Structure of the Anion-Transport Protein of the Human Erythrocyte Membrane

FURTHER STUDIES ON THE FRAGMENTS PRODUCED BY PROTEOLYTIC DIGESTION

By David G. WILLIAMS, Robert E. JENKINS and Michael J. A. TANNER

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(Received 6 February 1979)

The topology of the human erythrocyte membrane anion-transport protein (band 3) has been investigated by isolation and peptide 'mapping' of the major and minor fragments derived from proteolytic cleavage of the lactoperoxidase ^{125}I -labelled protein in erythrocytes and erythrocyte membranes. The content, in each fragment, of lactoperoxidase ^{125}I -labelled sites (which have a known location in the extracellular or cytoplasmic domain of the protein), together with the location of the sites of proteolytic cleavage yielding the fragments, has allowed us to determine the alignment of the fragments on the linear amino acid sequence and to infer the topology of the polypeptide in the membrane. The results suggest that a region in the C-terminal portion of the polypeptide forms part of the cytoplasmic domain of the protein in addition to a large N-terminal segment. The membrane-bound regions of the protein are located in the C-terminal two-thirds of the molecule. In this region the polypeptide chain traverses the membrane at least four times and an additional loop of polypeptide is either embedded in the membrane or also penetrates through it to the other surface. The location of the lectin receptors on the protein and the site of binding of an anion-transport inhibitor have also been studied.

Studies with inhibitors of erythrocyte anion transport have shown that the transport process is mediated by the major integral protein of the membrane (Cabantchik & Rothstein, 1974a; Lepke *et al.*, 1976; Ship *et al.*, 1977; Cabantchik *et al.*, 1978). This protein, described as band 3 (Steck, 1974) or polypeptide 3 (Jenkins & Tanner, 1977a), has an apparent subunit mol.wt. of 90000-100000, and spans the erythrocyte membrane (Bretscher, 1971; Boxer *et al.*, 1974). Studies on the protein have shown that it is glycosylated (Ho & Guidotti, 1975; Yu & Steck, 1975a; Tanner *et al.*, 1976) and contains receptors for concanavalin A and the lectins from *Phaseolus vulgaris* and *Ricinus communis* (Findlay, 1974; Tanner & Anstee, 1976; Jenkins & Tanner, 1977b), which are located in the C-terminal region.

The folding and disposition of the polypeptide chain of the protein into the membrane has been studied by chemical and enzymic cleavage. We have used the sites on the protein that are ^{125}I -labelled by lactoperoxidase in appropriate membrane preparations as markers for the cytoplasmic and extra-cellular location of regions of the protein (Boxer *et al.*, 1974). The content of these sites in the major fragments obtained by trypsin cleavage of the protein at an intracellular region led us to suggest

that the polypeptide chain traversed the membrane at least twice (Jenkins & Tanner, 1975, 1977a). Steck *et al.* (1976, 1978) have also studied the proteolytic fragmentation of this protein and have shown that a large soluble fragment that binds the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (Yu & Steck, 1975b) can be obtained from a cytoplasmic domain of the protein. In fragmentation studies on the isolated protein, Drickamer (1976, 1977) used additional markers for the extra- and intra-cellular segments of the anion-transport protein and concluded that the polypeptide chain may cross the membrane four times.

We have extended our studies on the topography of the anion-transport protein with further investigation of the major and minor products of proteolytic fragmentation at a known surface of the membrane. The order of these fragments in the polypeptide sequence, their origin and the topographical location of the lactoperoxidase-labelled sites contained in them suggest that the polypeptide chain contains at least four membrane-spanning segments and an additional loop that either also spans the membrane or is simply embedded in it. The general features of this model for the structure of the protein are similar to those suggested by Steck *et al.* (1978) and Grinstein *et al.* (1978) and differ from the model that we suggested previously (Jenkins & Tanner, 1975, 1977a) in not requiring the presence of a duplicated

Abbreviation used: DIDS, 4,4'-di-isothiocyanatostilbene-2,2'-disulphonic acid.

set of sites that are labelled by lactoperoxidase and in the location of the *N*-terminus of the protein.

