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ANION TRANSPORT ACROSS THE ERYTHROCYTE MEMBRANE, IN SITU PROTEOLYSIS OF BAND 3 PROTEIN, AND CROSS-LINKING OF PROTEOLYTIC FRAGMENTS BY 4,4′-DIISOTHIOCYANO DIHYDROSTILBENE-2,2′-DISULFONATE

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Summary

Extracellular chymotrypsin cleaves the 95 000 dalton protein that migrates in band 3 of SDS-polyacrylamide gel electropherograms of the erythrocyte membrane into fragments of 60 000 and 35 000 daltons, but not further. Minor components of band 3 that remain at the original 95 000 dalton location may be eluted from the membrane by 0.1 N NaOH, indicating that, in contrast to the major component and the chymotryptic fragments, they are not integral membrane constituents.

Incubation at neutral pH of chymotrypsinized erythrocytes with the bifunctional anion transport inhibitor 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid results in covalent binding of that inhibitor primarily to the 60 000 dalton fragment and some cross-linking of the 60 000 dalton fragment with the 35 000 dalton fragment. Increasing the pH to 9.5 leads to a cross-linking of virtually all of the pairs of chymotryptic fragments and thus to a reconstitution of band 3 with its typical diffuse appearance in the 95 000 dalton region of the SDS-polyacrylamide gels. This indicates that (1) each integral 95 000 dalton protein molecule is capable of binding at least one 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid molecule; (2) the 35 000 dalton fragment, though it is only weakly stained with Coomassie blue, is present in an amount that is equimolar with that of the 60 000 dalton fragment. Since the

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Abbreviations: H_2DIDS, 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid; SITS, 4-isothiocyano-4′-acetamido stilbene-2,2′-disulfonic acid; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone. For the designation of band 3 and other bands on sodium dodecyl sulfate polyacrylamide gels the nomenclature of Fairbanks et al. [19] was used.

number of 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid binding sites on the protein in band 3/cell is known to be close to the number of band 3 molecules/cell, it is suggested that the cross-linking takes place at a region of the band 3 molecule that is involved in the control of anion transport.

Like chymotrypsin, papain digests the band 3 protein from the outer membrane surface. Unlike chymotrypsin, however, papain digestion results in an inhibition of anion exchange. Papain produces a major fragment of 60 000 daltons that differs from the major chymotryptic fragment by at most six amino acid residues. The only detectable difference between the non-inhibitory action of chymotrypsin and the inhibitory action of papain on the band 3 protein is that papain is capable of partially digesting the 35000 dalton fragment. No reconstitution of band 3 by cross-linking of the fragments with 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid can be achieved. Since the 35 000 dalton fragment reacts with one of the two reactive groups of 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid and is also susceptible to digestion by the inhibitory papain, we suggest that a portion of this peptide participates, together with a portion of the 60 000 dalton fragment, in the control of anion transport.

Introduction

The most abundant integral membrane protein of the erythrocyte is the protein in band 3 [1]. It has a molecular weight of about 95 000 *, contains about 6—8% carbohydrate [2—5], and is believed to be involved in anion transport [2,6,7]. Like a carrier molecule, it seems to be capable of binding the anions to be transported. However, in contrast to the well-characterized small carrier molecules of the valinomycin type, the anion transport protein spans the membrane (e.g. Refs. 1, 3, 8, 9) and is incapable of moving the ion to be transported by translational or rotational diffusion [10] from one surface of the lipid bilayer to the other. Thus, it is likely that local conformational changes within the large protein molecule are responsible for the actual translocation of the bound anions across the permeability barrier.

During the last few years considerable attention has been paid to the study of the enzymatic degradation in situ of the band 3 protein. Much of this work

* The molecular weights of band 3 and of the fragments of band 3 produced by various proteolytic enzymes are used here primarily for the purpose of identification. We do not suggest that these values represent the true molecular weights of the various peptides. For example, the gel electrophoretic estimates of the molecular weight of the intact band 3 range from 88 000 to 106 000 [1]. The chymotryptic fragment that, in this paper, we refer to as 60 000 daltons has been reported as having a molecular weight of between 55 000 [12,16,23] and 70 000 [15,22]. The other chymotryptic fragment referred to as 35 000-dalton runs as a broad doublet, the most rapidly migrating component of which has an apparent molecular weight of about 38 000 in the gradient slab gels and 35 000 in the tube gels used here. This peptide is designated 38 000 daltons by Steck and coworkers [12,23]. The transmembrane peptide referred to here as 17 000 daltons has an apparent molecular weight of about 18—19 000 on the gradient slab gels in 0.1% sodium dodecyl sulfate, and about 16 000 on the cylindrical gels in 0.5% sodium dodecyl sulfate. It should be noted that the more or less arbitrary designation of the various bands is responsible for the fact that the sums of the molecular weights of the fragments do not always add up to the molecular weight of the parent peptides from which they are derived.

was aimed at obtaining smaller fragments of the molecule that can be used for a future elucidation of its primary structure. This work yielded interesting information on the disposition of the protein in the native membrane. It showed that the carbohydrates are largely and perhaps exclusively located at the outer surface of the membrane [4,11,12] on a segment of the peptide chain that has a molecular weight of about 35 000. This segment is followed by a strongly hydrophobic segment of a molecular weight of 17 000 that traverses the lipid bilayer. The rest of the peptide chain is again fairly hydrophilic, has a molecular weight of about 40 000 and resides at the inner membrane surface [12]. It has been reported by Tanner and Boxer [3] that the peptide chain traverses the lipid bilayer more than once. Recent results of two other groups [4,12] suggest that only the 17 000 dalton fragment passes all the way across the membrane.

Attempts to correlate enzymatic modification of band 3 protein with parallel changes of anion transport have been carried out in the hope of identifying those segments of the transport molecule that are primarily responsible for the mediation of transport. It has been shown that certain enzymes, like externally applied chymotrypsin and pronase (at low concentrations) [14,15], or internally applied trypsin [7,9,16] cleave the peptide chain at a number of well-defined loci without inhibiting anion transport. Others, notably papain and pronase at high concentrations, produce strong inhibition that is accompanied by the degradation of the band 3 protein into smaller fragments [14,15].

The present paper focuses on the effects of externally applied chymotrypsin and papain. It is shown that chymotrypsin severs the carbohydrate-carrying 35 000 dalton segment from the rest of the integral band 3 molecule without removing it from the membrane. This 35 000 dalton fragment can be cross-linked with the remaining 60 000 dalton fragment by the bifunctional reagent H_2DIDS, which is a powerful inhibitor of anion transport. Since the cross-linking reaction can be carried out under conditions where no more than the single H_2DIDS molecule that is necessary to inhibit anion transport is actually bound to a band 3 molecule, we suggest that the H_2DIDS-sensitive anion transport controlling site on the transport protein is formed by both fragments of the peptide chain. This conclusion is supported by the further observation that papain, which in contrast to chymotrypsin inhibits anion transport, also produces a fragment of about 60 000 daltons which differs from that produced by chymotrypsin by less than six amino acid residues but whose H_2DIDS binding capacity could not be related to the inhibition of transport. The only other detectable difference between the non-inhibitory action of chymotrypsin and the inhibitory action of papain is that papain partially digests the 35 000 dalton fragment.

Materials and Methods

Red blood cells. Blood (Type O, Rh^+) from apparently healthy donors was obtained from the Red Cross and stored at 4°C in acid/citrate/dextrose for 2—6 days before use. Cells were prepared for enzyme treatment by washing three times in 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (phosphate-buffered

saline, pH 7.4). Enzyme digestion of the outer membrane surface was carried out at 37°C for 1 h, at the indicated enzyme concentration, at a 50% hematocrit. The enzymes used were bovine pancreatic α-chymotrypsin (chymotrypsin A_4) and papain (from *Papaya carica*), both obtained from Boehringer Mannheim GmbH (Mannheim, F.R.G.). The papain was dialyzed at least 24 h against phosphate-buffered saline, pH 7.4, and was activated with 2 mM cysteine before addition to the cell suspension. After papain treatment, the enzyme was inhibited with 2 mM iodoacetic acid (10 min, 37°C). Following either the papain or chymotrypsin treatment, the cells were washed twice at room temperature in 10 vols. of phosphate-buffered saline, pH 7.4, containing 0.5% bovine serum albumin, and once in phosphate-buffered saline, pH 7.4. In some experiments, in which it was desired to make white ghosts directly after the digestion, 150 mM $NaHCO_3$ (pH 8.1) was used in the washes instead of phosphate-buffered saline, pH 7.4; it appears to be easier to remove the hemoglobin from the $NaHCO_3$-washed cells.

