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Major proteolytic fragments of the murine band 3 protein as obtained after in situ proteolysis

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Proteolytic fragments of murine band 3 were produced by exposure to extracellular chymotrypsin and intracellular trypsin. The ensuing proteolytic fragments were isolated, their N-terminal sequences were determined and their locations in the known amino acid sequence of murine band 3 established. Equivalents of the human 60, 35 and 17 kDa fragments were obtained though the cleavage sites were situated at locations that are not strictly homologous to the corresponding cleavage sites in human band 3, although all of them were near such sites. Exposure of the intact murine red cell to chymotrypsin leads to the formation of two fragments of 67 kDa and 41 kDa, which are equivalent to the 60 kDa and the 35 kDa fragments of the human band 3. Internal trypsin cleaves the chymotryptic 67 kDa fragment while the 41 kDa fragment appears essentially unaffected. The 67 kDa fragment is first degraded to 64 kDa, then further to 22 kDa and finally to 19 kDa. The anion transport inhibitor H_2DIDS (4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate) combines with murine band 3 protein as it does with human band 3. Anion transport is maximally inhibited when $5 \cdot 10^5$ H_2DIDS molecules per cell are bound to band 3. As in the human red cell, after exposure to high pH (9.0–9.5) of the H_2DIDS-labeled, chymotryptically cleaved band 3 intramolecular cross-linking takes place. This joins the 67 and 41 kDa chymotryptic pieces together to form a peptide of the original molecular mass of band 3 of 108 kDa. If cross-linking is performed after additional tryptic cleavage, the 19 and 22 kDa pieces join together with 41 kDa pieces to form overlapping bands that cover the molecular weight range from 60 to 63 kDa.

Introduction

Attempts to determine the amino acid sequence of band 3 protein have lead to the establishment of the complete sequence of erythroid band 3 from mouse [1,2], chicken [3] and man [4] and a non-erythroid band 3 derived from human K562 cells [2]. The sequences were derived from cloned cDNA's of the band 3 proteins of the various species. For the prediction of the folding of the peptide chains of the various anion transporting erythroid band 3 species hydrophobicity plots and helical wheels have been prepared. In view of the uncertainties associated with these predictions additional information is needed. One way of obtaining the additional information about the disposition of the protein in the lipid bilayer consists of studying the accessibility of the peptide chain to the action of proteolytic enzymes. Such studies have been performed extensively with human red cells in the past [5].

In the present paper we deal with the band 3 protein of the red blood cell of the mouse. Analogously to previous studies in human red blood cells we performed experiments about the susceptibility of the murine band 3 protein to in situ cleavage by the proteolytic enzymes chymotrypsin and trypsin, and a combination of the two enzymes. The fragments were isolated, their N-terminal sequences determined and their locations in the known sequence of the peptide chain established.

In the human red blood cell, anion transport can be inhibited by stilbenedisulfonates which combine with high specificity with the band 3 protein [6,7]. Our experiments on proteolysis of murine band 3 were supplemented by correlating the binding of the non-penetrating stilbenedisulfonate H_2DIDS to band 3 with transport. This enabled us to demonstrate that each

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; H_2DIDS, 4,4′-diisothiocyanodihydrostilbene-2,2′-disulfonate; SDS, sodium dodecylsulfonate; Tris, tris(hydroxymethyl)aminomethane; PITC, phenylisothiocyanate; PMSF, phenylmethylsulfonyl fluoride.

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band 3 protein molecule is actually involved in mediating anion transport, and to determine the number of band 3 molecules per cell. We further showed that, again similar to the human red blood cell [8], the two isothiocyanate groups of the H_2DIDS molecule are able to establish intramolecular cross-links. The results show that the behavior of the murine band 3 is essentially similar to that of human band 3 but not identical. This conclusion will become important for the comparison of the disposition of the band 3 in its natural environment and in the plasma membrane of *Xenopus* oocytes in which murine band 3 can be expressed after microinjection of mRNA that encodes the protein [15].

Materials and Methods

All experiments were performed with red blood cells obtained from anesthesized (diethyl ether) white mice (NMRI) by cardiac puncture. Before use, the cells were washed and equilibrated either with 'Cl^- medium' (130 mM NaCl, 1 mM Na_2SO_4, 20 mM EDTA (pH 7.4)) or with 'SO_4^{2-} medium' (108 mM Na_2SO_4, 20 mM EDTA (pH 7.4)).

