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The Anion-Transport Protein of the Human Erythrocyte Membrane

STUDIES ON FRAGMENTS PRODUCED BY PEPSIN DIGESTION

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We have studied the fragmentation by pepsin in 1 M-acetic acid of the erythrocyte anion-transport protein in erythrocyte membranes. The location of the fragments obtained was determined by radioiodinating the protein with the use of lactoperoxidase, and identifying the labelled peptides obtained in peptide 'maps' of thermolysin digests of the fragments. Three of the fragments were found to be related overlapping products, and shared a common *C*-terminus. The major site of pepsin cleavage leading to the *C*-termini of these fragments was shown to be close to the major site of extracellular cleavage of the protein by proteinases active at a neutral pH. Another two fragments were isolated and shown to be derived from the *C*-terminal portion of the protein. No well-defined large radioactive fragments of the protein were solubilized from the membrane by pepsin in 1 M-acetic acid, the bulk of the radioactivity attributable to the anion transport protein being recovered in very small fragments that could not be resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Our results suggest that the polypeptide chain of the anion-transport protein emerges at the extracellular face of the membrane 8000–13000 daltons on the *N*-terminal side of the major site of extracellular cleavage of the protein by proteinases that are active at a neutral pH.

The structure of the erythrocyte anion-transport protein (band 3; Steck, 1974) has been investigated by using various fragmentation methods (for recent reviews see Cabantchik *et al.*, 1978; Steck, 1978). Fragmentation by proteinases has been employed (Jenkins & Tanner, 1975, 1977*a*; Steck *et al.*, 1976, 1978; Grinstein *et al.*, 1978; Williams *et al.*, 1979) as well as by chemical protein-cleavage reagents (Drickamer, 1976, 1977; Steck *et al.*, 1976, 1978). Proteinases that are active at a neutral pH all yield rather similar fragments from the native protein in erythrocyte membranes. Cleavage in the extracellular domain of the protein with Pronase, chymotrypsin, thermolysin and subtilisin gives very similar membrane-bound fragments with an apparent mol.wt. of about 60000. Subsequent cleavage of this 60000-mol.wt. fragment in the intracellular domain by trypsin or chymotrypsin also gives rise to membrane-bound fragments that are similar to each other.

For structural studies on the protein it would be desirable to be able to cleave the protein at different groups of sites with different proteinases. We have studied the fragmentation of the protein with pepsin at an acid pH. Pepsin yields a group of fragments that differ from those produced by the proteinases active at a neutral pH. The locations of the regions of the polypeptide chain that give rise to the pepsin-digest fragments have also been investigated.

Abbreviations used: SDS, sodium dodecyl sulphate; DIDS, 4,4-di-isothiocyano-2,2-stilbenedisulphonic acid.

Methods

SDS/polyacrylamide-gel electrophoresis and determination of molecular weights

SDS/polyacrylamide-gel electrophoresis was done with the discontinuous buffer system of Laemmli (1970) with a ratio of acrylamide to *NN'*-methylenebisacrylamide of 1:30 (w/v) throughout the gel. The separating gels contained a gradient of acrylamide concentration as indicated. The stacking gels contained 5% (w/v) acrylamide. Samples for electrophoresis were dissolved with 1 vol. of a solution containing 5% (w/v) SDS, 10% glycerol, 20 mM-Tris/HCl buffer, pH 8.0, 5 mM-EDTA, pH 8.0, 5% (v/v) 2-mercaptoethanol, 2 mM-phenylmethanesulphonyl fluoride (Sigma Chemical Co.) and 0.1 mg of each of the marker dyes Bromophenol Blue and Xylene Cyanol/ml. The dissolved samples were immediately heated in boiling water for 2 min. Molecular weights were determined by using a marker peptide mixture (BDH Chemicals) covering the

mol.wt. range 2500–17000 and the marker protein mixture described by Williams *et al.* (1979).

Thermolysin and trypsin digestion of erythrocytes and erythrocyte 'ghosts'

Thermolysin or trypsin digestion of intact human erythrocytes, radioiodination of erythrocytes or erythrocyte 'ghosts' with $Na^{125}I$ and lactoperoxidase, the isolation of labelled fragments and the preparation of thermolysin peptide 'maps' were done as described previously (Boxer *et al.*, 1974; Jenkins & Tanner, 1975; Williams *et al.*, 1979). The preparation of DIDS and labelling of erythrocytes with DIDS were done as described by Cabantchik & Rothstein (1974), by using 9 μM-DIDS with a 25% (v/v) suspension of erythrocytes.

Pepsin digestion of erythrocyte 'ghosts'

'Ghosts' from 5ml of erythrocytes were suspended in 30ml of ice-cold 0.1M-acetic acid and centrifuged at 30000*g* for 40min. The pellet was suspended in 4ml of 1M-acetic acid and incubated at 37°C for 15 min with 0.5mg of pepsin (Sigma Chemical Co.)/ml. At the end of the reaction 4ml of 1M-NH_4HCO_3 was slowly added with gentle mixing, and the mixture was centrifuged at 30000*g* for 30min at 4°C. The solubilized material in the supernatant was freeze-dried and the membrane-bound material in the pellet was washed twice with 40ml of ice-cold diluted iso-osmotic sodium phosphate buffer, pH7.4 (iso-osmotic sodium phosphate buffer, pH7.4, diluted with 19vol. of deionized water) before being solubilized for SDS/polyacrylamide-gel electrophoresis.

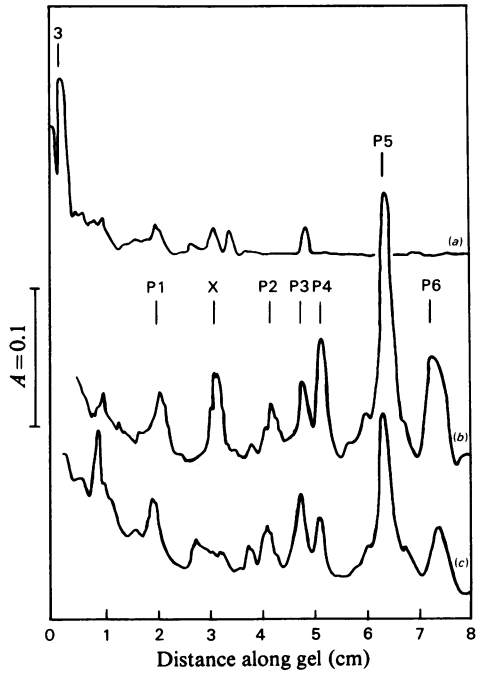

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of pepsin-treated erythrocyte 'ghosts'*

A scan of gel containing a gradient of 10–30% (w/v) acrylamide stained with Coomassie Blue is shown. (*a*) Erythrocyte 'ghosts' extracted with 0.1M-acetic acid; (*b*) pepsin-treated membranes from trypsin-treated erythrocytes; (*c*) pepsin-treated membranes from thermolysin-treated erythrocytes. 3 indicates intact anion-transport protein.