The H_2DIDS treatments (except those in Figs. 1 and 7, which were done as described previously [18]) were carried out at a 10% hematocrit, in phosphate-buffered saline (pH 7 or 7.4) containing 12 μM H_2DIDS. The unreacted H_2DIDS was removed by washing twice in 10 vols. of buffer containing 0.5% bovine serum albumin, and once in normal buffer. The subsequent incubations at high and low pH were performed as described in the legends to Figs. 2—4.

Erythrocyte ghosts. Hemoglobin-free erythrocyte ghosts were prepared essentially according to Fairbanks et al. [19] by lysis of cells at 0°C in 20 vols. of 5 mM sodium phosphate, pH 8. After each spin (10—15 min at 20 000 rev./min in a Sorvall SS 34 angle rotor), any material appearing as an opaque, cream-colored 'button' [19] at the bottom of the pellet was removed. For trypsin treatment of the unsealed ghosts, an aliquot of packed ghosts was resuspended in 20 vols. of phosphate-buffered saline, pH 7.4, containing 50 μg/ml porcine pancreatic trypsin (Boehringer Mannheim) and incubated at 37°C for 45 min. Following the incubation, 100 μg/ml of the trypsin inhibitor *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; Serva Heidelberg) was added, and the ghosts were centrifuged 15 min at 20 000 rev./min. The ghosts were then 'stripped' by adding 20 vols. of 0.1 N NaOH at 0°C [33], centrifuged as above, and washed once in 40 vols. of distilled water, also at 0°C.

Electrophoresis. Samples were prepared for polyacrylamide gel electrophoresis in sodium dodecyl sulfate as described previously [18] for the experiments in Figs. 1 and 7. For the rest of the experiments, 1 vol. (usually 50 μl) of packed ghosts was added to 1 or 2 vols. of a solution containing 4% sodium dodecyl sulfate, 20% glycerol, 80 mM dithiothreitol and 0.20 mg/ml bromophenol blue. This solution had been previously heated in a 100°C bath. The ghosts were added to the hot solution, mixed, and heated further for 3 min at 100°C. The resulting mixture was optically clear. Gel electrophoresis in cylindrical tubes was performed as described previously [20] using 5% acrylamide, 0.15% bisacrylamide, and 0.5% sodium dodecyl sulfate in 100 mM sodium phosphate, pH 7.1, as the running buffer. Slab gels (PAA 4/30, Deutsche Pharmacia GmbH, Freiburg, F.R.G.) were pre-run for 6 h at 40 V or overnight at 20 V in the running buffer (50 mM sodium phosphate, pH 7.1, 0.1% sodium dodecyl sulfate). Samples of 20—25 μl were applied, and the gels

were run 16—18 h at 20 V (22—25°C). Molecular weight standards were bovine serum albumin (67 000), rabbit muscle aldolase (39 500), beef erythrocyte carbonic anhydrase (29 000), horse myoglobin (17 200) and cytochrome *c* (12 300). Protein was determined according to the method of Lowry et al. [34].

Flux measurements. To measure sulfate equilibrium exchange, the erythrocytes were washed three times and subsequently equilibrated at 37°C for 90 min in a standard medium containing 20 mM sodium phosphate, 5 mM Na_2SO_4 and 122.5 mM NaCl, pH 7.4. During this incubation period, a trace amount of $^{35}SO_4^{2-}$ was present in the medium. After equilibration, extracellular $^{35}SO_4^{2-}$ was removed by three washes in standard medium at 0°C. Efflux was initiated by resuspending the washed erythrocytes in $^{35}SO_4^{2-}$-free standard medium at 37°C. For details of the procedure and the evaluation of the results, see the legends to Figs. 1 and 7 and Refs. 18 and 20. Chloride equilibrium exchange was measured at 8°C, using the inhibitor stop technique described by Ku et al. [21].

Results

The action of chymotrypsin on anion transport and the protein in band 3

It has been demonstrated in several laboratories that extracellular chymotrypsin cleaves the 95 000 dalton band 3 molecule into 60 000 and 35 000-dalton fragments [14,15,22,23]. In some reports, material has been found

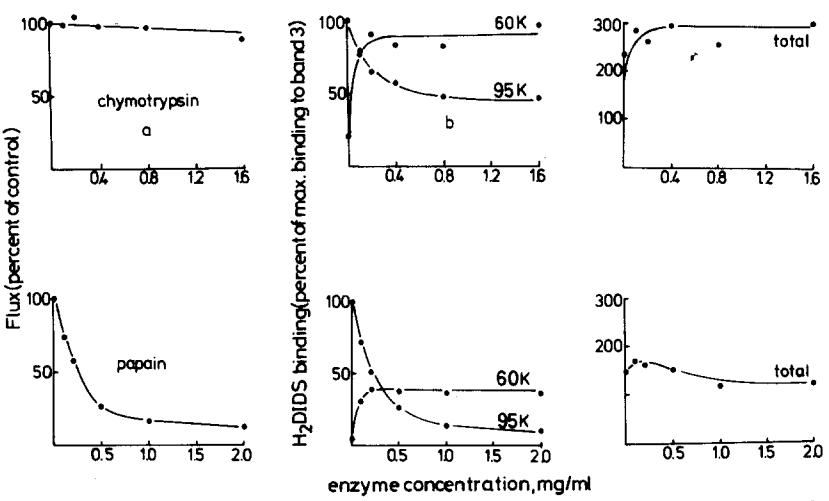

Fig. 1. Effects of increasing concentrations of chymotrypsin or papain on anion exchange (left) and the capacity to bind H_2DIDS to the outer membrane surface as a whole (right) or to the isolated protein in band 3 (95 000 daltons) and the 60 000 dalton peptide derived from that band by enzymatic hydrolysis (middle). Erythrocytes incubated at 37°C, pH 7.4, 10% hematocrit for 60 min in phosphate-buffered (20 mM) saline containing 5 mM Na_2SO_4 and the enzymes at the concentrations indicated on the abscissa. After removal of the enzyme by washing, the cells were subdivided for separate measurements of sulfate equilibrium exchange and $^3H_2$DIDS binding capacity. The latter was measured after 90 min of incubation at 37°C in the medium described above containing 25 μM $^3H_2$DIDS. The binding capacity of the specified peptides and of the erythrocyte membrane as a whole are expressed as a percent of $^3H_2$DIDS binding to band 3 in the absence of enzymatic treatment. 100% corresponds to $1.23 \cdot 10^6$ molecules H_2DIDS/cell. Same data as in preliminary reports of observations related to this work [15,32].

which migrates in the band 3 region and which is resistant to chymotryptic digestion [14,23,24]. At least part of this protease-resistant protein is known to consist of protein species which are totally unrelated to the bulk of band 3, but which happen to migrate in the same region of the gel (e.g. $(Na^+ + K^+)$-ATPase, Ref. 24). We also find that, even after treatment with 1 mg/ml chymotrypsin for 1 h at 37°C, a small amount of Coomassie blue-staining material is detected at 95 000 ± 5000 daltons. However, in agreement with previous work of Steck and Yu [33], virtually all of this material may be eluted from the membrane by treatment with 0.1 N NaOH. This indicates that the detectable chymotrypsin-resistant material in band 3 is not integral membrane protein, and thus differs fundamentally from the bulk of the band 3 protein. The integral membrane protein in band 3, then, behaves homogeneously with respect to digestion by extracellular chymotrypsin.

In contrast, if erythrocytes are first treated with H_2DIDS, a specific inhibitor of anion exchange, and then digested with chymotrypsin, a substantial fraction of the Coomassie-staining integral membrane protein continues to migrate on sodium dodecyl sulfate-polyacrylamide gel electropherograms at the original location of band 3, even after digestion at very high enzyme concentrations [15]. In such experiments, most of the band 3 protein is split into two fragments of 60 000 and 35 000 daltons, with most of the H_2DIDS counts in the 60 000 dalton fragment. However, a sizable fraction (10—35%) of the counts remain at the 95 000 dalton location, as found also by Cabantchik and Rothstein [14] and Grinstein et al. [16]. Even if the H_2DIDS is added after the exhaustive chymotrypsin treatment of the cells, a similar fraction of the band 3 protein continues to reside in the 95 000 dalton region (Fig. 1). The $^3H_2$DIDS, that is associated with this fraction, cannot be removed by NaOH. Thus, in cells treated with H_2DIDS, either before or after exposure to chymotrypsin, a significant fraction of the integral band 3 protein behaves like the undigested protein, while virtually all of this protein is found to be digested when the exposure to chymotrypsin is not preceded by H_2DIDS binding.