(1) Chymotryptic-tryptic digestion

Intact red cells or resealed ghosts were incubated with 1 mg/ml TLCK-Chymotrypsin-A_4 (Sigma) in Cl^- medium for 60 min at 37°C. Chymotrypsin was inhibited by PMSF (final concentration 150 μM). The cells were extensively washed to remove the chymotrypsin. Trypsin treatment was performed after hemolyzing the cells or ghosts in 5 mM sodium phosphate (pH 8.0 at 0°C). The ensuing membranes are freely permeable. They were exposed to trypsin for 30 min at 37°C, 10% hematocrit in 130 mM NaCl, 20 mM EDTA (pH 7.4). Trypsin was inhibited by adding trypsin inhibitor from soya beans (Sigma, 100 μg/ml) and aprotinin (Trasyloi®, Bayer Leverkusen, 100 μg/ml). In some experiments the treated membranes were 'stripped' with 10 mM NaOH at 0°C for 2 min to remove peripheral proteins. The washed membranes were dissolved in sample buffer (see below).

(2) Gel electrophoresis according to Laemmli

Washed membranes of hemolysed red cells were dissolved in 4% SDS, 60% glycerol, 0.25 g DTT/10 ml, 0.1 M Tris-HCl (pH 6.8) and heated for 5 min at 100°C. Electrophoresis was performed on Laemmli gels with a 5% stacking gel and a 12.5% separation gel [9]. Gels were stained in a solution of 45% methanol, 5% acetic acid and 50% water, containing 0.5% Coomassie blue. They were destained in the same solution without the dye. Dried gels were autoradiographed on Kodak XRP5 films for 4–6 days.

(3) Blotting and sequenching

After electrophoresis, the separated proteins were blotted on Immobilon™-Membranes [10], using a 100 mM Tris-acetate buffer, (pH 8.3) containing 0.2% SDS and 20% ethanol, in a Semi-Dry Blotting Apparatus at 200 mA, 20°C for 2 h. The Immobilon-Membrane was stained for 10–15 s with either 0.5% Coomassie blue or 0.5% Amido black in the staining solution described in the preceding section. The membrane was destained in the same solution without the dye for 10 to 15 min. Stained bands were cut out and sequenced in an Applied Biosystems 470 A Gas Phase Sequencer using the standard (03CPTH) program. The phenylthiohydantoin derivatives were analysed by an on-line PTH analyser (model 120A, Applied Biosystems).

(4) Flux measurements

Sulfate equilibrium exchange was measured in SO_4^{2-} medium as described by Lepke et al. [7] and chloride equilibrium exchange in Cl^- medium by the inhibitor stop technique described by Ku et al. [14].

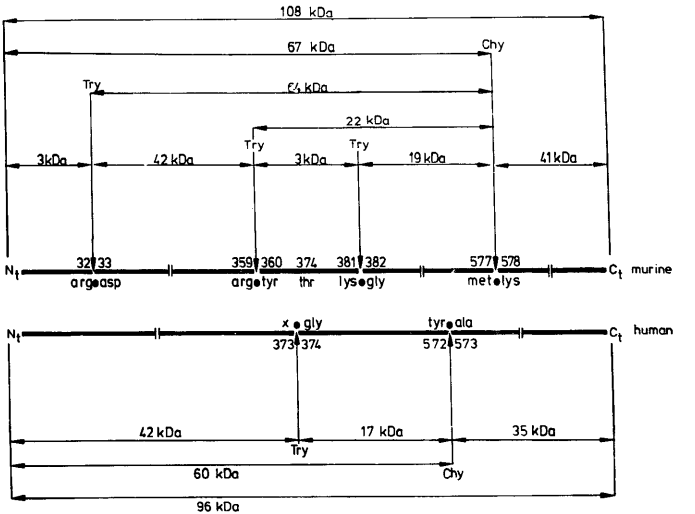
(5) Determination of H_2DIDS binding to band 3

H_2DIDS and $^3H_2$DIDS were synthesized as described earlier [7]. The H_2DIDS binding was calculated from the $^3H_2$DIDS bound to the band 3 region on SDS-polyacrylamide gel electrophoretograms. The specific activity of the $^3H_2$DIDS was determined as described in Ref. 17. The amount of membrane protein placed on the gels was estimated by the method of Lowry. Determinations of the amount of membrane protein per cell were made by combining measurements of $^3H_2$DIDS binding per cell with measurements of $^3H_2$DIDS binding per mg of membrane protein, derived from the known number of cells. The conversion factor obtained was $2.68 \cdot 10^7$ cells/mg membrane protein.