Results and Discussion

Pepsin treatment of erythrocyte 'ghosts'

Erythrocyte 'ghosts' were washed with 0.1M-acetic acid to remove the peripheral proteins and subsequently treated with pepsin in 1M-acetic acid. The membrane-bound fragments were separated by discontinuous SDS/polyacrylamide-gel electrophoresis in a concentration gradient of acrylamide (Fig. 1). Several membrane-bound fragments were obtained with pepsin-treated 'ghosts' from trypsin-treated cells (denoted P1–P6; Fig. 1*b*). The heterogeneous band X (Fig. 1*b*) was not present in pepsin-treated 'ghosts' from untreated erythrocytes and was probably not derived from the anion-transport protein. Bands P1–P6 probably represent membrane-bound fragments of the erythrocyte anion-transport protein. Inspection of the gels showed that bands P1 and P2 each consisted of at least two poorly resolved bands.

Bands P3 and P4 were obtained in variable amounts in different experiments, very little of these two bands being recovered in some digests. Bands P5 and P6 were consistently observed after pepsin treatment. The mobility and sharpness of bands P5 and P6 on SDS/polyacrylamide-gel electrophoresis was dependent on the amount of digest loaded on the gel. Sharp bands and reproducible electrophoretic mobilities were obtained only when small amounts of digest were applied to the gel. Larger amounts of sample caused a decrease in the electrophoretic mobilities of bands P5 and P6 and broadening of the bands. The apparent mol.wts. of bands P3, P4, P5 and P6 on SDS/polyacrylamide-gel electrophoresis were 21000, 18500, 12500 and 4000 respectively. Lower apparent mol.wts. were obtained when 6M-urea was present in the gel (17000, 16500, 8000 and 3500 for bands P3, P4, P5 and P6 respectively). Since these are membrane-bound fragments, it is likely that they bind the detergents in a micellar fashion, a mode of binding of SDS different from that which occurs with the soluble marker peptides used for calibrating the gels (Tanford & Reynolds, 1976). The different values obtained on electrophoresis in the presence and

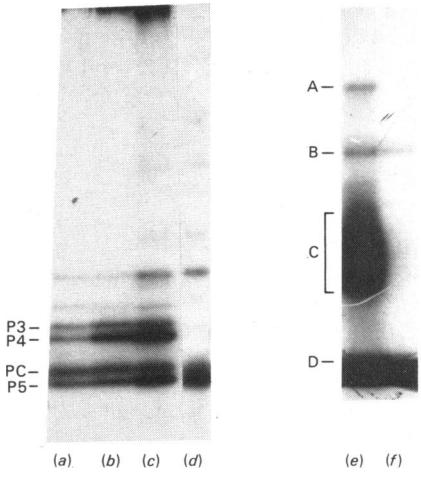

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of pepsin-digest fragments of the anion-transport protein*

(*a*)–(*d*) Membrane-bound fragments. A radioautograph of gel containing a gradient of 10–25% (w/v) acrylamide is shown. The samples in tracks (*a*)–(*c*) were from the same batch of trypsin-treated erythrocytes that had been radioiodinated with lactoperoxidase and $Na^{125}I$. (*a*) Membrane-bound fragments from pepsin-digested 'ghosts' derived from radioiodinated trypsin-treated erythrocytes. (*b*) Membrane-bound fragments from pepsin-digested 'ghosts' derived from thermolysin-treated radioiodinated trypsin-treated erythrocytes. (*c*) Membrane-bound fragments obtained after pepsin digestion of trypsin-treated 'ghosts' from radioiodinated trypsin-treated erythrocytes. The 'ghosts' were treated with 0.1 mg of trypsin (1-chloro-4-phenyl-3-tosylamidobutan-2-one-treated)/ml at 37°C for 15 min and washed with 0.1 M-acetic acid before pepsin digestion. (*d*) Sample from an experiment that used the same procedure as that for track (*a*) but done on a different occasion. (*e*) and (*f*) Fragments solubilized from the membrane by pepsin. A radioautograph of unstained gel containing a gradient of 10–25% (w/v) acrylamide is shown. 'Ghosts' from trypsin-treated erythrocytes were digested with pepsin and the material solubilized from the membranes was isolated as described in the Methods section. The results of a parallel experiment with the same batch of erythrocytes are shown. In (*e*) the 'ghosts' from the trypsin-treated erythrocytes were radioiodinated with lactoperoxidase and $Na^{125}I$. In (*f*) the trypsin-treated erythrocytes were radioiodinated. The D band could be distinguished as a discrete sharp band within the broad area of radioactivity migrating with the Bromophenol Blue marker dye and could be clearly distinguished on short exposures of radioautographs.

absence of urea probably reflect different effects of urea on the two types of binding processes. These apparent molecular weights are unlikely to be accurate, since soluble proteins were used to calibrate the gels, but are useful in indicating the relative sizes of the fragments.

Erythrocytes were treated with DIDS, a specific inhibitor of erythrocyte anion transport (Cabantchik & Rothstein, 1974), and incubated with either trypsin or thermolysin before lysis. The erythrocyte 'ghosts' were digested with pepsin and the membranes were separated by gel electrophoresis. The DIDS bound to the fragments was detected by its fluorescence (Williams *et al.*, 1979). Bands P3 and P4 were found to contain the fluorescent DIDS label, and some fluorescence was found in the low-molecular-weight P6 band.