A possible explanation for these seemingly contradictory results is that H_2DIDS, a bifunctional covalently binding reagent, is able to 'repair' the digested band 3 by forming a covalent intramolecular cross-link between the 60 000 and 35 000-dalton fragments. The experiment in Fig. 2 supports this interpretation, and shows that under proper conditions, H_2DIDS can repair nearly 100% of the band 3. In this experiment, cells were first digested with chymotrypsin. A portion of the cells was then treated with 12 μM H_2DIDS for 1 h at pH 7.4. After the unreacted H_2DIDS was washed off, the cells were further subdivided into two parts and incubated for another hour at either pH 6 or pH 9.5. The electrophoresis pattern for the membrane proteins of the H_2DIDS-treated cells that had been incubated at pH 6 (sample 2) is very similar to that of cells that had not been treated with H_2DIDS (sample 1). Most of the band 3 is digested into a sharp, darkly stained 60 000 dalton peptide and a diffuse, weakly stained 35 000 dalton peptide which is difficult to visualize in samples 1 and 2 because of overlap with other bands. The 35 000 dalton band is much more apparent after removal of most other membrane proteins by 'stripping' (sample 5).

The gel pattern for ghosts made from the cells incubated at pH 9.5 (sample 3)

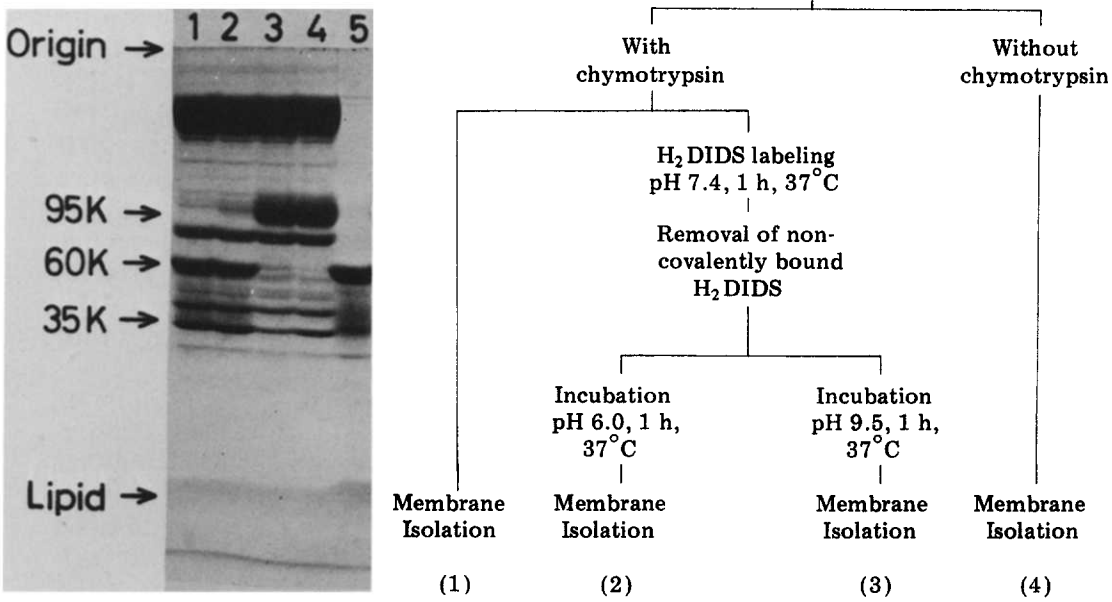

Fig. 2. Gradient slab gel (4—30% acrylamide, 0.1% sodium dodecyl sulfate) of (1) ghosts from chymotrypsin-treated erythrocytes, 1 mg/ml, 1 h, 37°C. (2) Ghosts from the same chymotrypsin-treated erythrocytes subsequently labeled with 12 μM H_2DIDS (pH 7.4, 1 h, 37°C) and then incubated for 1 h at pH 6.0 in phosphate-buffered saline after washing off the reversibly bound H_2DIDS. (3) Ghosts from the same cells as in sample 2 except that the incubation after removing the reversibly bound H_2DIDS was in 150 mM $NaHCO_3$, pH 9.5. (4) Control ghosts, no chymotrypsin, no H_2DIDS. (5) Ghosts from chymotrypsinized erythrocytes not treated with H_2DIDS, same as (1), except that the ghosts are stripped with 0.1 N NaOH (not included in the flow diagram).

is entirely different. The 60 000 and 35 000-dalton fragments are no longer present, but the 95 000 dalton band appears to be regenerated. Indeed, the gel from these ghosts is nearly identical to that for ghosts made from cells which had not been treated with chymotrypsin (sample 4) *. Obviously, the incubation at pH 9.5 has resulted in the regeneration of virtually all of the band 3, and the band 3 thus formed is identical in shape and mobility to the band 3 in untreated ghosts. Fig. 3 shows the distribution pattern of $^3H_2$DIDS on gels made from the same ghosts as in samples 2 and 3. For the ghosts made from cells that had been labeled and then incubated at low pH (Fig. 3, left), most of the counts are in the 60 000 dalton fragment, with small amounts remaining in the 95 000 dalton band and some also in the 35 000 dalton region. For the ghosts from cells that had been incubated at pH 9.5 (Fig. 3, right), however, the overwhelming majority of the counts resides in the band 3 region, with very few remaining in the 60 or 35 000-dalton regions. Thus, both Coomassie blue-staining material and H_2DIDS counts return to the location of the original 95 000 dalton band. The obvious interpretation of these findings is that at neutral pH, one of the -N=C=S groups of the H_2DIDS molecule reacts with a

* One detectable difference is that band 6, which is the monomer of glyceraldehyde-3-phosphate dehydrogenase [1], is reduced in the ghosts made from cells incubated at high pH. This elution of band 6 in the high pH-treated cells is independent of whether or not H_2DIDS is present.

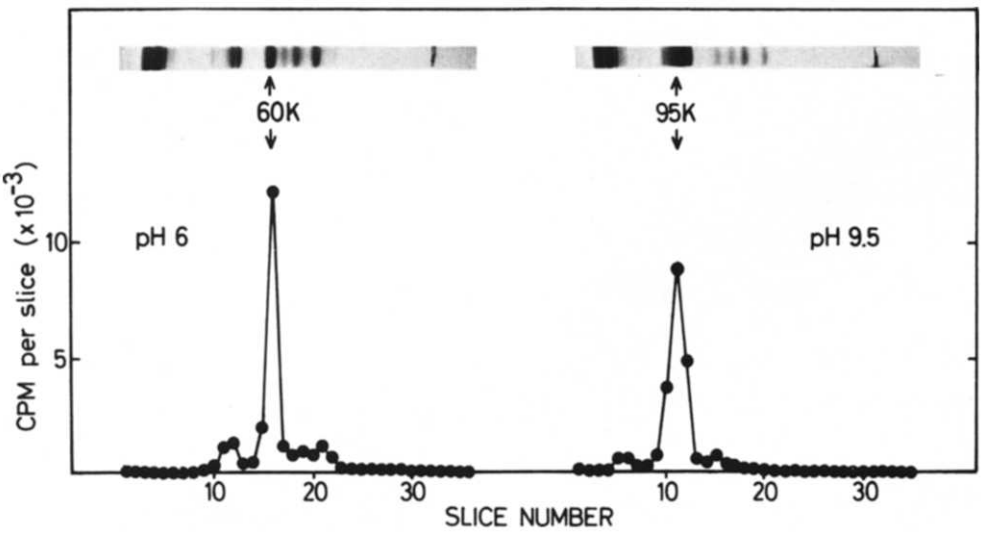

Fig. 3. Reappearance of Coomassie blue staining and $^3H_2$DIDS at the 95 000 dalton location after treatment of the chymotrypsinized erythrocyte membrane with $^3H_2$DIDS. The ghosts used in these two gels were the same as those in Fig. 2, gels 2 and 3, respectively. They were made from erythrocytes that had first been chymotrypsinized, labeled subsequently with H_2DIDS, washed to remove reversibly bound H_2DIDS, and then incubated at pH 6 (left) or pH 9.5 (right). Two parallel gels (5% acrylamide, 0.5% sodium dodecyl sulfate) were run. One was stained with Coomassie blue (shown above); the other was sliced and counted. The number of $^3H_2$DIDS molecules bound/cell was the same for the two gels, and amounted to $1.2 \cdot 10^6$.

site on the 60 000 dalton peptide. The other —N=C=S group reacts with the 35 000 dalton fragment only slowly at neutral pH and even more slowly at pH 6, while at pH 9.5 the reaction with the 35 000 dalton fragment is driven to completion. Under the labeling conditions applied in previously published work [14,15], this cross-linking reaction should be only partially complete. Thus, although all of the band 3 protein had actually been split into two fragments, the cross-linking made it appear as if there existed a chymotrypsin-resistant fraction of band 3.

