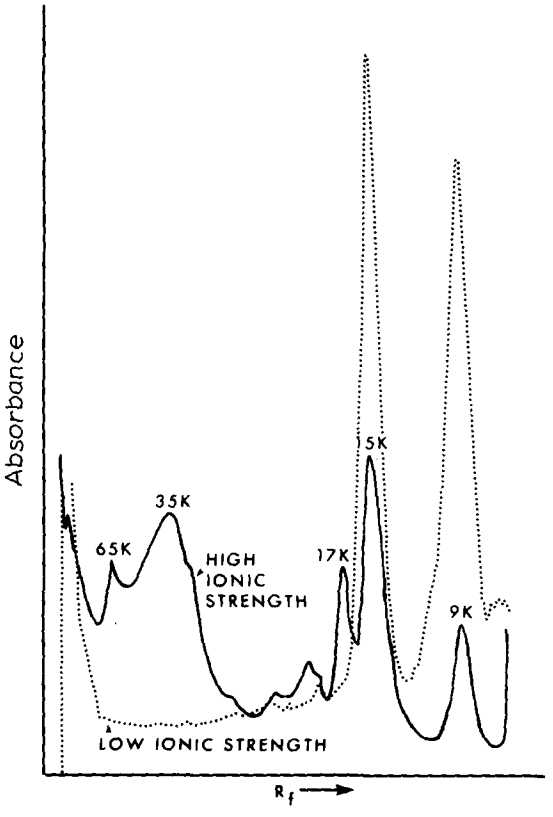

Fig. 2. Scans of stained peptides from alkali-stripped ghosts treated with chymotrypsin at low and high ionic strength. The ghosts were treated with 1.5 mg/ml chymotrypsin for 1.5 h at 37°C in the presence of 5 mM sodium phosphate, pH 8 (low ionic strength), or 5 mM sodium phosphate plus 150 mM NaCl, pH 8 (high ionic strength). The peptides were separated by urea-SDS-polyacrylamide electrophoresis.

The degree of proteolysis of band 3 in ghosts can be controlled by adjusting the chymotrypsin concentration [15], but since the production of the 15- and 9-kdalton segments under these circumstances appear to proceed in parallel, it would not be possible to determine which of the additional cleavages are inhibitory. It was found, however, that increasing the ionic strength during chymotrypsinization of ghosts resulted in a substantial inhibition of proteolysis. With 150 mM NaCl, large amounts of uncleaved 60- and 35-kdalton peptides remained. Consequently, the amounts of 15- and 9-kdalton fragments were greatly reduced, and some intermediate fragments, e.g., the 17-kdalton segment, were evident in the stained gels (Fig. 2, 'high ionic strength'). The effect is due to ionic strength per se, rather than the osmolarity, since 150 mM KCl also affords protection against proteolysis, whereas 300 mM sucrose does not.

Chymotrypsin cleavages in media of increasing ionic strength are illustrated by the acrylamide gel patterns of Fig. 3. With increasing ionic strength the 60- and 35-kdalton segments are present in larger quantities. The amount of 17-kdalton segment, which is negligible at very low ionic strength, is substantial at high ionic strength. A band of about 22 kdaltons of unknown origin behaves in parallel to the 17-kdalton segment.

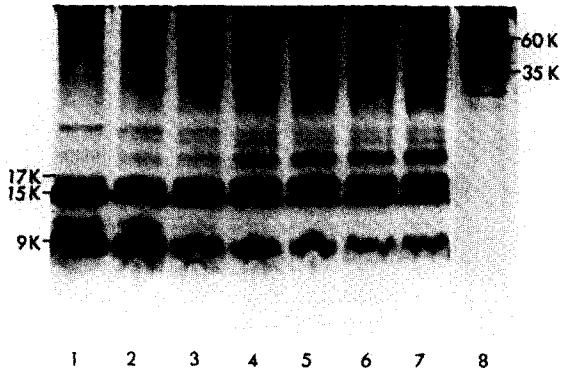

Fig. 3. Urea SDS-polyacrylamide electrophoresis of peptides from alkali-stripped ghosts treated with chymotrypsin in the presence of varying ionic strengths. Ghost suspensions were treated with 1.5 mg/ml chymotrypsin for 1.5 h at 37°C in the presence of 5 mM sodium phosphate, pH 8 and the indicated concentrations of NaCl. Gel 1, no NaCl; Gel 2, 25 mM NaCl; Gel 3, 50 mM NaCl; Gel 4, 75 mM NaCl; Gel 5, 100 mM NaCl; Gel 6, 125 mM NaCl; Gel 7, 150 mM NaCl; Gel 8, control (no chymotrypsin), no NaCl.