Methods

Thermolysin or trypsin digestion of intact erythrocytes, labelling of erythrocytes or erythrocyte 'ghosts' by using Na^{125}I and lactoperoxidase, the isolation of labelled peptides and the preparation of thermolysin peptide 'maps' were done as described previously (Boxer *et al.*, 1974; Jenkins & Tanner, 1975). After proteinase digestion of the erythrocytes, the cells were washed three times with 0.2% bovine serum albumin in 0.15M-NaCl, three times with 0.15M-NaCl and once with iso-osmotic sodium phosphate buffer, pH 7.4 or 8.0, as indicated, before lysis. Lysis at pH 7.4 was done into cold hypo-osmotic sodium phosphate buffer, pH 7.4 (iso-osmotic sodium phosphate buffer, pH 7.4, diluted with 19 vol. of deionized water) as described by Dodge *et al.* (1963). 'Ghosts' were depleted of bands 1, 2, 5 and 6, and digested with trypsin at low ionic strength as described by Steck *et al.* (1976). Trypsin treatment was terminated by adding di-isopropyl phosphorofluoridate (2 μl /mg of trypsin) and/or soya-bean trypsin inhibitor (1mg/mg of trypsin). The preparation and labelling of erythrocytes with DIDS was carried out as described by Cabantchik & Rothstein (1974a) by using 9 μM -DIDS.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis in the presence of 0.1% sodium dodecyl sulphate was done in slab gels with the buffers described by Fairbanks *et al.* (1971) or Laemmli (1970) as indicated. The separating gels contained a linear gradient of acrylamide concentration and were prepared by using a gradient maker. The ratio of acrylamide monomer to *NN'*-methylenebis(acrylamide) was maintained at 1:30 throughout the gel. Protein stained gels containing a high concentration of acrylamide tended to crack on drying. This cracking was decreased by soaking the gel for about 3 h in a solution containing 40% (v/v) methanol and 5% (v/v) glycerol before drying between two sheets of water-permeable Cellophane (a gift from British Cellophane) on a porous polyethylene plate under vacuum. After this treatment the dried gels tended to be hygroscopic and were best stored in a desiccator.

For the detection and elution of labelled fragments the unstained wet gels were covered with wrapping film and radioautographed when exposures of less than 8 h were used. The gel overlay was first removed to avoid artefactual blackening of the film because of the reducing agent in the samples. For longer

exposures the unstained gel was dried before radioautography.

The fluorescence of DIDS-labelled bands in the gels was visualized under u.v. illumination after soaking the unstained gel in 40% (v/v) methanol for 2 h. The fluorescent bands were marked by puncturing the gel with a needle and gels were subsequently stained for protein.

The marker protein mixture used for calibrating apparent molecular weights of the fragments was described by Jenkins & Tanner (1977b), except that myoglobin (mol.wt. 17000) replaced cytochrome *c*. A commercial molecular-weight-marker peptide mixture (BDH) covering the mol.wt. range 17000–2500 was also used for low-molecular-weight fragments.

Carboxypeptidase A digestion of erythrocytes

Packed washed ^{125}I -labelled erythrocytes or thermolysin-treated erythrocytes (0.2 ml in each case) were washed with 0.15M-NaCl containing 0.1M- NH_4HCO_3 . A 25% (v/v) suspension of the packed cells in the same solution was treated with 0.15 mg of carboxypeptidase A (Worthington) at 37°C for 10 min. The cells were washed three times with 0.15M-NaCl containing 0.2% bovine serum albumin and three times with iso-osmotic sodium phosphate buffer, pH 7.4, before lysis.

Results and Discussion

Previous studies have shown that when the human erythrocyte anion-transport protein was ^{125}I -labelled in leaky erythrocyte membranes by using lactoperoxidase, isolated and digested with thermolysin, 14 major radioactive peptides were obtained that could be distinguished by their mobility on peptide 'maps'. Five of these peptides (numbered 1 to 5; see Figs. 2a and 4a) were shown to be derived from sites located in the extracellular region of the protein, whereas the remaining nine peptides (numbered 6 to 14; see Figs. 2a and 5a) were from sites in the cytoplasmic domain of the anion-transport protein (Boxer *et al.*, 1974; Jenkins & Tanner, 1975, 1977a).