The experiment in Fig. 4 shows more clearly that both the 60 000 and the 35 000-dalton chymotryptic fragments disappear as a result of the H_2DIDS/pH 9.5 treatment. Sample 1 is from chymotrypsin-treated cells, subsequently incubated at pH 9.5 for 1 h. Sample 2 is from the same cells, but H_2DIDS treated after the chymotryptic digestion, with subsequent incubation at pH 9.5. Sample 3 consists of membranes from cells treated with neither chymotrypsin nor H_2DIDS. The samples on the right were prepared from the ghosts in samples 1, 2 and 3, respectively, and stripped with 0.1 N NaOH. As in Fig. 3, H_2DIDS 'regenerates' virtually all of the band 3. It is clear, especially in the stripped ghosts, that the 35 000 dalton band disappears, concomitant with the 60 000 dalton band, as a result of the H_2DIDS treatment (Fig. 4, sample 2), and that, again, the regenerated band 3 appears identical with the original band 3 that had never been exposed to chymotrypsin (sample 3).

It has been suggested by Grinstein et al. [16] that the 35 000 dalton fragment, in contrast to the 60 000 dalton fragment, is susceptible to degrada-

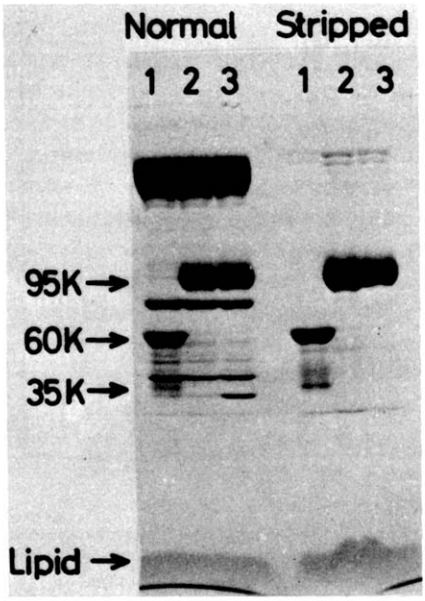

Fig. 4. Cross-linking of 35 000 dalton chymotryptic fragment with 60 000 dalton fragment by H_2DIDS. The formation of the 35 000 dalton band after chymotrypsination and its disappearance after H_2DIDS treatment of the chymotrypsinized erythrocytes is illustrated by the electropherograms of stripped and unstripped ghosts obtained from the treated erythrocytes. (1) Chymotrypsin treatment (1 mg/ml, 1 h, 37°C) with subsequent incubation at pH 9.5. (2) Chymotrypsin treatment followed by exposure to H_2DIDS (12 μM, pH 7.0, 1 h) and subsequent incubation at pH 9.5. (3) Control, untreated erythrocyte membranes.

tion by chymotrypsin. This would imply that it should be impossible to obtain a complete reconstitution of band 3 since the number of 35 000-dalton fragments that are available for cross-linking should be less than the number of 60 000-dalton fragments. At least under our experimental conditions this does not seem to be the case, since all of the 60 000-dalton fragments disappear after cross-linking at pH 9.5. Nevertheless, we felt it necessary to study the survival of the 35 000 dalton band during exposure to a range of chymotrypsin concentrations, up to the excessively high concentration of 4.0 mg/ml. We incubated the erythrocytes either for 1 h at 37°C, as in the present work, or overnight at room temperature as in the work of Grinstein et al. [16]. However, in contrast to Grinstein et al. the prolonged incubation was performed in the presence of an antibiotic (to reduce bacterial growth and hemolysis) and the washings that preceded the isolation of the membrane for gel electrophoresis were carried out in media containing 0.5% serum albumin. As may be seen in Fig. 5, the gels obtained after incubation under these conditions are virtually indistinguishable. It is clear, therefore, that the externally applied chymotrypsin A_4 that had been used in the present work does not progressively degrade the 35 000 dalton fragment.

An alternative explanation for the cross-linking experiments that could come to mind is the possibility that H_2DIDS cross-links two chymotryptic 60 000-dalton peptides into one dimer that migrates in approximately the same place

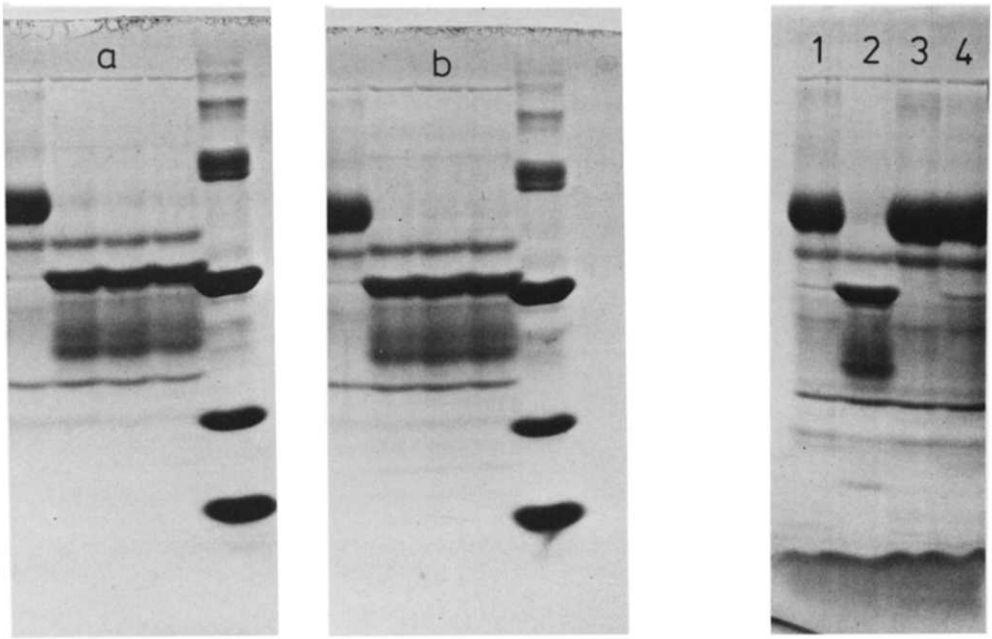

Fig. 5. Survival of 35 000 dalton fragment after treatment of intact erythrocytes at a range of chymotrypsin concentrations. (a) Treatment at 37°C for 30 min. (b) Treatment as in (a); subsequently the treatment is continued at room temperature overnight. Chymotrypsin concentrations (from left to right) 0, 1, 2, 4 mg/ml, hematocrit 10%, pH 7.0. At the end of the respective incubation periods the erythrocytes were washed twice in phosphate-buffered saline, pH 7.0, containing 0.5% bovine serum albumin and once in the same medium without bovine serum albumin. Membranes were isolated and 'stripped' as described under Materials and Methods. The standard proteins (extreme right) are (from bottom) cytochrome *c*, chymotrypsin and bovine serum albumin.

Fig. 6. Effects of chymotrypsin on band 3 after intramolecular cross-linking with H_2DIDS at pH 9.5. Intact erythrocytes were incubated at 37°C, pH 9.5, for 90 min in the absence (samples 1 and 2) or presence (samples 3 and 4) of 10 μM H_2DIDS. Hematocrit 10%. After two washes in phosphate-buffered saline, pH 7.0, containing 0.5% bovine serum albumin and one wash in phosphate-buffered saline, pH 7.0, without albumin, the cells were incubated for 30 min at 37°C, pH 7.0, in the absence (samples 1 and 3) or presence (sample 2 and 4) of 1 mg/ml chymotrypsin. Subsequently, membranes were isolated and 'stripped' as described under Materials and Methods.

as the original band 3. Three pieces of evidence argue against such an interpretation (Fig. 6).

First, if a cross-linking of two 60 000-dalton fragments would be possible, one would expect that H_2DIDS treatment at pH 9.5 of erythrocytes with intact band 3 protein should yield band 3 dimers. This has not been observed. Second, chymotrypsination after cross-linking in the intact membrane with H_2DIDS should still lead to a release of the 35 000-dalton fragments. No such fragments could be detected. Finally, cross-linking of the 60 000 dalton fragment into a covalent dimer by Cu^{2+}-orthophenanthrolin treatment [23] leads to the formation of a sharp band that contrasts markedly to the diffuse band 3 and the band formed by the H_2DIDS-reconstituted material. This band migrates considerably more slowly than the band 3 or the reconstituted product (not shown, cf. Ref. 33).