Location of the membrane-bound pepsin-digest fragments in the protein amino acid sequence

The origin of the membrane-bound pepsin-digest fragments of the anion-transport protein was examined by lactoperoxidase radioiodination of the anion-transport protein in intact erythrocytes and erythrocyte 'ghosts'. To label the extracellular region of the protein, erythrocytes were radioiodinated with lactoperoxidase and treated with trypsin to remove radiolabel associated with the major sialoglycoprotein. 'Ghosts' prepared from these erythrocytes were digested with pepsin and the membrane-bound fragments were separated by SDS/polyacrylamide-gel electrophoresis (Fig. 2*a*). Co-electrophoresis with the unlabelled samples shown in Fig. 2 showed that the fragments P3, P4 and P5 were strongly labelled, whereas fragment P6 was weakly labelled. In addition, a strongly labelled band (PC) was obtained, as well as labelled material with a slower electrophoretic mobility than that of band P3. Band PC corresponded in position to the band, staining weakly for protein, migrating just more slowly than band P5 in the sample shown in Fig. 1(*b*). Apparent mol.wts. of 13500 and 8000 were obtained for band PC on gel electrophoresis in the absence and presence of 6 M-urea. Fragments P3, P4, PC and P5 contain radioiodinated sites that are located in the extracellular region of the protein.

When both the intracellular and extracellular domains of the protein were labelled by radioiodination of the 'ghosts' derived from trypsin-treated erythrocytes, the pattern of radioactivity in the pepsin fragments shown in Fig. 3(*a*) was obtained. Fig. 3(*b*) shows the profile obtained when only the extracellular region of the protein was radioiodinated and the protein was digested with pepsin under the same conditions. In this experiment bands P4, P6 and P5 were obtained, but band P3 was much diminished and only discernible as a shoulder on the P4 band. Strong labelling corresponding to the band P6 staining for protein was obtained in the sample that had been radioiodinated in both intracellular and

extracellular regions (Fig. 3*a*), and this radioactivity extended as a broad shoulder to the front of the P6 band (P7 in Fig. 3).

Thermolysin digestion of the radioiodinated anion-transport protein yields 14 major labelled peptides that have characteristic mobilities on peptide 'maps'. Peptides 1–5 (see Fig. 4*a*) are located in the extra-cellular domain of the protein, whereas peptides 6–14 are located in the cytoplasmic domain of the protein (Boxer *et al.*, 1974). The positions of many of these sites on the linear amino acid sequence of the protein are also known (Williams *et al.*, 1979). The pepsin fragments derived from 'ghosts' prepared from radioiodinated trypsin-treated erythrocytes were isolated by SDS/polyacrylamide-gel electrophoresis. Thermolysin digests of the pepsin-digest fragments were prepared and the radioactive peptides were separated on peptide 'maps'. Fragment P4 gave the labelled peptides 2, 2A and 3 (Fig. 4*b*), and fragment P5 contained peptides 4 and 5 (Fig. 4*c*). Hydrophobic radioactive spots with a high mobility in the chromatography solvent were also evident on the 'maps', and some of these were also detected in the peptide 'maps' of the intact protein (Fig. 4*a*). Peptide 'maps' of fragment PC gave mainly peptides 2, 2A and 3, but also contained small amounts of peptides 4 and 5. The last-mentioned peptides are probably derived from contamination by fragment P5, which is poorly resolved from fragment PC. Conversely, traces of peptides 2, 2A and 3 were found in the 'maps' of fragment P5 and probably resulted from contamination by fragment PC. Fragment P6 gave very weak peptide 'maps' containing several faint spots with a predominant component that migrated near the chromatography solvent front but did not migrate in the electrophoresis dimension. The weak labelling of the P6 band was probably due to a small amount of radioactivity incorporated into lipids together with very minor fragmentation products of the protein. No fragment P3 was obtained in this experiment, but fragment P3 isolated from other experiments gave the same peptide 'map' as fragment P4, containing only peptides 2, 2A and 3 (Fig. 4*d*).

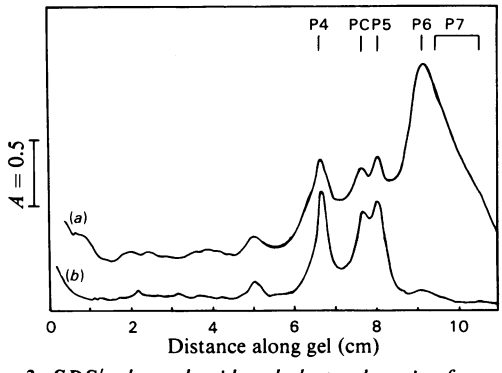

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of pepsin-treated membranes from radioiodinated erythrocytes and radioiodinated erythrocyte 'ghosts'*

A scan of radioautograph of gel containing a gradient of 10–25% (w/v) acrylamide is shown. 'Ghosts' from trypsin-treated erythrocytes were treated with pepsin and the membrane-bound fragments were isolated as described in the Methods section. The results of a parallel experiment with the same batch of erythrocytes are shown. In (*a*) the 'ghosts' from trypsin-treated erythrocytes were radioiodinated with lactoperoxidase and $Na^{125}I$, and in (*b*) the trypsin-treated erythrocytes were radioiodinated with lactoperoxidase and $Na^{125}I$.

The pepsin-digest fragments were also isolated from radioiodinated 'ghosts' from trypsin-treated erythrocytes in a parallel experiment, in which the protein was labelled in both intracellular and extracellular domains. The peptide 'maps' obtained for fragments P3, P4, PC and P5 in this case were the same as the 'maps' of the corresponding fragments obtained when the protein was labelled in the extracellular region alone. None of the characteristic labelled peptides that originate from the cytoplasmic domain of the protein were present in the peptide 'maps' of fragments P3, P4, PC and P5.

The peptide 'maps' of fragments P6 and P7, which were strongly radioiodinated when the protein was labelled in both extracellular and cytoplasmic domains (Fig. 3*a*), contained large amounts of radioactivity (probably labelled lipids) that migrated as a broad smear in the chromatography solvent but did not migrate or gave a slow-moving smear in the electrophoresis dimension. Interference on the peptide 'maps' by this material was minimized by extraction of the P6 and P7 bands, which had been eluted from the polyacrylamide gel, with butan-1-ol. The peptide 'map' of fragment P7 (Fig. 5*a*) contained strong spots corresponding to the labelled peptides 8, 9 and 10, which are located in the cytoplasmic domain of the protein. The 'map' of fragment P6 was similar except for the additional presence of the weakly labelled spots found in 'maps' of fragment P6 derived from the protein labelled in the extracellular region alone. These results suggest that the P7 band contains a membrane-bound fragment of the protein that carries only the intracellular sites which gave rise to the labelled peptides 8, 9 and 10. The group of fragments P3, P4, PC and P5 contain only the extracellular sites that can be radioiodinated in the protein with lactoperoxidase. It is clear that fragments P3, P4 and PC, which all contain peptides 2, 2A and 3, are related overlapping fragments distinct from fragment P5, which contains only peptides 4 and 5.