The comparative rates of cleavage of 60- to 17- to 15-kdalton segments and of 35- to 9-kdalton segments are difficult to quantify precisely. The best estimate can be made by measuring the relative amounts of the 17-, 15- and 9-kdalton segments since they are present in the gels in relatively sharp discrete bands. The production of the 9-kdalton segment is inhibited to a greater extent by high ionic strength than the production of the 15-kdalton segment. Thus the ratio of the 15- to 9-kdalton segments is about one to one at low ionic strength (5 mM) and about two to one at high ionic strength (155 mM), based on estimates of the areas under the respective peaks in the gel scans of Fig. 2. The same trend is evident for the series of gels from ghosts treated with chymotrypsin at varying ionic strengths (Fig. 3). The estimated areas under the peaks in gel scans are presented in Fig. 4. High ionic strength (155 mM) inhibits all of the cleavages to some extent, but to different

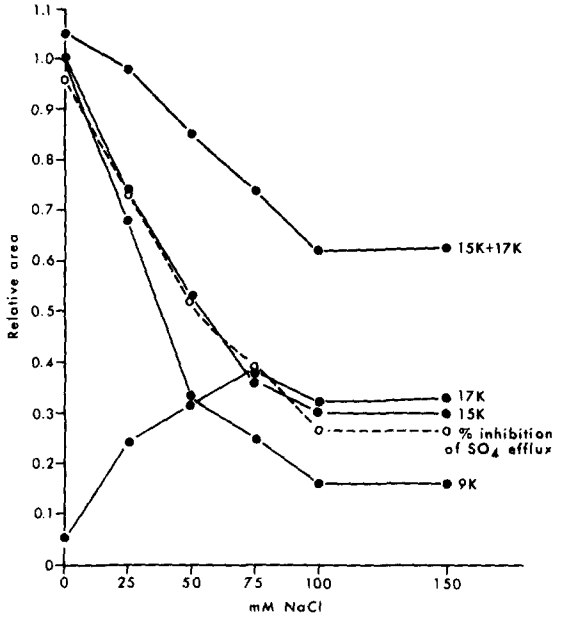

Fig. 4. Relative inhibition of anion transport and amounts of 17-, 15- and 9-kdalton segments produced by proteolysis of alkali-stripped ghosts at different ionic strengths. The inhibition curve for SO_4^{2-} efflux is calculated from the data shown in Fig. 5. The gel series shown in Fig. 3 were scanned and the areas under the peaks of the 15- and 9-kdalton peptides were estimated and normalized to values at the lowest ionic strength (assumed to represent complete proteolysis). The amount of 17-kdalton segment is expressed as a fraction of the maximal amount of 15-kdalton segment.

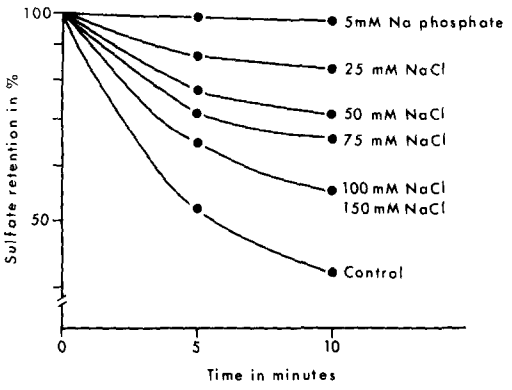

Fig. 5. Sulfate retention by vesicles derived from alkali-stripped ghosts treated with chymotrypsin at varying ionic strengths. The DIDS-insensitive component is subtracted in each case. All experiments were performed in the presence of 5 mM sodium phosphate. The concentration of NaCl present during chymotrypsinization is indicated.

degrees. The cleavage of the 60-kdalton segment proceeds in the sequence 60 to 17 to 15 kdaltons [15]. The extent of the first cleavage in the sequence is therefore represented by the total of 15 plus 17 kdaltons. This cleavage is least affected by high ionic strength, being over 70% complete in the 1.5 h of proteolysis (assuming that proteolysis is complete at low ionic strength). In contrast, the cleavage of 17 to 15 kdaltons is only 30% complete, and the cleavage of 35 to 9 kdaltons is the most protected at 15% completion. As the ionic strength is reduced, all of the cleavages proceed at faster rates. Consequently the intermediate segment of 17 kdaltons disappears as the amount of 15-kdalton segment increases.

The specific (DIDS-sensitive) sulfate efflux is also protected by increasing the ionic strength during the chymotrypsinization (Fig. 5). The inhibitions calculated from data presented in Fig. 5 are plotted in Fig. 4 as a function of ionic strength. The resulting curve is parallel to those for the relative amounts of 15- and 9-kdalton segments.