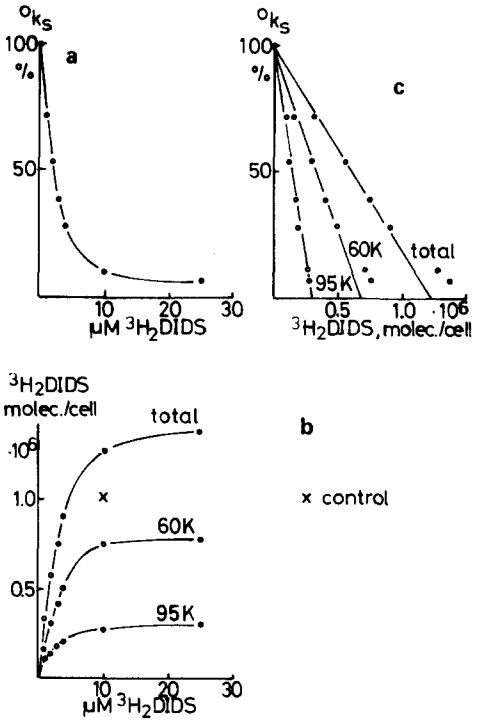

Fig. 7. (a) Effect of H_2DIDS on $^{35}SO_4^{2-}$ efflux from chymotrypsin-treated erythrocytes. Prior to the exposure to H_2DIDS at the concentrations indicated on the abscissa (90 min, pH 7.4), the cells had been treated with 0.2 mg/ml chymotrypsin for 1 h. The flux measurements were performed after removal of reversibly bound H_2DIDS by washing in albumin-containing media. All reactions and the flux measurements were carried out at 37°C in a medium containing 5 mM Na_2SO_4, 122.5 mM NaCl, and 20 mM Tris-HCl, pH 7.4. Ordinate: sulfate efflux as percent of efflux from chymotrypsin-treated control. This control value was indistinguishable from efflux from intact erythrocytes that had not been exposed to the enzyme. (b) Irreversible binding of H_2DIDS to chymotrypsin-treated erythrocytes as a function of H_2DIDS concentration in the medium. Same experiment as in (a). Control: H_2DIDS binding to erythrocytes that had not been exposed to chymotrypsin. Total, 95K, and 60K refer, respectively, to binding to the membrane as a whole, to the chymotrypsin-'resistant' (repaired) 95 000 dalton peptide, and to the 60 000 dalton fragment of chymotryptic digestion. Abscissa: as in (a). (c) Relationship between sulfate efflux and irreversible H_2DIDS binding to chymotrypsin-treated erythrocytes. Data from (a) and (b) plotted against each other. Ordinate: SO_4^{2-} efflux. Abscissa: H_2DIDS binding. Both axes are expressed as percent of control value. Same data as in Ref. 32.

Since all of the band 3 can be regenerated by H_2DIDS treatment at high pH, it appears that:

1. All of the integral membrane protein in band 3 can bind at least one H_2DIDS molecule, and

2. The weakly staining 35 000 dalton band and the strongly staining 60 000 dalton band are present in equimolar amounts even after extensive chymotrypsin treatment. The weak staining of the 35 000 dalton band is related in part to the fact that the dye is smeared out over a wide molecular weight range, from 35 000 to nearly 60 000 daltons. Moreover, the carbohydrate content of this band may reduce its capacity of binding the dye.

Fig. 1 shows that after chymotryptic cleavage of band 3 the total amount of

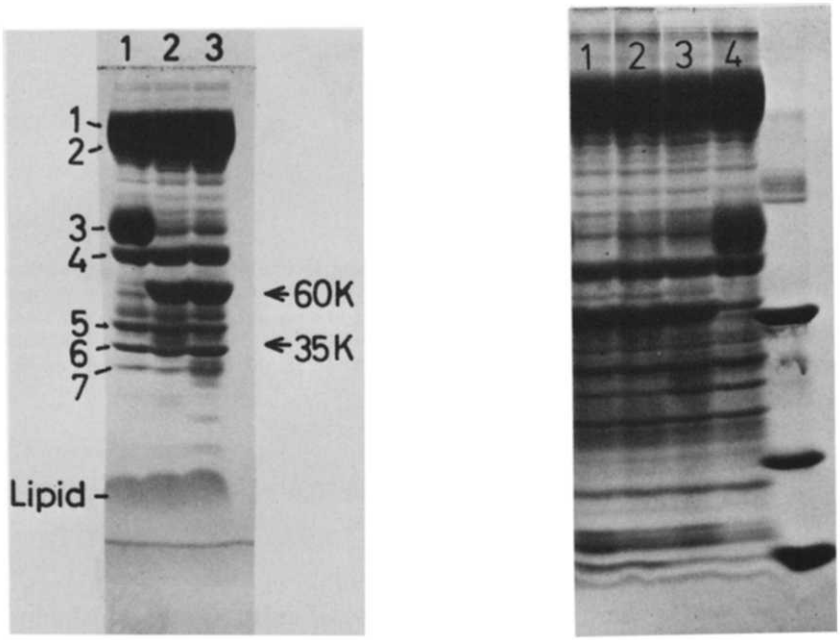

Fig. 8. Comparison of the action of chymotrypsin and papain on the outer surface of the erythrocyte membrane. Sodium dodecyl sulfate gradient slab gels of ghosts from: (1) untreated cells; (2) chymotrypsin-treated cells, and (3) papain-treated cells. The enzyme treatments were at 1 mg/ml, 1 h, 37°C, pH 7.4. The major bands of the untreated ghosts are numbered according to Fairbanks et al. [19]. The positions of the products of chymotryptic digestion of band 3 (60 000 and 35 000 daltons) are indicated. The diffuse band referred to as 35K runs slightly behind band 6 (glyceraldehyde-3-phosphate dehydrogenase, molecular weight 36 000) and overlaps with band 5.

Fig. 9. (1, 2) Failure of H_2DIDS to reconstitute band 3 after papain treatment. Intact erythrocytes were exposed to papain (1 mg/ml) and cysteine (2.0 mM) in phosphate-buffered saline (pH 7.0) for 30 min at 37°C, hematocrit 10%. After inhibition of the enzyme by iodoacetate (2 mM, 30 min, pH 7.0) the cells were washed twice in phosphate-buffered saline, pH 7.0, exposed to H_2DIDS at 37°C, hematocrit 10%, for 60 min. This leads to covalent binding of H_2DIDS to the 60 000 dalton fragment. After removal of excess H_2DIDS by two albumin washes and one wash without albumin the cells were subdivided into two samples (1 and 2). Sample 1 was incubated at pH 9.5, sample 2 at 6.0 (60 min, 37°C). Subsequently the membranes were isolated for electrophoresis. (3, 4) Failure of papain to cleave band 3 after intramolecular cross-linking at pH 9.5. Intact erythrocytes were first exposed to H_2DIDS (10 μM) at 37°C, pH 7.0, hematocrit 10% for 60 min. This leads to covalent binding of H_2DIDS to the 60 000 dalton fragment. After two albumin washes and one wash without albumin at pH 7.0 they were subdivided into two samples. Sample 3 was incubated at pH 6.0, sample 4 at pH 9.5 (37°C, 60 min). Subsequently each sample was resuspended at pH 7.0 and incubated with 1 mg/ml papain at 37°C for 30 min. After inhibition of the enzyme by iodoacetate and three washes the membranes were isolated and used for the electrophoresis. Same standard proteins as in Fig. 5.

H_2DIDS molecules bound/cell increases by about 30%. However, the sum of the binding sites on the surviving (or, more correctly, 'repaired') band 3 and on the 60 000 dalton fragment changes little if at all. Anion transport is not inhibited by chymotryptic cleavage of band 3, and covalent H_2DIDS binding to the enzymatically modified cells still produces inhibition. If the maximally chymotrypsinized erythrocytes are exposed to increasing concentrations of H_2DIDS, a linear relationship is obtained between the reduced rates of transport and the number of H_2DIDS molecules that are bound to the peptides that migrate at the 95 000 and the 60 000-dalton locations (Fig. 7). This implies

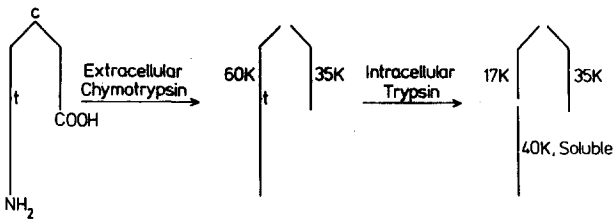

Fig. 10. Simplified schematic diagram of the disposition of the major tryptic and chymotryptic peptides of band 3, adopted from Steck et al. [12]. Chymotrypsin, acting at an extracellular site c, cleaves the protein into 60 000 and 35 000-dalton peptides. Trypsin cleaves the protein at an intracellular site t and releases a water-soluble peptide of 40 000 daltons from the membrane. The integral fragments remaining after treatment with extracellular chymotrypsin and intracellular trypsin are the 17 000 dalton transmembrane fragment and the 35 000 dalton exofacial fragment.

that all H_2DIDS binding sites on the chymotrypsin-treated band 3 continue to function in anion transport as in the untreated cells.