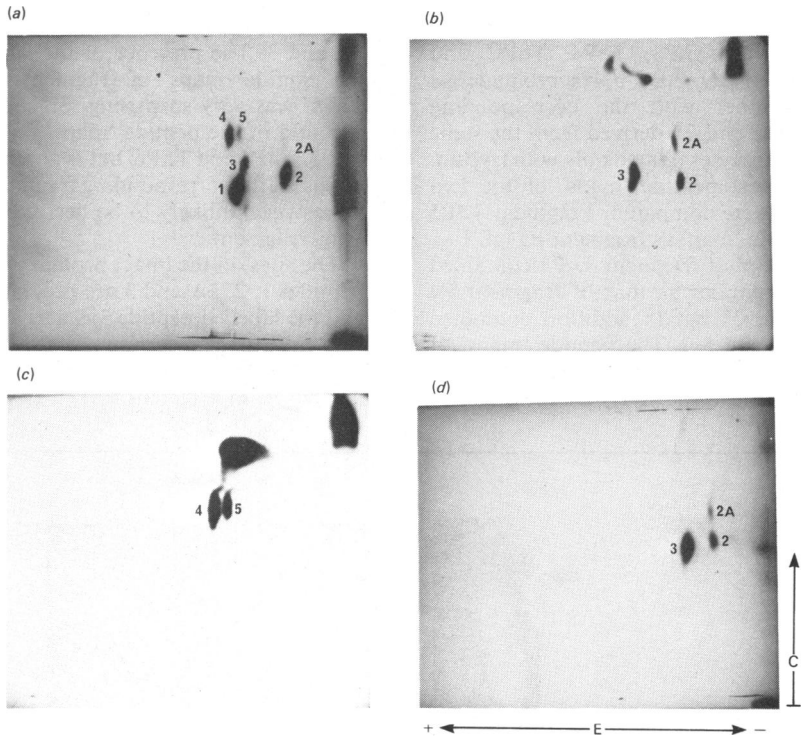

Fig. 4. *Radioautographs of peptide 'maps' of thermolysin digests of membrane-bound pepsin-digest fragments of the anion-transport protein*

Fragments were obtained by pepsin digestion of 'ghosts' from radioiodinated trypsin-treated erythrocytes. (*a*) Intact anion-transport protein obtained from a control in which pepsin digestion was omitted. (*b*) Fragment P4 obtained from the sample shown in Fig. 3(*b*). (*c*) Fragment P5 obtained from the sample shown in Fig. 3(*b*). (*d*) Fragment P3 obtained from the sample shown in Fig. 2(*a*). The samples in (*a*)–(*c*) were derived from the same batch of labelled trypsin-treated erythrocytes and peptide 'maps' were prepared in parallel. The sample in (*d*) was from a different experiment and the peptide 'map' was prepared on a different occasion. The mobilities of the labelled peptides in (*d*) are not directly comparable with those in (*a*)–(*c*). The labelled peptides in (*d*) were assigned by use of a peptide 'map' of the intact protein that was prepared in parallel. The labelled peptide 2A is a minor peptide, derived from a site in the extracellular domain of the protein, that is evident in the peptide 'maps' of some of the fragments. C, Chromatography dimension; E, electrophoresis (pH 3.5) dimension.

Pepsin treatment of 'ghosts' from thermolysin-treated erythrocytes

Thermolysin treatment of erythrocytes results in extracellular cleavage of the anion-transport protein to yield a major fragment (Th1) that has a *C*-terminal tyrosine residue (Jenkins & Tanner, 1977*b*). The related chymotrypsin fragment (ChyA) also has *C*-terminal tyrosine (Drickamer, 1976). The site yielding the labelled peptide 3 on peptide 'maps' is located near the *C*-terminus of fragment Th1 (Williams *et al.*, 1979). Since fragments P3, P4 and PC contain the labelled peptide 3, it was decided to determine the effects of previous cleavage by thermolysin (near the site that yields labelled peptide 3 in thermolysin peptide 'maps') on the fragmentation by pepsin, so as to define the location of these fragments more precisely.

Fig. 1(*c*) shows that pretreatment of erythrocytes with thermolysin did not result in any detectable change in the mobility of bands P3, P4, P5 and P6, although there were differences in the higher-molecular-weight bands. When the experiment was done with radioiodinated erythrocytes the same result was obtained (Fig. 2*b*). The labelled band PC was also obtained after pretreatment of erythrocytes with thermolysin. Electrophoresis of control samples showed that the thermolysin treatment of the erythrocytes in this experiment resulted in the almost total cleavage of the anion-transport protein to yield the fragment Th1. The labelled fragments from pepsin

digestion of 'ghosts' from thermolysin-treated radioiodinated erythrocytes (ThP3, ThP4, ThPC and ThP5) were isolated by SDS/polyacrylamide-gel electrophoresis together with the corresponding fragments P3, P4, PC and P5 derived from the same radioiodinated erythrocytes treated only with trypsin. The thermolysin-digest peptide 'maps' of the two series of fragments were compared. Fragment ThP5 gave the same peptide 'map' as fragment P5 (cf. Fig. 4*c*). The peptide 'map' of fragment ThP4 contained the same labelled peptides as that of fragment P4 (peptides 2, 2A and 3) but in addition contained peptide 1 (Figs. 5*b* and 5*c*). The peptide 'maps' of fragment ThP3 and ThPC were also similar to those of fragments P3 and PC respectively except that they both contained peptide 1 in addition to peptides 2, 2A and 3. The presence of the labelled peptide 1 in the peptide 'maps' of fragments ThP3, ThP4 and ThPC was very surprising. Since labelled peptide 1 is found in the peptide 'maps' of each of fragments ThP3, ThP4 and ThPC but is absent from the peptide 'maps' of the corresponding fragments P3, P4 and PC, it was very unlikely to be derived from a contaminating fragment.