Results and Discussion

(1) Proteolytic fragments of band 3 and their disposition in the lipid bilayer (Fig. 1)

(a) Action of extracellular chymotrypsin. In the human red blood cell, treatment with extracellular chymotrypsin leads to a cleavage of the band 3 protein at a single location, yielding fragments of 60 kDa and 35 kDa. The former represents the whole of the hydrophilic domain, including the N-terminus of the band 3 protein and a segment of 17 kDa of the hydrophobic domain. The latter represents the remaining part of the hydrophobic domain (35 kDa), including the C-terminus. The cleavage site has been identified and it has been shown that the C-terminus of the 60 kDa fragment most likely is homologous to Tyr-572 of the murine band 3 (Fig. 1, for a review, see Ref. 11). In spite of the cleavage of the primary structure, anion



peptide kDa	N - terminal sequences(Edman) of murine band 3 peptides	comments
64	Asp Leu Leu Ile Pro Val Thr Glu Met Gln Asp Pro Glu Ala Leu	hydrophilic
22	Tyr Leu Pro Ser Pro Ala Lys Pro Asp Pro Asn Leu Tyr Asn Thr Leu	hydrophobic
19	Gly Gly Pro Gly Asp Glu Asp Asp Pro Leu Arg Arg Thr Gly	hydrophobic
41	Lys Pro Lys Pro Gln Gly Pro Val Pro Asn Thr Ala Leu	hydrophobic

Fig. 1. Disposition of the human and murine band 3 in the lipid bilayers of their respective red cell membranes as infered from proteolytic digestion by external chymotrypsin and internal trypsin. Digestion of murine band 3 yields from some band 3 molecules fragments of 3, 42, 22 and 41 kDa; from others fragments of 3, 45, 19 and 41 kDa. The fragments of 3, 42 kDa and 45 kDa are water soluble and lost during the preparation of the membranes for SDS-polyacrylamide gel electrophoresis. On the gels we observe three hydrophobic peptides of 19, 22 and 41 kDa (see Figs. 2, 3). For as yet unknown reasons, the relative quantities of the 19 and 22 kDa peptides obtained in different experiments vary considerably, indicating that the accessibility of the two tryptic cleavage sites varies. The table at the bottom of the figure indicates the N-terminal amino acid sequences determined in the isolated peptide fragments of the murine band 3. The four partial sequences agree completely with corresponding sequences deduced from cDNA clones by Kopito and Lodish [1] or Demuth et al. [2]. The human N-terminal sequence at Gly-374 is represented in Table II. Numbering of amino acid residues according to Kopito and Lodish [1]. Note that the figure is not to scale. Note also that molecular weights indicated in the figure are the apparent values derived from their location on the SDS-polyacrylamide gel electrophoretograms. They are not necessarily identical to the molecular weights derived from amino acid sequence. Adding them up does, therefore, not necessarily lead to 108 kDa, the molecular weight of the band 3 protein (including its carbohydrate moiety) observed by gel electrophoresis.

transport remains unchanged [16], indicating that the tertiary structure stabilizes the disposition of the modified protein in the membrane.

On SDS-polyacrylamide gel electrophoretograms, the murine band 3 is located at 108 kDa. Extracellular chymotrypsin cleaves the protein at one single site, leading to the formation of fragments of 67 kDa and 41 kDa, which are equivalent to the 60 kDa and 35 kDa

TABLE I

Effects of external chymotrypsin on band 3 protein-mediated sulfate transport in murine red cell ghosts

^0k_s =, rate constant for sulfate transport; σ_M, = standard error of the mean (at 30 °C $n = 4$, at 0 °C $n = 3$). Intact murine red cells were treated with chymotrypsin (1 mg/ml) for 1 h at 37 °C. The enzyme was removed by washing first with albumin-containing (0.5%) Cl^- medium and subsequently with Cl^- medium without albumin. Next, the cells were incubated for 90–105 min without or with 50 μM H_2DIDS (pH 9.0). Subsequently, the H_2DIDS was removed by washes with albumin-containing Cl^- medium and the cells were used to prepare resealed ghosts in Cl^- medium (pH 7.4). The ghosts were fluxed with $^{35}SO_4^{2-}$ in that medium (no H_2DIDS present) at 30 °C or 0 °C (pH 7.4). The measurements at 0 °C were included to demonstrate that lowering the temperature to the temperature at which Cl^- exchange is usually measured does not increase the stability of the chymotrypsinized transport protein.