The action of papain on anion transport and the protein in band 3

Fig. 8 illustrates the effects on the band 3 protein of extracellular papain and contrasts the effects with those of chymotrypsin. Sample 1 represents the band pattern of undigested ghosts; samples 2 and 3 represent the patterns of ghosts made from cells that had been treated with chymotrypsin and papain, respectively (1 h, 37°C, 1 mg enzyme/ml). Papain produces a 60 000 dalton fragment which appears indistinguishable from that produced by chymotrypsin. However, the 35 000 dalton peptide that appears after chymotrypsin is not seen after papain; instead a new band, possibly a product of the 35 000 dalton peptide, appears at about 30 000 daltons, and coincides roughly with the preexisting band 7. If cross-linking with H_2DIDS at pH 9.5 precedes enzyme treatment, so far as visual inspection can tell, band 3 remains unaltered, regardless of whether chymotrypsin or papain is used *. However, the reconstitution by cross-linking with H_2DIDS of band 3 that had first been exposed to the proteolytic enzymes, was only seen after treatment with chymotrypsin but not after papain (Fig. 9). The comparison between the effects produced by chymotrypsin and papain is of interest, because chymotrypsin does not inhibit anion exchange [14,15], whereas papain treatment strongly inhibits both Cl^- [21] and SO_4^{2-} self-exchange [15,17,21].

Comparison between the 60 000-dalton peptides produced by chymotrypsin and papain

To improve the resolution of the comparison between the 60 000-dalton fragments produced by chymotrypsin and papain, a water-soluble peptide of approximately 40 000 daltons [23] was removed from the inside surface with trypsin, acting on unsealed white ghosts prepared from cells that had been pretreated with chymotrypsin or papain. The purpose of this pretreatment may be understood more easily by consulting the diagram in Fig. 10, which represents a simplified version of the current knowledge of the disposition in the mem-

* A loss of the 5—10 000 dalton papain fragment (see p. 517) would probably remain undetected.

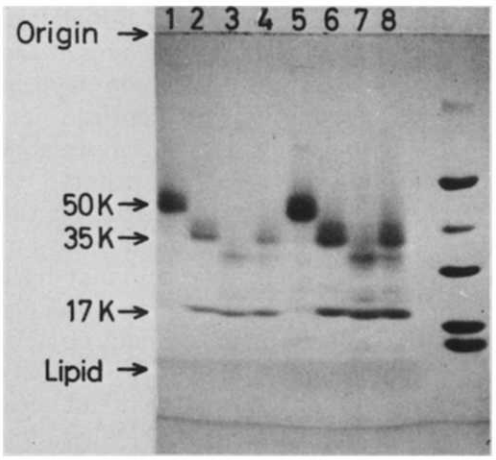

Fig. 11. Comparison of the actions of external papain and chymotrypsin on band 3. Sodium dodecyl sulfate gradient slab gel of ghosts, stripped after trypsin treatment. (1) Ghosts from untreated erythrocytes. (2) Ghosts from chymotrypsin-treated erythrocytes (1 mg/ml, 1 h, 37°C). (3) Ghosts from papain-treated erythrocytes (1 mg/ml, 1 h, 37°C). (4) A one-to-one mixture of the samples applied to 2 and 3. 5—8 are the same as 1—4, respectively, except twice as much protein was put on the gel. The molecular weight standards on the right are, from the bottom, cytochrome *c*, myoglobin, carbonic anhydrase, aldolase, and bovine serum albumin. Digestion of the ghosts was carried out in a medium containing 150 mM NaCl, 10 mM sodium phosphate, pH 7.4, and 50 μg/ml trypsin for 45 min at 37°C.

brane of the tryptic and chymotryptic fragments of band 3 [12].

Fig. 11 shows the peptides that remain in trypsin-treated unsealed ghosts after stripping with NaOH. Trypsin alone (sample 1) produces a double band at the 50 000 dalton location, with a sharp leading edge and a more diffuse trailing edge, very similar to that seen by Jenkins and Tanner and others [4,9, 25]. Both constituents of this double band are products of band 3, and both bind SITS [8]. Ghosts prepared in the same manner, but from chymotrypsin-treated cells (sample 2), show two major bands. One is a diffuse band of about 35 000 daltons, which is also an apparent double band with a diffuse trailing edge. This band is identical in appearance with the 35 000 dalton band produced by chymotrypsin alone (Fig. 2), indicating that the 35 000 dalton band is resistant to internal trypsin. The other band has an apparent molecular weight of 17 000, and is a sharp, single band. This band represents what is left of the 60 000 dalton chymotryptic fragment after a fragment of approximately 40 000 daltons has been removed from the inner surface by trypsin (see Fig. 10). Therefore, if the 60 000 dalton papain fragment is actually smaller than the 60 000 dalton chymotryptic fragment, then this difference should be observed as a difference in the 17 000 dalton fragment, since the same 40 000 dalton fragment presumably has been removed from the inner surface of each by trypsin. Sample 3 shows that the 17 000 dalton fragment produced by external papain plus internal trypsin, is very similar to that produced by external chymotrypsin plus internal trypsin. Sample 4 contains a 1 : 1 mixture of the protein in samples 2 and 3. If the 17 000-dalton fragments in samples 2 and 3 were indeed different, the 17 000 dalton band in the mixture should appear broader; it does not. On these gels, the resolution is such

that a difference of about 600 in the molecular weight of the 17 000-dalton bands in samples 2 and 3 would be readily detectable as a broadening in the mixture. Therefore, the 60 000-dalton fragments produced by chymotrypsin and papain differ by at most six amino acid residues, and may be identical.

In the experiments presented in Fig. 11, the chymotrypsin- or papain-treated erythrocytes were first converted into leaky ghosts and then exposed to trypsin. In intact cells, extracellular trypsin has no detectable effect on the band 3 protein. However, after pretreatment of the exofacial membrane surface with either chymotrypsin or papain, subsequently added trypsin may have gained access to a trypsin-susceptible peptide bond that is not normally accessible. This could make it appear that the effects of papain and chymotrypsin are indistinguishable. To rule out this possibility, in two experiments (not shown) the 40 000 dalton fragment was removed by trypsin that had been incorporated into resealed erythrocyte ghosts under conditions [7,9] where the outer surface of the membrane is not affected by that enzyme. Subsequent treatment of the still resealed ghosts with extracellular chymotrypsin or papain leads to the formation of 17 000-dalton fragments that were indistinguishable from each other. The 35 000 dalton exofacial fragment is, of course, still intact after chymotrypsin treatment, but not after papain. These experiments indicate that the products of band 3 resulting from the action of extracellular chymotrypsin or papain, plus intracellular trypsin, are independent of the order of the enzymatic treatments. It therefore appears that even after treatment of the external membrane surface with chymotrypsin or papain, the action of trypsin on band 3 in unsealed ghosts is confined to the inner membrane surface.