The sites on the intact protein yielding the labelled peptides 1, 2, 2A and 3 are present in fragment Th1, and the labelled peptide 3 is derived from a site at or very close to the *C*-terminus of the fragment (Williams *et al.*, 1979). As fragments ThP3, ThP4 and ThPC are derived from fragment Th1, it is very likely that the

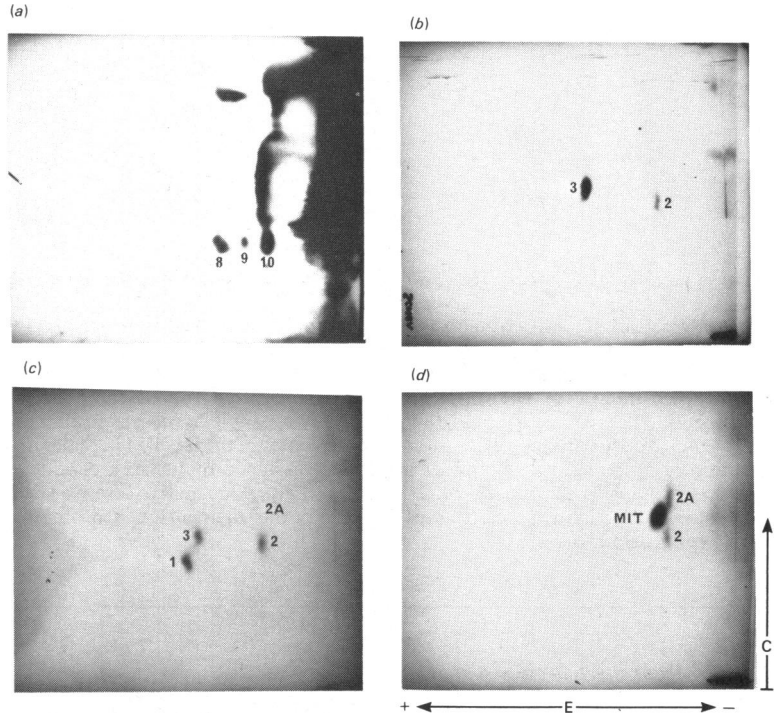

Fig. 5. *Radioautographs of peptide 'maps' of thermolysin digests of fragments of the anion-transport protein*
In each case the labelled peptides were assigned by comparison with a peptide 'map' prepared in parallel of the intact protein derived from the same sample of radioiodinated erythrocyte membranes. (*a*) Fragment P7 derived from pepsin-treated radioiodinated erythrocyte 'ghosts' (same sample as that shown in Fig. 3*a*). After elution of the fragment from the gel and removal of SDS by gel filtration on Sephadex G-25 (fine grade) as described by Jenkins & Tanner (1975), the fragment sample was freeze-dried. Then 1 ml of water was added to the dry sample and it was extracted with 1.5 ml of butan-1-ol with vigorous shaking for 30s. The phases were separated by centrifugation at 1000 rev./min for 20min in an MSE Mistral centrifuge. Approx. 70% of the radioactivity was extracted into the organic phase. The aqueous phase was freeze-dried, digested with thermolysin and the labelled peptides were separated on peptide 'maps' as described by Boxer *et al.* (1974). (*b*) Fragment P4 from a sample similar to that shown in Fig. 2(*a*). (*c*) Fragment ThP4 from a sample similar to that shown in Fig. 2(*b*). This sample was derived from the same radioiodinated erythrocyte preparation as that used to prepare the fragment P4 sample in (*b*). (*d*) Carboxypeptidase A-treated fragment ThP4. Half of the purified fragment ThP4 sample shown in (*c*) was digested with carboxypeptidase A. For experimental details see the legend to Table 4. C, Chromatography dimension; E, electrophoresis (pH 3.5) dimension.

C-termini of all these fragments are coextensive with fragment Th1. The pepsin cleavage that gives the *C*-termini of fragments P3, P4 and PC must also be on the *C*-terminal side of the site yielding peptide 3. If the site yielding peptide 1 were on the *N*-terminal side of the sites yielding peptides 2 and 3, then the fragments with corresponding molecular weights would have different *N*- and *C*-termini. Since fragment ThPC contains site 1, whereas fragments P3, P4 and PC do not, the *C*-termini of fragments P3 and P4 would have to extend substantially (at least 7500–9000 daltons and 5000–8500 daltons respectively) to the *C*-terminal side of the site yielding the labelled peptide 3. If this were the case, it would be extremely unlikely that the different sites of pepsin cleavage that lead to the fragments of corresponding molecular weight would fortuitously yield fragments of the same molecular weight for three pairs of fragments, each pair having different sites of cleavages. It seemed much more likely that the corresponding pairs of fragments were generated by the same pepsin cleavages to yield their *N*-termini, and, since fragments ThP3, ThP4 and ThPC share common *C*-termini, pepsin cleavage of the untreated protein occurred sufficiently close to the position of the *C*-termini of fragments ThP3, ThP4 and ThPC that the two groups of fragments were not resolved by SDS/polyacrylamide-gel electrophoresis. In this case the labelled peptide 1 could not be derived from a site on the protein that is different from that yielding either peptide 2 or 3.

Table 1. *Radioactivity in labelled peptides* 1, 2 *and* 3 *in thermolysin-digest peptide 'maps' of pepsin-digest fragments from the untreated and thermolysin-treated anion-treated protein*

Fragment samples were obtained as described in the legend to Table 2 and peptide 'maps' were prepared. The radioactivity in each peptide on the 'maps' was determined as described by Jenkins & Tanner (1975) after detection by radioautography. The amounts of radioactivity in the spots from each peptide 'map' were normalized for comparison by making the radioactivity in peptide 2 = 1.0. The intact protein and fragment Th1 were appropriate controls from the same sample of radioiodinated erythrocytes used to obtain the pepsin-digest fragments.