	^0k_s (10^{-4} min^{-1}), mean ± σ_M	
	temp. 30 °C	temp. 0 °C
Control	1300 ± 120	7.34 ± 0.31
Control + H_2DIDS	468 ± 40	3.58 ± 0.44
H_2DIDS-sensitive flux	830	3.76
Chymotrypsin	923 ± 120	5.03 ± 0.39
Chymotrypsin + H_2DIDS	358 ± 30	3.17 ± 0.69
H_2DIDS-sensitive flux	570	1.86
Inhibition of H_2DIDS-sensitive flux by chymotrypsin	31%	51%

fragments seen in human band 3 (Figs. 1, 3). The cleavage site could be determined by sequencing the first 13 N-terminal amino acid residues of the 41 kDa fragment (Fig. 1). It is located between Met-577 and Lys-578, i.e. 5 amino acid residues further towards the C-terminal end of band 3 than in the human red cell. Cleavage is associated with a partial inhibition of SO_4^{2-} equilibrium exchange (Table I). Thus the cleavage site of the murine band 3 is either closer to the transport site or the tertiary structure of the protein is less stable in the murine red cell than in the human red cell.

(b) Combined actions of extracellular chymotrypsin and intracellular trypsin. When human red cells are first treated with chymotrypsin, ghosted and then exposed to trypsin, the 60 kDa chymotryptic fragment is further cleaved to yield two fragments of 42 kDa and 17 kDa (Fig. 1). The former consists of the hydrophilic domain of band 3 and includes the N-terminus. The latter represents part of the hydrophobic domain. The tryptic cleavage site is located at the inner membrane surface since extracellular trypsin produces no degradation of band 3. The hydrophilic 42 kDa fragment is released from the membrane as the (leaky) ghosts are washed. Thus, on SDS-polyacrylamide gel electrophoretograms of the membranes isolated after treatment with chymotrypsin and trypsin one observes only two bands that are derived from the band 3 protein: the chymotryptic 35 kDa band and the 17 kDa chymotryptic-tryptic band. These two fragments comprise the whole of the hydrophobic domain of band 3 and hence that portion of the protein that is required for the execution of the transport function (Fig. 1, for reviews, see Refs. 5, 11, 12).

Treatment of the murine red cell membrane with chymotrypsin and trypsin analogously to the treatment of the human red cell membrane followed by washes to remove water-soluble fragments of the hydrophilic domain of band 3, first leads to the reduction of the 67 kDa fragment to 64 kDa. This is due to the release of a tryptic 3 kDa piece from the N-terminal end, as demonstrated by the determination of the 15 N-terminal amino acid residues of the isolated 64 kDa fragment (Figs. 1, 3 and 4). The cleavage site was localized as being between Arg-32 and Asp-33. Upon further digestion hydrophilic fragments of 45 kDa or 42 kDa and 3 kDa are released. On the gel there appear three proteolytic fragments, rather than the two fragments observed in the human red cell membrane (Fig. 1). The molecular weights of the fragments are 41, 22 and 19 kDa. It was found that the first 16 amino acid residues of the N-terminus of the 22 kDa fragment coincided with the amino acid residues of the cDNA derived sequence of murine band 3, beginning with Tyr-360. The first 14 amino acid residues of the 19 kDa fragment agreed with the amino acid residues beginning at Gly-382. In the 41 kDa fragment, the first 13 amino acid residues beginning at Lys-388 were found to be identical with those of the published sequence. From these data one may conclude that the 41 kDa fragment is identical to the 41 kDa fragment obtained after chymotryptic cleavage without subsequent treatment with trypsin. The two smaller peptides represent overlapping pieces of the amino acid sequence in the hinge region between the hydrophilic and the hydrophobic domains of murine band 3. The overlap indicates that they cannot originate from one and the same peptide chain. They are most likely derived from identical peptide chains of different band 3 molecules. In some of the band 3 molecules, the cleavage site Arg-359/Tyr-360 is hydrolyzed by trypsin faster than the cleavage site Lys-381/Gly-382. In others, the situation is inverse: the cleavage site Lys-381/Gly-382 is split faster than the cleavage site Arg-359/Tyr-360. Perhaps the two cleavage sites are located in two different protomers of the same dimer and the protomer, in which the cleavage site Arg-359/Tyr-360 is exposed protects the cleavage site Lys-381/Gly-382 on the other protomer, and vice versa.