In the present paper we have studied the fragmentation of the protein by the action of proteinases at a known side of the membrane. By using membranes that have been ^{125}I -labelled by lactoperoxidase, and determining which of the radioactive peptides are present in thermolysin digests of the labelled fragments, it is possible to deduce both the topographical location of the fragments in the membrane and their location in the amino acid sequence of the polypeptide chain. The presence of the labelled peptides characteristic of the anion-transport protein in the peptide 'maps' of the thermolysin digest of the fragments also provides unambiguous evidence that they are derived from this protein, since thermo-

lysin digestion of the remaining major intrinsic membrane protein, the major sialoglycoprotein (which is also strongly ^{125}I -labelled by lactoperoxidase), gives rise to radioactive peptides that differ in mobility on peptide 'maps' from those of the anion-transport protein. However, to simplify the identification and purification of fragments from the anion-transport protein the ^{125}I -labelled membranes were 'depleted' or 'stripped' of extrinsic membrane proteins or soluble fragments, as appropriate, by using the techniques described by Steck *et al.* (1976). ^{125}I -labelled erythrocytes were also trypsin-digested to remove the radioactivity associated with the major erythrocyte sialoglycoprotein.

Soluble tryptic fragments of the anion-transport protein

Although the extracellular region of the anion-transport protein is resistant to the action of trypsin under iso-osmotic ionic conditions, the cytoplasmic regions of the protein are susceptible to this proteinase under these conditions. Digestion of leaky erythrocyte 'ghosts' with trypsin at iso-osmotic ionic strengths yields a major membrane-bound fragment (T1; Jenkins & Tanner, 1975, 1977a). Steck *et al.* (1976) have also shown that fragments of the protein

are also solubilized from the protein during trypsin digestion of leaky erythrocyte membranes at low ionic strengths. We have studied the composition and origin of the soluble tryptic fragments released from the anion-transport protein on digestion of membranes at iso-osmotic and at low ionic strengths. Erythrocyte 'ghosts' were oxidized with the Cu^{2+} /phenanthroline reagent to form a disulphide-linked dimer of the anion-transport protein and depleted of peripheral proteins as described by Steck *et al.* (1976). The oxidized membranes were digested with trypsin at low ionic strength as described by Steck *et al.* (1976), and the fragments released from the membrane were separated by gel electrophoresis in

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of fragments solubilized from anion-transport protein by trypsin

Erythrocyte 'ghosts' were depleted of peripheral proteins and the anion-transport protein cross-linked by incubation at 0°C for 30 min with $100\text{ }\mu\text{M}$ - CuSO_4 , and $20\text{ }\mu\text{M}$ -*o*-phenanthroline in 5 mM-sodium phosphate, pH 8.0, as described by Steck *et al.* (1976). The 'ghosts' were digested with trypsin as indicated and digestion was terminated by adding $1\text{ }\mu\text{l}$ of diisopropyl phosphorofluoridate and further incubation at 0°C for 10 min. After centrifugation at 40000g for