	Radioactivity in labelled peptide			
Peptide ... Fragment	1	2	3	1+3
Intact protein	2.9	1.0	0.6	3.5
Th1	2.7	1.0	1.6	4.3
P3	—	1.0	3.6	3.6
ThP3	1.7	1.0	1.5	3.2
P4	—	1.0	3.5	3.5
ThP4	1.4	1.0	1.2	2.6
PC	—	1.0	3.5	3.5
ThPC	0.9	1.0	0.7	1.6

Table 2. *Recovery of labelled peptides in thermolysin-digest peptide 'maps' of labelled fragments of anion-transport protein after carboxypeptidase A treatment*

Pepsin-treated membranes from trypsin-treated radioiodinated erythrocytes and pepsin-treated membranes from thermolysin-digested trypsin-treated radioiodinated erythrocytes were separated by SDS/polyacrylamide-gel electrophoresis (compare with Figs. 2*a* and 2*b*), together with control samples from which the pepsin digestion was omitted. The intact protein, fragment Th1, and the two groups of pepsin fragments were eluted and freeze-dried. The samples were dissolved in 0.2ml of 5mM-NH_4HCO_3 and desalted on a column (0.5cm×20cm) of Sephadex G-25 (fine grade) (Pharmacia) equilibrated with the same buffer. The radioactive material eluted at or near the void volume was collected and divided in half. One portion of each fragment sample was used as a control, and peptide 'maps' were prepared as below but with the carboxypeptidase A treatment omitted. The data in Table 1 were derived from these samples. Ammonium acetate (2M) was added to the remaining portions of the fragments to give a final concentration of 20mM, and each sample was incubated with 100μg of carboxypeptidase A (Worthington Biochemical Corp.) at 37°C for 20min. The digests were made 0.5mM with respect to EDTA and heated at 100°C for 5min to denature the carboxypeptidase A. The samples were thermolysin-digested and peptide 'maps' were prepared as described by Boxer *et al.* (1974). The amounts of radioactivity in the labelled peptides in each peptide 'map' were normalized for comparison by making the radioactivity in peptide 2 = 1.0. The recovery of each labelled peptide in each fragment was calculated by dividing the normalized radioactivity in the labelled peptide from the peptide 'map' of the carboxypeptidase A-treated fragment by the normalized radioactivity in the corresponding peptide from the peptide 'map' of the untreated fragment. For fragments P5 and ThP5 the radioactivities were normalized so that peptide 4 = 1.0. —, Peptide not detected or weak in the peptide 'map' of untreated or carboxypeptidase A-treated sample; 0, peptide not detected in the peptide 'map' of carboxypeptidase A-treated sample.

	Recovery after carboxypeptidase A treatment of labelled peptide				
Peptide ... Fragment	1	2	3	4	5
Intact protein	1.0	1.0	1.1	0.8	0.6
P3	—	1.0	0	—	—
P4	—	1.0	0	—	—
PC	—	1.0	0	—	—
P5	—	—	—	1.0	0.8
Th1	0.4	1.0	0.1	—	—
ThP3	0.1	1.0	0	—	—
ThP4	0	1.0	0	—	—
ThPC	0	1.0	0	—	—
ThP5	—	—	—	1.0	1.0

Table 1 shows the relative amounts of radioactivity in peptides 1, 2 and 3 in the peptide 'maps' of the two series of fragments. Fragments P3, P4 and PC all gave over 3 times more radioactivity in peptide 3 than in peptide 2. In contrast, fragments ThP3, ThP4 and ThPC contained nearly equal amounts of radioactivity in peptide 3 and peptide 2, in addition to substantial amounts in peptide 1. A value of 3–4 was obtained for the ratio of the sum of the radioactivity in peptide 1 + peptide 3 relative to that of peptide 2 for controls not treated with pepsin and for both series of fragments, although the value obtained for fragment ThPC was rather lower. These results suggested that the labelled peptides 1 and 3 may be related thermolysin-digestion products containing the same labelled tyrosine residue of the intact protein.

Origin of labelled peptides 1 *and* 3

To further define the origin of the labelled peptides 1 and 3, portions of the purified labelled fragments P3–P5 and ThP3–ThP5 were treated with carboxypeptidase A before thermolysin digestion and peptide 'mapping'. The purified labelled fragments were the same as those used in the experiment shown in Table 1. In addition to monoiodotyrosine, the peptide 'maps' of carboxypeptidase A-treated fragments P3, P4 and PC contained peptides 2 and 2A but did not contain peptide 3 (Table 2). Peptide 3 was also absent and peptide 1 was very weak or absent in the peptide 'maps' of fragments ThP3, ThP4 and ThPC (Fig. 5*d* and Table 2). Thus the sites giving rise to peptides 1 and 3 are on the *C*-terminal side of the site giving rise to peptide 2.

In a further experiment fragment Th1 and the pepsin-digest fragments ThP3, ThP4 and ThPC were isolated from 'ghosts' derived from thermolysin-treated radioiodinated erythrocytes that were untreated or had been treated with carboxypeptidase A. Peptide 'maps' were obtained for the two series of pepsin fragments. In this case the comparison was between fragments ThP3, ThP4 and ThPC derived from untreated fragment Th1 and from carboxypeptidase-treated fragment Th1. The results are shown in Table 3. Carboxypeptidase A treatment of fragment Th1 resulted in the substantial loss of peptide 3 and a small increase in peptide 1. Similarly the pepsin-digest fragments originating from carboxypeptidase A-treated fragment Th1 all showed a decrease in peptide 3 and an increase in peptide 1. This result, together with the results in Tables 1 and 2, suggests that peptides 1 and 3 are different subfragments derived from the same labelled tyrosine site in the protein.

We conclude that the series of fragments P3, P4 and PC differ from the corresponding fragments ThP3, ThP4 and ThPC only at their *C*-termini, and it is this difference that gives rise to the different peptide 'maps' of the series of pepsin fragments from untreated or thermolysin-treated erythrocytes. The probable basis of this difference is illustrated in Fig. 6. The pepsin cleavage of the intact protein that yields the *C*-termini of fragments P3, P4 and PC results in the generation of only the labelled peptide 3 during the thermolysin digestion used to prepare the peptide 'maps'. Thermolysin treatment of the native protein in erythrocytes results in cleavage at two sites that are close together, yielding two fragment Th1 species containing slightly different *C*-

Table 3. *Effects of carboxypeptidase A treatment of thermolysin-digested radioiodinated erythrocytes on labelled peptides in thermolysin-digest peptide 'maps' of pepsin-digest fragments of anion-transport protein*

A sample of washed thermolysin-digested radioiodinated erythrocytes was divided into two portions. One portion was retained as a control, and the remainder was treated with carboxypeptidase A after being washed with 0.15M-NaCl containing 0.1M-ammonium acetate. A 25% (v/v) suspension of cells in the same buffer was incubated with 100μg of carboxypeptidase A (Sigma Chemical Co.) at 37°C for 15min. After being washed three times in 0.15M-NaCl containing 0.2% bovine serum albumin, twice with 0.15M-NaCl and once with iso-osmotic sodium phosphate buffer, pH7.4, the control and carboxypeptidase A-treated cells were lysed and the 'ghosts' were digested with pepsin as described in the Methods section. Portions of the 'ghosts' were retained before pepsin digestion for the isolation of fragment Th1 and carboxypeptidase A-treated fragment Th1. The two groups of fragments were isolated by SDS/polyacrylamide-gel electrophoresis, thermolysin-digested and peptide 'maps' were prepared. The radioactivity in each labelled peptide of each peptide 'map' was measured. For each peptide 'map' the radioactivity in each labelled peptide was normalized so that the radioactivity in peptide 2 = 1.0. To calculate the recovery of peptides for each fragment, the normalized radioactivity of the peptide in the 'map' of the fragment from carboxypeptidase A-treated thermolysin-digested cells was divided by the radioactivity in the 'map' of the fragment from thermolysin-digested cells.