(2) H_2DIDS binding to band 3 and the localisation of the binding sites on proteolytic fragments of band 3

The anion transport inhibitor H_2DIDS combines fairly selectively with the band 3 protein of the red cells of man [6,7] and mouse (Fig. 2). In the human red cell, the 17 kDa and 35 kDa chymotryptic-tryptic fragments

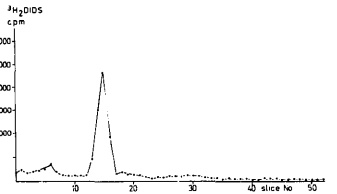


Fig. 2. Distribution profile of $^3H_2$DIDS on a SDS-polyacrylamide gel electrophoretogram of the isolated murine red cell membrane.

can be cross-linked by H_2DIDS to form the 55 kDa fragment, which comprises the hydrophobic domain of the band 3 protein [8]. Analogous cross-linking experiments were performed with murine red cells. The intact red cells were first exposed to chymotrypsin, then to H_2DIDS at pH 7.4 to achieve the covalent reaction of one of the two isothiocyanate groups of the cross-linker with one of the two fragments of the cleaved band 3 molecules. Subsequently the cells were ghosted and the membranes exposed to trypsin. Next, the membranes were subdivided into two batches. One was incubated at pH 9.0, 37 °C for 90 min to establish the cross-links. The other was kept at pH 7.4, 0 °C for the same length of time. The membranes were then subjected to SDS gel electrophoresis. The electrophoretograms show that after cross-linking in place of the 3 bands at 41, 19 and 22 kDa in the controls only one band appears that extends from 60 to 63 kDa (Figs. 3 and 4). This indicates that 41 kDa fragments combine with either 19 or the 22 kDa fragments to form two bands of 60 kDa and 63 kDa, which overlap and hence cannot be distinguished on the gels. We conclude, therefore, that the tryptic cleavage of the chymotryptic 67 kDa fragment leads to the formation of two 41 kDa fragments, one fragment of 19 kDa, and one fragment of 22 kDa, all of which are predominantly hydrophobic and are seen on the gels. The remaining peptides (Fig 1) are lost during the washes of the membranes prior to gel electrophoresis.

The results described provide independent evidence for the conclusion drawn above that tryptic cleavage may take place simultaneously at different locations of distinct band 3 molecules. It shows in addition that

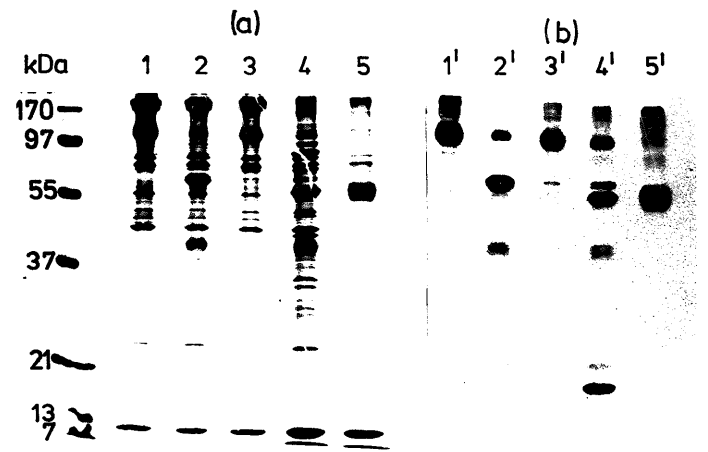


Fig. 3. (a) Effect of proteolytic digestion in situ on the $^3H_2$DIDS labeled murine band 3 protein. Lane 1: control. Lane 2: external chymotrypsin, 1 mg/ml. Lane 3: cross-linking after cleavage by 1 mg/ml chymotrypsin. Lane 4: external chymotrypsin (1 mg/ml) and internal trypsin (30 μg/ml). Lane 5: cross-linking after cleavage by external chymotrypsin (1 mg/ml) and internal trypsin (30 μg/ml) (membranes not stripped). (b) Lanes 1'–5': Autoradiogram of the lanes represented in Fig. 3 (a). Lanes 2 and 2' show the 67 kDa and 41 kDa fragments together with some undigested band 3. Lanes 4 and 4' represent full length band 3, 67, 64, 41, 22 and 19 kDa pieces.