The action of papain on the 35 000 dalton chymotryptic peptide

As shown in Fig. 8 and demonstrated more clearly in Fig. 11, the 35 000 dalton peptide that is seen after chymotryptic digestion of band 3 is not observed after papain treatment. Instead, there appears as the only visible product a diffuse band, the leading edge of which migrates at about 30 000 daltons. Smaller fragments must also be produced by papain, but these are not detected on the gels. The experiment in Fig. 11 represents one way in which to compare the integral fragments of band 3 produced by extracellular chymotrypsin and papain. Another way is first to treat erythrocytes with chymotrypsin, and then digest the thus-formed products with papain. This type of experiment is also useful for the study of the mechanism of inhibition of exchange by papain. Chymotrypsin treatment of band 3 leads to the formation of the 60 000 and 35 000-dalton fragments, without inhibition of anion transport. Subsequent digestion with papain results in inhibition of anion exchange. The experiment in Fig. 12 is an attempt to correlate this inhibition with changes produced by papain in the integral chymotryptic fragments. Membranes were prepared as in Fig. 11. The ghosts from cells pretreated with chymotrypsin and subsequently with 0, 0.1, or 0.3 mg/ml papain were prepared, and then treated with trypsin. As in Fig. 11, the major integral peptides remaining after external chymotrypsin and internal trypsin are the 17 000 and 35 000-dalton products of band 3. In the ghosts made from cells with papain after the chymotrypsin, the 17 000 dalton transmembrane frag-

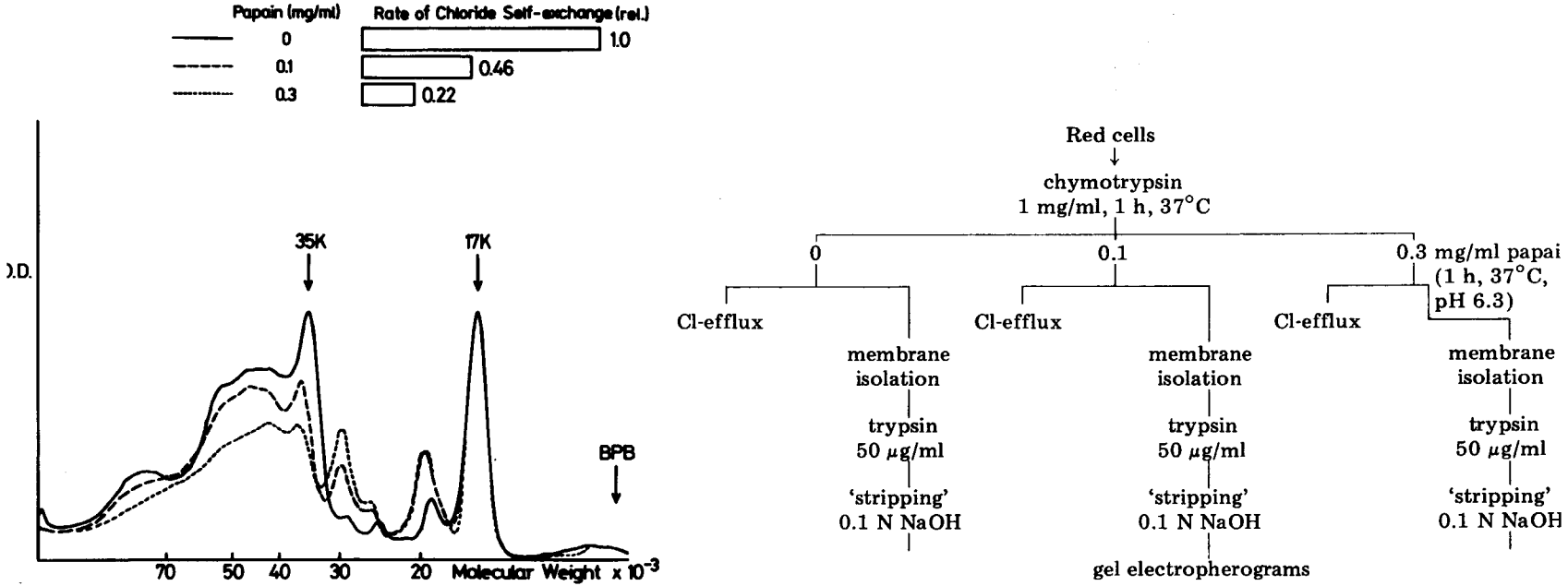

Fig. 12. Effect of papain on chloride self-exchange and the chymotryptic 35 000 dalton fragment of band 3. In all cases the erythrocytes were digested first with 1 mg/ml chymotrypsin (1 h, 37°C, pH 7.4), and subsequently incubated with 0, 0.1 or 0.3 mg/ml papain (1 h, 37°C, pH 6.3). A portion of the cells was retained in each case for measurement of the $^{36}Cl^-$ efflux, the relative rates of which are shown in the upper portion of the figure. Another portion was used for the preparation of ghosts. To increase the resolution of the gels, these ghosts were treated with trypsin (50 µg/ml, 1 h, Ph 7.4, 37°C) and subsequently stripped with 0.1 N NaOH. After solubilization in sodium dodecyl sulfate, electrophoresis was performed in 7.5% polyacrylamide gels (0.5% sodium dodecyl sulfate). The gel scans shown have been normalized so that the height of the 17 000 dalton band is the same in all scans. The peak heights in the original gels are (in absorbance units at 550 nm) 0.45, 0.52, and 0.59, respectively, for the ghosts from cells treated with 0, 0.1 and 0.3 mg/ml papain. The total amounts of protein (Lowry) in the three gels are, respectively, 37, 39, and 42 µg. The logarithmic molecular weight scale on the horizontal axis was derived from the position of molecular weight standards bovine serum albumin, aldolase, carbonic anhydrase and myoglobin, run on a parallel gel.

ment is not detectably altered. The 35 000 dalton band, however, is reduced by the papain treatment, with progressive appearance of the 30 000 dalton fragment *. The digestion of the 35 000 dalton peptide by the papain increases with increasing inhibition of the rate of Cl^- self-exchange (Fig. 12, upper). However, it is not clear whether or not a parallel relation exists between the digestion of the 35 000 dalton band and the inhibition of anion exchange, because

(1) The 35 000 dalton band is quite broad and rather poorly defined; the diffuse trailing edge extends to an apparent molecular weight of over 50 000. It is not obvious to what extent other proteins, unrelated to band 3, contribute to the Coomassie blue staining in the broad band.

(2) The 30 000 dalton band produced by papain overlaps with the 35 000 dalton chymotryptic band, making it difficult to assign a numerical value to the extent of digestion of the 35 000 dalton band by papain.

There is, however, a clearly detectable change in the 35 000 dalton band, and this change increases with papain concentration in the same range in which the Cl^- transport is progressive inhibited. At the same time, there is no detectable change in the 17 000 dalton band. This suggests that the inhibitory action of papain may be related to the digestion of the 35 000 dalton peptide.

Discussion

A proposition

The experiments presented here constitute evidence for the proposition that portions of the exofacial 35 000 dalton fragment of band 3 produced by extracellular chymotrypsin may cooperate with exofacial portions of the chymotryptic 60 000 dalton fragment in the control of anion exchange. The experimental results on which this proposition is based may be summarized as follows:

When the non-penetrating inhibitor H_2DIDS is added to exhaustively chymotrypsinized erythrocytes it first reacts covalently with a single site on the 60 000 dalton fragment and thereby inhibits anion transport. Subsequent incubation at elevated pH leads to the establishment of a covalent cross-link with the 35 000 dalton fragment. This indicates a close juxtaposition of exofacial portions of the two fragments that are near the anion transport controlling binding site for H_2DIDS (Fig. 13).

Extracellular chymotrypsin does not affect anion transport. Exposure of the chymotrypsinized erythrocytes to papain leads to inhibition. Under these conditions papain produces no detectable change in the chymotryptic 60 000 dalton fragment, but it does digest the chymotryptic 35 000 dalton fragment into a 30 000 dalton fragment that is not further digested, and into one or several smaller fragments that could not be detected on the gels. This supports the suggestion that a portion of the chymotryptic 35 000 dalton fragment cooperates with a portion of the 60 000 dalton fragment in the control of anion transport.

* Another new band, of about 20 000 daltons, is also seen when the cells have been treated with papain. The amount of material in this band, however, does not depend on the papain concentration, and may result from papain adsorption to the membrane. It is unknown why such adsorbed papain should remain after the extensive washing and stripping procedures used.

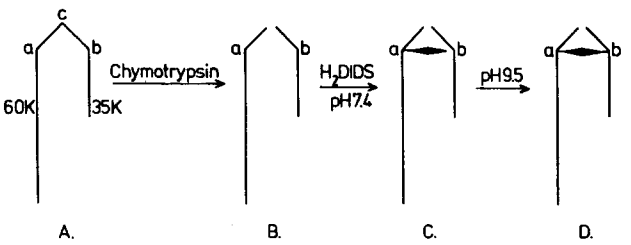

Fig. 13. Cross-linking of the chymotryptic fragments of band 3 by H_2DIDS.