	Relative recovery in fragments after carboxypeptidase A treatment in labelled peptide		
Peptide ...	1	2	3
Fragment			
Th1	1.2	1.0	0.3
ThP3	1.5	1.0	0.4
ThP4	1.5	1.0	0.3
ThPC	1.6	1.0	0.4

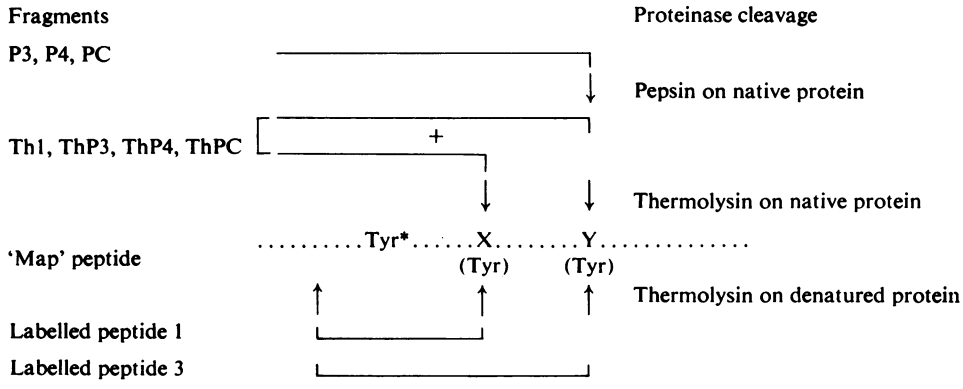

Fig. 6. *Relationship between labelled peptides* 1 *and* 3

The polypeptide chain of the intact protein is indicated by the dotted line. The arrows above the dotted line representing the polypeptide chain show the sites of cleavage by pepsin and thermolysin on the native protein in erythrocyte membranes that give rise to the fragments indicated. The arrows below the dotted line indicate the sites of cleavage by thermolysin on the denatured protein or fragments isolated after SDS/polyacrylamide-gel electrophoresis and that give rise to the labelled peptides 1 and 3 on peptide 'maps'. The parentheses indicate that residues X and Y may be tyrosine residues. The asterisk indicates the tyrosine residue that is ^{125}I-labelled by lactoperoxidase. This residue may be identical with residue X.

termini. One site of cleavage is the same as that of pepsin on the intact protein, and this Th1 species yields labelled peptide 3 on subsequent thermolysin digestion of the denatured protein to prepare peptide 'maps'.

The other site of extracellular thermolysin cleavage on the native protein is to the *N*-terminal side of the pepsin cleavage site, and this Th1 species yields the labelled peptide 1 during the subsequent thermolysin digestion for preparation of peptide 'maps'. Fragments ThP3, ThP4 and ThPC retain the heterogeneous *C*-termini of fragment Th1 and give both labelled peptides 1 and 3 in thermolysin-digest peptide 'maps'. Since thermolysin-digest peptide 'maps' of the denatured intact protein contain both labelled peptides 1 and 3 (see, e.g., Fig. 4*a*), it is clear that thermolysin also cleaves the denatured intact protein at the two sites that yield fragment Th1 from the native protein. We previously suggested that fragment Th1 has a *C*-terminal sequence of Tyr-Tyr, since carboxypeptidase A treatment of the purified fragment yielded 1.6mol of tyrosine/mol of polypeptide (Jenkins & Tanner, 1977*b*). This result would also be consistent with the two species of fragment Th1 having *C*-terminal sequences of -Tyr and -Tyr-Tyr. If this were the case, labelled peptides 1 and 3 would differ by the presence of an additional tyrosine residue at the *C*-terminus of peptide 3. The relative mobilities on peptide 'maps' of peptides 3 and 1 are consistent with this relationship. Although suggestive, the above evidence is not in itself conclusive, and other variants of this *C*-terminal sequence could give rise to the observed relationship between peptides 1 and 3.

The tyrosine present at the *C*-terminus of peptide 3 does not appear to be significantly radioiodinated by lactoperoxidase, since the results in Tables 1 and 3 show that a decrease in the radioactivity in peptide 3 is associated with an increase in the radioactivity of peptide 1. A tyrosine residue in peptide 1 is radioiodinated, and this could be located at the *C*-terminus of peptide 1. It is apparent from the peptide 'maps' of fragments P3, P4 and PC that once formed, peptide 3 is not cleaved to peptide 1 even under the conditions of exhaustive thermolysin digestion used to prepare the peptide 'maps'.

Alignment of the membrane-bound pepsin-digest fragments

Fig. 7 shows the disposition of the pepsin-digest fragments on the linear sequence of the intact protein. Because the relative order of the groups labelled sites 4 and 5 and sites 8, 9 and 10 is not yet defined, there are two possible locations for fragments P5 and P7.

Trypsin treatment of leaky erythrocyte 'ghosts' results in cleavage of the anion-treatment protein at two points around the labelled site 14 (see Fig. 7), producing a large membrane-bound fragment that contains the *C*-terminal half of the protein (Steck *et al.*, 1976, 1978; Jenkins & Tanner, 1977*a*; Williams *et al.*, 1979). Pepsin treatment of trypsin-treated erythrocyte 'ghosts' also gave fragments P3, P4, PC and P5 (Fig. 2*c*), as would be expected.