Discussion

Proteolytic enzymes have been particularly useful tools in determining the arrangement of band 3 protein in the bilayer, the location of particular binding sites in the primary structure, and the functional importance of different parts of the peptide

[24,25]. The application of chymotrypsin to the outside of the intact cell results in the cleavage of band 3 into membrane bound segments of 60 and 35-kdaltons without loss of anion transport activity [4]. A second cleavage at the cytoplasmic face of the membrane, produced by chymotrypsin treatment of inside-out vesicles [7], is also without effect on transport [11], even though a soluble cytoplasmic segment of band 3 of over 40 kdaltons is removed from the membrane. The residual membrane-bound segments are 17 and 35 kdaltons. The further cleavage of band 3 segments by chymotrypsin treatment of ghosts, however, can result in the complete loss of specific transport activity*. At least three additional cleavages occur. A 15 000 dalton segment is derived from the 17 000 dalton segment, and a 9 000 dalton segment [15] and a carbohydrate-containing segment of undefined molecular weight (Ramjeesingh, M. and Rothstein, A., unpublished observations) are derived from the 35 000 dalton segment**. One or more of these cleavages results in loss of transport activity. The cleavages are differentially inhibited by high ionic strength, with the production of thr 15-kdalton segment being less affected than the production of the 9-kdalton segment. Although the degree of inhibition correlates best with production of the 15-kdalton segment (Fig. 4), the overlap is considerable and quantification by gel scanning too imprecise to allow hard conclusions.

Unlike chymotrypsin, which has no effect on transport in intact cells, papain is inhibitory [5,6]. The major difference between chymotrypsin and papain digestion of cells is that the latter enzyme partially digests the 35-kdalton fragment, reducing it to a 25-kdalton fragment, suggesting that the 35-kdalton peptide might be functionally important [6]. It cannot be ruled out, however, that removal of a small (undetectable by molecular weight determinations) segment from the 60-kdalton fragment may be responsible for the inhibitory action of papain.

Other evidence that bears on the functional importance of different segments of band 3 is based on the localization of inhibitory probes. The 17-kdalton segment contains the covalent binding sites for the affinity probes, DIDS [11] and NAP-taurine [26], which appear to specifically inhibit anion transport by binding to functional ligands, defined by kinetic analysis as the transport [27] and modifier sites [28], respectively. The DIDS site is retained in the 15-kdalton segment [29].

Recently, it has been demonstrated that DIDS can cross-link between its site on the 17-kdalton segment and another on the 35-kdalton segment, indicating that the two segments are close neighbors [6], and it has been suggested that both segments may be contributing ligands that are important in the transport process [30]. The close association of the two segments is also evident by their comigration during centrifugation after extraction in Triton X-100 [31]. The daughter segments (15 and 9 kdaltons) also seem to be associated. They co-extract in Triton X-100 and copurify on filtration columns [15]. The architectural arrangement of band 3 and the role of its various segments is not entirely clear, but present evidence suggests that both the 17- and 35-kdalton segments are important elements of the transmembrane portion of the protein and may contribute to the structure of the specific functional sites.

* In a preliminary report from this laboratory [16] vesicles from chymotrypsinized ghosts were found to retain some residual DIDS-sensitive anion transport activity. It was presumed that the chymotrypsin treatment (0.2 mg per ml for 1.5 h at 37°C) resulted in complete proteolysis. The published gel in the preliminary report [16] appears to contain only 15- and 9-kdalton segments but appearances of published gels depends on many factors such as the amount of protein loaded on the gel and the photographic treatment. The completeness of proteolysis can only be assessed by a careful comparative study of parallel gels with the same amount of peptide applied, with staining and destaining simultaneously in the same solution and with the use of an optical scanner, as for example, Figs. 3 and 4. A similar series at low ionic strength but with increasing concentrations of chymotrypsin indicates that complete proteolysis (measured by the maximal production of the 15- and 9-kdalton segments, or by maximal inhibition) requires 1.5 mg per ml of chymotrypsin for 1.5 h at 37°C. On the basis of the present more detailed study can be concluded that the proteolysis in the preliminary study [16] was substantial, but not complete.

** The carbohydrate-containing fragment, like the other two fragments (15 and 9 kdaltons) is membrane bound. It does not stain on acrylamide gels but it can be labelled by coupling of radioactive moieties to the sugar amino groups. It does not form a discrete band but is spread over the gels in the molecular weight range of about 5 to 20 kdaltons. This behaviour is presumably due to the known heterogeneity of the carbohydrate moiety of band 3, which apparently dominates the molecular weight behavior of the glycopeptide fragment.

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