20 min, the supernatant was mixed with 0.25 vol. of 5% sodium dodecyl sulphate, 50 mM-Tris/HCl buffer, pH 8.0, 5 mM-EDTA, pH 8.0, 20% glycerol and 2 mM-phenylmethanesulphonyl fluoride and heated at 100°C for 2 min. The reduced samples were made 40 mM with respect to dithiothreitol and heated at 100°C for 2 min before electrophoresis. Gels (a)–(e) show the results of trypsin digestion by incubation for 1 h at 0°C with $0.5\text{ }\mu\text{g}$ of trypsin/ml in 5 mM-sodium phosphate buffer, pH 7.0. (a) and (b) show dried Coomassie Blue-stained gels of unreduced (a) and reduced (b) trypsin-solubilized material. (c) shows a radioautograph of a dried gel of a reduced sample from trypsin-solubilized material derived from erythrocyte 'ghosts' ^{125}I -labelled by using lactoperoxidase. Electrophoresis was on the same slab gel as that shown in (a) and (b). (d) and (e) show radioautographs of dried gels of unreduced (d) and reduced (e) samples of trypsin-solubilized material from erythrocyte 'ghosts' ^{125}I -labelled by using lactoperoxidase. The samples were derived from a different experiment from that shown in (c). (f) and (g) show the results of trypsin digestion by incubation at 37°C for 15 min with 0.5 mg of trypsin/ml. Digestion at iso-osmotic ionic strength was carried out in 5 mM-sodium phosphate, pH 8.0, containing 0.15 M-NaCl, whereas digestion at low ionic strength was carried out in 5 mM-sodium phosphate, pH 8.0, alone. Both samples were derived from the same preparation of erythrocyte 'ghosts' that had been ^{125}I -labelled by using lactoperoxidase. (f) shows a radioautograph of material solubilized by trypsin at iso-osmotic ionic strength. (g) shows a radioautograph of material solubilized by trypsin at hypo-osmotic ionic strength. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was done by using slab gels containing a gradient of acrylamide concentration with the discontinuous buffer system of Laemmli (1970). The stacking gels all contained 5% acrylamide and the separating gels contained acrylamide (w/v) concentration gradients of 8–25% amide (sample a–c), 10–25% acrylamide (samples d and e), and 5–15% acrylamide (samples f and g). The position of the various fragments is indicated on the gels.

the presence or absence of reducing agent. Fig. 1(b) shows that three major fragments of the anion-transport protein were obtained (T2s, T3s and T4s, with apparent mol.wts. of 40000, 23000 and 20000 respectively) on electrophoresis in the presence of reducing agent. The additional band X of apparent mol.wt. 43000 was unlikely to be derived from the anion-transport protein since it was also obtained when no trypsin was added to the 'ghosts'. When no reducing agent was present in the electrophoresis samples (Fig. 1a) fragments T2s and T4s were obtained in much lower yield and new bands of apparent mol.wt. 80000 and 38000 were obtained, which represent disulphide-linked dimers of fragments T2s and T4s respectively. Further new bands of apparent mol.wt. 58000 and 17000 were also obtained, which are probably disulphide-linked complexes of fragments T2s and T4s, and of low-molecular-weight fragments respectively. These results are in agreement with those of Steck *et al.* (1976).

The same experiment was carried out with intact erythrocytes that had been ^{125}I -labelled with lactoperoxidase and were then trypsin-digested to remove the radioactivity associated with the major sialoglycoprotein. Trypsin treatment of membranes derived from these cells did not release any radioactivity in the soluble peptides. When the experiment was performed with leaky erythrocyte 'ghosts' that had been ^{125}I -labelled with lactoperoxidase, electrophoresis in the presence of reducing agent showed that fragments T2s and T3s contained radioactive label (Figs. 1c and 1e), whereas no label was found in fragment T4s. Lower-molecular-weight radioactive fragments were also present. When electrophoresis was done in the absence of reducing agent fragment T2s was obtained in much decreased yield and its disulphide-linked dimer (fragment T2s₂) appeared (Figs. 1d and 1e). The same fragments were obtained if digestion was done under the same conditions, but at iso-osmotic ionic strength rather than at hypo-osmotic ionic strength.

When the temperature of digestion and the trypsin concentration were increased more extensive digestion occurred. Figs. 1(f) and 1(g) show the results obtained by using ^{125}I -labelled erythrocyte 'ghosts' and digestion with 0.5mg of trypsin/ml of 'ghosts' at 37°C for 15min at hypo-osmotic and iso-osmotic ionic strength. No fragment T2s was obtained and the yield of fragment T3s was low. Digestion at iso-osmotic ionic strength produced further major radioactive fragments T5s, Y, and T6s, as well as other minor fragments (Fig. 1f). Fragments Y and T6s migrated with the Bromophenol Blue dye front on discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on gradients of acrylamide up to 25% (w/v), and both these and fragment T5s migrated in front of the lowest-molecular-weight marker peptide (mol.wt.