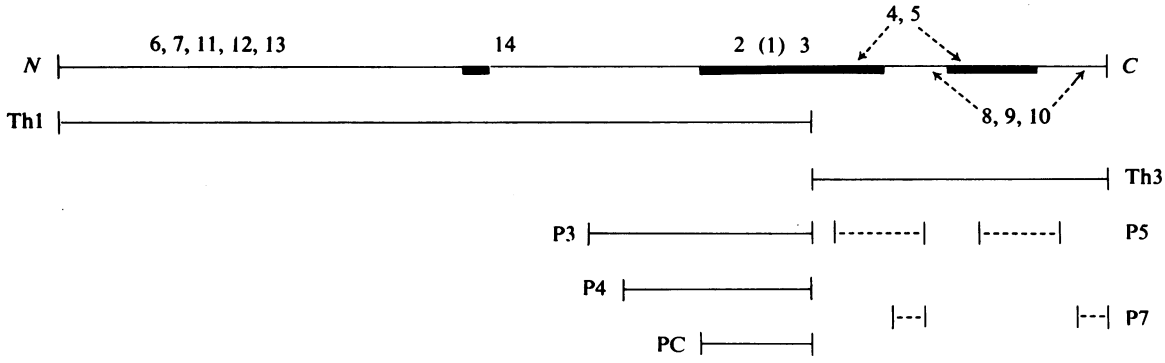

Fig. 7. *Alignment of pepsin-digest fragments on the sequence of the anion-transport protein*
The numbers represent the positions of the ^{125}I-labelled sites in the protein that give rise to the corresponding numbered radioactive peptides in thermolysin-digest peptide 'maps' of the anion-transport protein (Williams *et al.*, 1979). The broken lines indicate alternative positions of two of the groups of ^{125}I-labelled sites and alternative locations for the pepsin-digest fragments P5 and P7. The thickened portions of the line representing the intact protein indicate regions of the protein that are in the extracellular domain of the protein.

Fragment PC is the smallest of the series of membrane-bound fragments P3, P4 and PC, coextensive with a point close to the *C*-terminus of one species of fragment Th1. In some digestions no fragments P3 and P4 and only small amounts of fragment PC were observed (Fig. 2*d*). Fragments smaller than fragment PC that contain the radioactive label probably do not remain membrane-bound. A region of the polypeptide some 17000–20000 daltons from the *C*-terminus of fragment Th1 is known to be at the cytoplasmic face of the membrane (Grinstein *et al.*, 1978; Steck *et al.*, 1978; Williams *et al.*, 1979). The occurrence of fragment PC suggests that the polypeptide chain must emerge at the extracellular face of the membrane within 8000–13000 daltons from the *C*-terminus of fragment Th1. The present experiments do not allow us to distinguish whether the *N*-terminus of fragment PC is on the cytoplasmic or the extracellular side of the membrane. Drickamer (1977) has also suggested that the polypeptide crosses the membrane 7000–11000 daltons from the *C*-terminus of fragment Th1 on the basis of experiments with chemical cleavage reagents. It is not clear whether the sequence to the *N*-terminal side of fragment PC that is present in fragments P3 and P4 is in a part of the cytoplasmic domain of the protein that is relatively resistant to proteinase digestion or traverses the membrane again to form a loop at the extracellular surface of the membrane. Proteinases such as chymotrypsin and thermolysin, which are active at a neutral pH, yield a major fragment with *C*-termini close to that of fragments P3, P4 and PC and *N*-termini close to the *N*-terminal side of fragment P3 (Grinstein *et al.*, 1978; Steck *et al.*, 1978; M. J. A. Tanner, unpublished work).

Fragment P5 was also obtained when the extracellular region of the protein had been cleaved by thermolysin to give fragment Th1 and the complementary fragment Th3 (Fig. 7). Fragment P5 must be derived from pepsin cleavage of fragment Th3, which contains the radioiodinated sites 4 and 5 and sites 8, 9 and 10 (Williams *et al.*, 1979). The sharpness of the P5 band suggests that it does not carry the oligosaccharides that are responsible for the diffuse banding patterns of fragment Th3 and the intact protein. Since fragment P5 is membrane-bound, these oligosaccharides must be located either between the *C*-terminus of fragment Th1 and the *N*-terminus of fragment P5 or in a separate extracellular loop of the polypeptide chain.

Fragments of the anion-transport protein solubilized from the membrane by pepsin digestion

The material solubilized by pepsin digestion of 'ghosts' from trypsin-treated erythrocytes was separated by SDS/polyacrylamide-gel electrophoresis. When the membranes were labelled in both extracellular and intracellular domains four major radioactive bands were found (A–D; Fig. 2*e*), but when labelling of the membrane was restricted in the extracellular region only two of these bands, B and D, were labelled (Fig. 2*f*). The radioactivity in the B band was lost on staining of the gels for protein, and only part of the radioactivity in the D band was retained after this procedure. The components of bands A and B had apparent mol.wts. of 41000 and 22000 on SDS/polyacrylamide-gels. Material of band C gave a broad band with an apparent mol.wt. range of 3000–8000. Band D migrated with the marker dye on gels containing a gradient of 10–25% (w/v) acrylamide. The material in this band was clearly of very low molecular weight.

The bands were isolated and characterized by thermolysin-digest peptide 'mapping' to determine whether they contained any of the labelled thermolysin-digest peptides characteristic of the anion-transport protein. The peptide 'maps' of components of bands A and B did not contain any of the major labelled peptides characteristic of the anion-transport protein. The peptide 'map' of the component of band B contained large amounts of radioactivity in a smear around the origin, a region characteristic of glycopeptides. This band may be derived from the blood-group-Ss-active sialoglycoprotein (δ; Anstee *et al.*, 1979), which resists trypsin digestion of erythrocytes. The components of band C gave a complicated peptide 'map' that contained the major labelled peptide 7 derived from the anion-transport protein and several other peptides, some of which are present as minor components in peptide 'maps' of the intact anion-transport protein. The broadness of the C band and the complicated peptide 'map' suggests that it contains a heterogeneous mixture of peptides, some of which are derived from the anion-transport protein. The peptide 'map' of the components of D contained peptides 6, 8, 9, 10, 11, 12, 13 and 14, nearly the total complement of the labelled peptides derived from sites located in the intracellular domain of the anion-transport protein. This band clearly contains a number of very small unresolved soluble fragments of the anion-transport protein. Under these conditions of pepsin digestion no large labelled fragments of the anion-transport protein were released from the erythrocyte membrane.

Pepsin treatment of the anion-transport protein at an acid pH produces a series of membrane-bound fragments that differ from those obtained with neutral proteinases. Fragment P5 is likely to be particularly useful for structural studies, as it is derived from a region of the molecule from which it has proved difficult to obtain well-defined fragments.

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