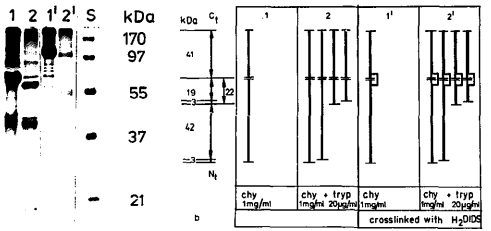


Fig. 4. (a) SDS-polyacrylamide gel electrophoretogram of murine red cell membranes treated with extracellular chymotrypsin (1 mg/ml) or extracellular chymotrypsin (1 mg/ml) and intracellular trypsin, without and with cross-linking by H_2DIDS. Lanes 1 and 1': Lanes 2 and 2': Lane S: 0 µg/ml trypsin; 20 µg/ml trypsin; standard proteins. Lanes 1, 2: without H_2DIDS; Lanes 1', 2': 25 µM H_2DIDS. After proteolysis for 1 h at 30 °C, the samples were incubated at pH 9.0 for 90 min. (to achieve cross-linking in the H_2DIDS-treated samples) and stripped in 0.1 M NaOH for 2 min at 0 °C. The polyacrylamide concentration was 12.5%. (b) Interpretation of the band pattern shown in the SDS polyacrylamide gel electrophoretograms in Fig. 4(a). This interpretation is based on the identification of the four peptides of 64, 41, 22 and 19 kDa by N-terminal amino acid sequence analysis (see Fig. 1). The bracket ([) indicates cross-linking of fragments by H_2DIDS.

both chymotryptic-tryptic fragments of 19 and 22 kDa are equivalent to the single 17 kDa fragment obtained from the human band 3. It may be added that we were able to confirm [13] that the isolated 17 kDa fragment of the human band 3 yields a single N-terminal sequence (Table II) and hence that the narrow 17 kDa band on the SDS-polyacrylamide gel electrophoretogram represents in fact a single peptide. It should be noted that neither one of the two tryptic peptides of 19 and 22 kDa of the murine band 3 is strictly homologous to the human 17 kDa fragment. The latter commences with its N-terminus at a location which is homologous to Thr-374 (or at Leu-375 according to Ref. 2), which resides 8 amino acid residues below Gly-382, the N-terminus of the tryptic 19 kDa fragment of the murine band 3, and 15 amino acid residues above Tyr-360, the N-terminal amino acid of the murine 22 kDa fragment.

Prolonged exposure of chymotrypsinized murine red cell membranes to trypsin leads to an accumulation of the 19 kDa fragment at the expense of the 22 kDa fragment, and to a degradation of the 41 kDa fragment. A further description of these changes is outside the scope of the present paper.

(3) Correlation between H_2DIDS binding to band 3 and inhibition of anion exchange

In the human red blood cell there exists a linear relationship between binding of H_2DIDS to band 3 and the inhibition of anion equilibrium exchange [7]. Inhibition is complete when each band 3 molecule has bound one H_2DIDS molecule. At complete inhibition of transport, $(1–1.1) \cdot 10^6$ H_2DIDS molecules are bound per cell [7], a number which agrees with independent estimates of the number of band 3 molecules per human red blood cell [5].

In the murine red blood cell, the relationship between H_2DIDS binding to band 3 and inhibition of anion transport is also linear. Inhibition is maximal

TABLE II

Comparison of amino acid sequence of N-terminal region of human chymotryptic-tryptic 17 kDa fragment (H) with homologous portion of murine sequence (M)

The murine sequence is derived from cDNA sequence [1,2]. The human band 3 sequence (established by Edman degradation) encompasses the sequence from Leu-375–GLy-380 previously determined by Mawby and Findlay [13].