The stoichiometry of the reaction between H_2DIDS and the band 3 protein

Since all of the band 3 may be regenerated by H_2DIDS after chymotrypsin digestion, the stoichiometry of the reaction between band 3 and H_2DIDS cannot be less than one-to-one. However, although it is clear that at least one H_2DIDS molecule must be bound/band 3 molecule to achieve the observed cross-linking of all chymotryptic fragments, it still remains to be asked whether or not the cross-link is established by an H_2DIDS molecule that is acutally involved in inhibition of anion transport. At complete inhibition H_2DIDS binding to the band 3 protein reaches saturation. Under these conditions, about $1—1.2 \cdot 10^6$ molecules of H_2DIDS/cell are bound to the band 3 protein [18,27]. This number agrees well with the only existing estimate [1] of the number of band 3 molecules/cell. Although the estimate is based on a rather crude method, it should be accurate enough to rule out that two or more H_2DIDS molecules are bound to band 3. Since it has been shown that the majority, if not all of the H_2DIDS molecules that are covalently bound to band 3 are involved in the inhibition of anion transport [31], it may be concluded that the H_2DIDS molecules that participate in cross-linking are most likely the same molecules that are responsible for the inhibition of anion transport. Since there is one functioning unit of anion exchange for each H_2DIDS binding site, one may further conclude that, although band 3 may exist as a non-covalent dimer in the membrane [23,26], the functional unit of anion exchange is a monomer of band 3. The H_2DIDS cross-linking results also argue that external chymotrypsin acts at a single site on band 3.

The observation that chymotryptic cleavage does not lead to an inhibition of anion transport and that H_2DIDS still continues to act as an inhibitor in the chymotrypsinized erythrocyte suggests that the disposition of the chymotryptic 60 000 and 35 000-dalton fragments remains as in the native protein from which they are derived. The results presented here certainly do not prove that this is the case. However, they are compatible with such a suggestion. The -N=C=S group of H_2DIDS which can react with the 35 000 dalton fragment at high pH appears to be buried deep in the protein, where it is incapable of reacting with extracellular added reagents. After the initial reaction of one of the -N=C=S groups with the 60 000 dalton fragment, the other -N=C=S group cannot be inactivated by the small molecule β-mercaptoethanol (50 mM, 20 min, 37°C, pH 7), which should react irreversibly with the -N=C=S group if it were accessible. After the β-mercaptoethanol treatment, raising the pH to 9.5 still results in a nearly complete reaction with the 35 000 dalton fragment

(greater than 95%). It is worth pointing out that the intramolecular cross-linking was demonstrated here with H_2DIDS, and not DIDS. It is unknown whether or not DIDS, a more rigid molecule, can also cross-link the 60 000 and 35 000-dalton peptides. Differences between H_2DIDS and DIDS could conceivably explain the finding of Rothstein et al. [28] that DIDS bound to band 3 still appears to have a reactive -N=C=S group which can attach to ferritin. In our hands, no added agent, either -SH or $-NH_2$ containing, reacts readily with the H_2DIDS after one of the -N=C=S groups is bound to the membrane.

The necessity to raise the pH to 9.5 in order to complete the reaction between the H_2DIDS and the 35 000 dalton fragment could have two origins. (1) The chemical group with which the -N=C=S reacts is much more reactive at high pH than the neutral pH. (2) The high pH produces a conformational change in the protein which results in access of the unreacted -N=C=S group of the H_2DIDS to a reactive group in the 35 000 dalton fragment. So far we have been unable to distinguish between these possibilities, but it is significant that between pH 7 and 11 (0°C) the rate of Cl^--Cl^- exchange is independent of pH [29]. It may therefore be argued that no major conformational changes result from raising the pH from 7 to 9.5.

The segments of the peptide chain that are involved in the control of anion transport

Erythrocyte anion transport is inhibited by a large variety of amino reactive reagents. Thus, the unique specificity of the H_2DIDS molecule is not simply brought about by its reactivity towards amino groups. The specificity is obviously related to a specific environment where the reversible binding takes place that precedes the covalent bond formation and the concomitant transition from reversible to irreversible inhibition [35]. Our cross-linking experiments with chymotrypsinized erythrocytes indicate that portions of both the 60 000 and the 35 000-dalton fragments contribute to this environment.

The 60 000 dalton fragment produced by papain is very similar to that produced by chymotrypsin. No difference could be detected between the two fragments even though by the technique used here the resolution of the gel electrophoresis was improved by removing the large (40 000 dalton) water-soluble portion of the 60 000 dalton fragment from the inner membrane surface by trypsin (see Figs. 10 and 11). The difference, if any, between the two 60 000-dalton peptides is less than six amino acid residues. The similarity is further demonstrated by the observation that papain does not visibly digest the chymotryptic 60 000 dalton fragment. In contrast, the chymotryptic 35 000 dalton fragment is converted by papain into a 30 000 dalton fragment that is indistinguishable from the 30 000 dalton fragment that is obtained after papain treatment of the intact band 3 protein. This indicates that the final 30 000 dalton product of papain digestion represents a major portion of the chymotryptic 35 000 dalton fragment. It is attractive, therefore, to believe that the relatively small portion (5—10 000 daltons) of the chymotryptic 35 000 dalton fragment that can be digested by papain is responsible for the papain-induced inhibition. However, it remains possible that papain inhibits the anion transport by removing a very small (less than six amino acid residues)

segment from the 60 000 dalton chymotryptic fragment.

Although our experiments suggest that the 35 000 dalton chymotryptic fragment of band 3 plays a role in anion exchange it is certainly not suggested that the anion exchange is carried out by that fragment alone. Recently, Grinstein et al. [16] have considered the possibility that both the anion exchange and H_2DIDS binding sites are solely confined to the 17 000 dalton fragment, which is transmembrane segment of the 60 000 dalton fragment (see Fig. 10). This suggestion is based in part on the observation that cleavage of the band 3 protein by external chymotrypsin and removal of the intrafacial 40 000 dalton segment by trypsin does not lead to inhibition and that most of the H_2DIDS now resides on the 17 000 dalton fragment. All of these findings agree with previously published observations [9,15] on the effect of external chymotrypsin and intracellular trypsin on anion transport and band 3. Combined with the present observation that H_2DIDS is capable of cross-linking the 60 000 dalton fragment (which encompasses the 17 000 dalton fragment) with the 35 000 dalton fragment and that papain inhibits anion transport without a visible effect on the 17 000 dalton fragment but with a concomitant hydrolysis of the 35 000 dalton fragment, these aspects of the experimental results of Grinstein et al. [16] are also compatible with the present suggestion that both the 35 000 and the 60 000 dalton fragment participate in the formation of the environment in which H_2DIDS produces an inhibition of anion exchange. It should be pointed out, however, that Grinstein et al. also report a degradation of the 35 000 dalton fragment by chymotrypsin that is not accompanied by an inhibition of anion transport. They conclude, therefore, that this fragment does not participate in the control of transport. This aspect of the work of these authors is clearly not compatible with our findings. It is necessary, therefore, to emphasize that we always observed the 35 000 dalton fragment after chymotryptic digestion and that we do not find any degradation of that fragment even after long exposure to exorbitantly high concentrations of the enzyme. The reasons for the discrepancy are not clear, since the techniques employed are essentially similar except that we used different gel systems for the detection of the bands and added bovine serum albumin to the washing media to remove residual proteolytic activity prior to the isolation of the cell membrane for electrophoresis.

Heterogeneity of band 3

On sodium dodecyl sulfate-polyacrylamide gels, band 3 extends over a molecular weight range of about 15 000 [1]. This is commonly attributed to a heterogeneity of the population of band 3 molecules [1] which is thought to reside in differences of carbohydrate composition [5,25]. The two different bands near 55 000 daltons that emerge after removal by internal trypsin of the water-soluble 40 000 dalton fragment provided further proof of this heterogeneity [8] and differences in the carbohydrates in the two bands were in fact demonstrated by Jenkins and Tanner who showed that the glycoproteins in the two bands bind different lectins [25].

The heterogeneity of the tryptic bands is reflected by the band pattern obtained after their digestion by extracellular chymotrypsin. The products observed are one single band at 17 000 daltons and a diffuse double band with an

extended trailing edge near 35 000 daltons (Fig. 11). The 17 000 daltons fragment is likely to be homogeneous both from its appearance as a single sharp band on the gel and from the fact that it has a single C-terminal amino acid, tyrosine [12]. The observed heterogeneity of the 35 000 dalton band could arise solely on the basis of difference of carbohydrate content. However, it has not been demonstrated that this band consists of a single polypeptide. In any case, the heterogeneity of the band 3 appears to reside in this fragment of the molecule, at least in most donors (see Ref. 30). The structural heterogeneity of the band 3 protein does not seem to result in a functional heterogeneity. All of the integral protein in band 3 can bind H_2DIDS, as evidenced by the complete regeneration by H_2DIDS of previously digested band 3 (Figs. 2–4) and, under the conditions of H_2DIDS labeling used, nearly all of the covalent H_2DIDS binding to band 3 is related to inhibition of anion exchange [31].

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