2512), which was present in the marker-peptide mixture used for molecular-weight calibration. Fragment T5s yielded an extrapolated apparent mol.wt. of 1000. Although no estimates of the molecular weights of fragments Y and T6s could be made, they were clearly very small fragments. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on gradients of acrylamide concentration (8–15% acrylamide) with the buffers described by Fairbanks *et al.* (1971) gave the markedly different value for the apparent mol.wt. of fragment T5s of 15000, but fragments Y and T6s migrated as a single band with the Bromophenol Blue dye front in this system. When the gels were stained with the Coomassie Blue stain no radioactivity corresponding to fragments T6s and Y was detected and the amount of radioactivity corresponding to fragment T5s was markedly decreased. These fragments do not remain fixed in the gel after the protein-staining procedure.

The radioactive fragments from the anion-transport protein were isolated from leaky erythrocyte membranes that had been ^{125}I -labelled with lactoperoxidase and digested with trypsin by using the conditions of Figs. 1(c) and 1(f). The isolated fragments were digested with thermolysin and the peptides separated on peptide 'maps' (Fig. 2). Fragments T2s, T3s and T5s all gave the same peptide 'map'. These contained only the labelled peptides 6, 7, 11, 12 and 13, all of which are derived from sites located in the cytoplasmic region of the protein, and some minor radioactive peptides that are also derived from the anion-transport protein (Figs. 2b and 2c). Fragment T6s (Fig. 2d) gave a peptide 'map' that contained only the labelled peptides, 8, 9 and 10, which are also derived from sites that are located in an intracellular domain of the protein. The peptide 'map' of fragment Y did not contain any of the labelled peptides characteristic of the anion-transport protein and is probably not derived from this component.

It is evident that fragment T2s corresponds to the soluble 41000mol.wt. tryptic fragment reported by Steck *et al.* (1976, 1978), whereas fragments T3s and T4s correspond to the mol.wt. 22000 and 16000 fragments also described by these workers. The cause of the discrepancy in apparent molecular weight that we obtained for fragment T4s (20000), and the value of 16000 obtained by Steck and his co-workers is not clear, but it may be related to the different acrylamide-gel system that we have used. Steck *et al.* (1978) suggested that fragments T3s and T4s are subfragments of fragment T2s. Our results confirm that this is indeed the case since fragments T2s and T3s share the same set of ^{125}I -labelled sites and fragments T2s and T4s both contain the cysteine residue involved in the oxidative dimerization of the anion-transport protein. The very small fragment T5s is the product of extensive digestion of fragment

T3s and contains all the ^{125}I -labelled sites present in fragment T3s. It is clear that these sites (peptides 6, 7, 11, 12 and 13) must be clustered in a small region of fragment T3s.

Steck *et al.* (1978) concluded that the fragment corresponding to T2s contains the blocked *N*-terminus of the protein and represents a major cytoplasmic domain of the anion-transport protein. They also showed that the fragment corresponding to T3s contains the *N*-terminus of the intact protein and of fragment T2s, whereas the fragment corresponding to T4s is derived from the *C*-terminal region of fragment T2s (see Fig. 8a). This alignment places the major cytoplasmically located labelled sites 6, 7, 11, 12, 13 within 23000 daltons from the *N*-terminus of the protein and more than 16000–20000 daltons from the point where the polypeptide enters the membrane. The cytoplasmically located sites 8, 9, 10 are also clustered together in the very small fragment T6s, but these are located in different cytoplasmic regions not present in the large fragment T2s.

Major and minor membrane-bound fragments of the anion-transport protein from thermolysin digestion of intact erythrocytes

Thermolysin digestion of intact erythrocytes yields a major membrane-bound fragment of the anion-transport protein of approx. mol.wt. 60000 (fragment 3f; Jenkins & Tanner, 1975). Fragments of indistinguishable mobility on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis are also produced by a variety of other proteinases including chymotrypsin (fragment Chy A; Drickamer, 1976; Steck *et al.*, 1976), subtilisin and Pronase (Bender *et al.*, 1971; Jenkins & Tanner, 1975). There have been several reports in the literature that treatment of intact erythrocytes by this group of enzymes also produces a fragment from the anion-transport protein of apparent mol.wt. approx. 40000 (Cabanchik & Rothstein, 1974b; Jenkins & Tanner, 1975; Steck *et al.*, 1976). This fragment migrated as a diffuse band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and was obtained in variable and low yields. Higher yields of the fragment