	381										
M	Thr	Leu	Asp	Leu	Asn	Gly	Gly	Lys	Gly	Gly	Pro	Gly	Asp	Glu
H	Gly	Leu	Asp	Leu	(Asn)	Gly	Gly	Pro	Asp	Asp	Pro	X	Gln	Glu

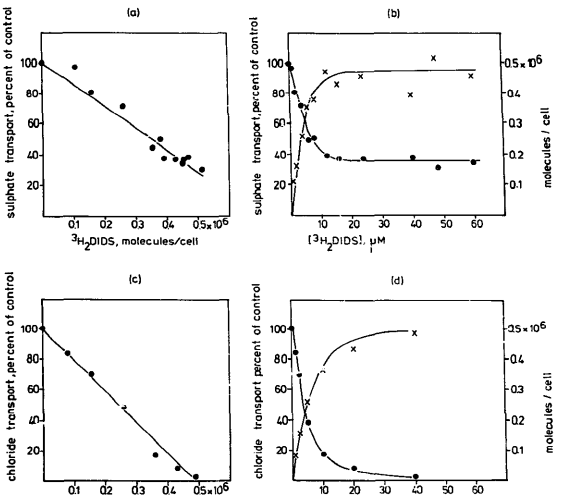


Fig. 5. Effect of H_2DIDS binding to the band 3 protein on sulfate (a, b) and chloride (c, d) equilibrium exchange in SO_4^{2-} and Cl^- medium, respectively (composition see p. 292). Cl^- flux measured at 0 °C (pH 7.4), SO_4^{2-} flux at 30 °C (pH 7.4). In (a) and (c) ordinate: equilibrium exchange as a percent of the untreated controls. Abscissa: Number of H_2DIDS molecules bound to band 3, per cell. In (b) and (d) left ordinate: equilibrium exchange as a percent of untreated controls (●); right ordinate: H_2DIDS binding to band 3, molecules/cell (×). Abscissa: H_2DIDS concentration in the medium prior to the addition of the cells. Note that maximal inhibition of SO_4^{2-} flux does not lead to complete inhibition.

when $5 \cdot 10^5$ H_2DIDS molecules are bound per cell (Fig. 5). Binding to band 3 cannot be increased beyond this number. When this number of band 3 molecules is occupied by H_2DIDS, intramolecular cross-linking (after previous exposure to chymotrypsin) is just complete (not shown), confirming a 1 : 1 stoichiometry for the relationship between binding and inhibition.

Comments

(1) In spite of the high sequence homology of the erythroid band 3 proteins of mouse and man, notably in the hydrophobic domains, none of the proteolytic cleavage sites established in the present experiments coincide exactly. At least in the case of the chymotryptic cleavage site, this does not only reflect differences of primary structure but also differences of the disposition in the membrane (see p. 294). This needs to be considered when one tries to interpret the kinetics of anion transport as mediated by the band 3 proteins of the two species.

(2) The differences of the cleavage patterns by intracellular trypsin suggest that the hinge region between the hydrophobic and the hydrophilic domains of murine and human band 3 differ noticeably. The failure of trypsin to split the human band 3 at the location homologous to the splitting site in the murine band 3 (lys 381) is due to a substitution of the amino acid residue by a proline residue in man. This residue may exert a rather profound effect on the structure of the hinge region, and our results suggest that in the two species these regions differ not only with respect to amino acid sequence but also with respect to folding.

(3) It is gratifying to know that the N-terminal amino acid sequence as determined by Edman degradation of four peptides of the murine band 3 (13–16 amino acids per peptide) agree completely with the corresponding sequences derived from cloned cDNA, obtained by

Kopito and Lodish [1] and Demuth et al. [2]. This is in accord with the view that the cloned cDNA is representative for the bulk of band 3 protein in murine red cells. However, since only 58 out of 929 amino acid residues have been checked, the possibility still exists that in murine band 3, similar to the chicken band 3 [3], there may exist closely related band 3 molecules which differ by minor pieces of amino acid sequence. Such differences would not necessarily need to be the result of the expression of different band 3 genes but may be the result of differences of the processing of hnRNA in the cell nucleus.

Appendix (added in proof)

The turnover number of band 3-mediated Cl^- equilibrium exchange in the red cell of the mouse

Assuming $^{\circ}k_{Cl} = 0.117 \pm 0.012\ s^{-1}$ ($n = 10$) (at 0 °C), the existence of $5 \cdot 10^5$ band 3 molecules per cell, a cell volume of $53.2 \pm 4.0\ \mu m^3$ ($n = 5$), a dry weight of 34%, a Donnan ratio of 0.75 (pH 7.4), one arrives at a turnover number of 490 ions · (band 3)$^{-1} \cdot s^{-1}$. In the human red cell, Brahm (J. Gen. Physiol. (1977) 70, 283–306) obtained a turnover number of 230 ions · (band 3)$^{-1} \cdot s^{-1}$ (at 0 °C). Our estimate pertains to a Cl^- concentration of 130 mM, that of Brahm to 150 mM.

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