Fig. 2. Thermolysin peptide 'maps' of soluble trypsin fragments of anion-transport protein

Radioautographs of soluble tryptic fragments derived from erythrocyte 'ghosts' ^{125}I -labelled with lactoperoxidase. Electrophoresis was done so that labelled peptide 14 migrated off the 'maps' (compare with Fig. 5a) so as to obtain better resolution of the region around peptides 6, 11, 12 and 13. (a) Intact protein. (b) Fragment T3s is isolated from the sample shown in Fig. 1(c). (c) Fragment T5s isolated from the sample shown in Fig. 1(f). (d) Fragment T6s isolated from the sample shown in Fig. 1(g). Abbreviations: C, chromatography dimension; E, electrophoresis (pH 3.5) dimension.

Scheme 1. Isolation of fragments Th1-Th5

were obtained when enzyme treatment was terminated by an inhibitor and when the treated cells were washed with serum albumin solutions (Cabantchik & Rothstein, 1974b).

The fragmentation products from thermolysin cleavage of the anion-transport protein were studied by thermolysin digestion of lactoperoxidase-labelled erythrocytes followed by serum albumin washing of the cells (preparation A, Scheme 1). Pretreatment of the erythrocytes with trypsin was not used because a small amount of digestion of the anion-transport protein occurred under these conditions yielding traces of fragments that interfered with the sub-

sequent analysis of the thermolysin-digestion products. Treatment of erythrocytes with thermolysin alone (Figs. 3b and 3c) caused partial digestion of the major sialoglycoprotein and fragments derived from the sialoglycoprotein were present in these digests, but these could be distinguished by the characteristic mobility on thermolysin peptide 'maps' of labelled peptides from the sialoglycoprotein. In addition to the major fragment Th 1 (the fragment referred to as 3f or Chy A above), a number of minor bands were obtained in 'ghosts' from thermolysin-treated erythrocytes (fragments Th2-Th5; Figs. 3a-3d). The fragment Th2 region was an ill-

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of membrane-bound fragments obtained after extracellular thermolysin cleavage of the anion-transport protein

Radioautographs of portions of dried slab gels. (a) shows the membrane-bound fragment from thermolysin-treated ^{125}I -labelled erythrocytes (preparation A, Scheme 1). The control sample (b) from the same preparation was treated in the same way, but thermolysin digestion was omitted. Separation was on a gel containing a gradient of 5–15% (w/v) acrylamide with a 4% acrylamide overlay by using the buffers described by Fairbanks *et al.* (1971). (c) and (d) show samples from an experiment by the same procedure as the samples in (a) and (b) separated on a gel containing a higher concentration of acrylamide. Separation of the control (c) and membranes from thermolysin-digested ^{125}I -labelled erythrocytes (d) was on a gel containing a gradient of 8–25% (w/v) acrylamide and a 4% acrylamide overlay, with the buffers described by Fairbanks *et al.* (1971). No fragment Th5 was detected in this experiment and the small amounts of fragment Th4 obtained were detected on more extended exposure of the radioautographs. (e) and (f) show membrane-bound fragments obtained on thermolysin digestion of resealed ^{125}I -labelled erythrocyte 'ghosts'. Erythrocytes were washed with iso-osmotic sodium phosphate buffer, pH 6.0, and were lysed in cold iso-osmotic sodium phosphate buffer, pH 6.0, which had been diluted with 19.5 vol. of deionized water. The 'ghosts' were washed with hypo-osmotic phosphate buffer, pH 7.4, ^{125}I -labelled by using lactoperoxidase, and washed three times with cold hypo-osmotic phosphate buffer, pH 7.4. The 'ghosts' were resealed by adding 20 vol. of 0.15 M-NaCl containing 4 mM-MgSO₄ and incubated at 37°C for 45 min. After centrifugation at 40000g for 20 min, the 'ghosts' were suspended in 2 vol. of 0.15 M-NaCl containing 4 mM-MgSO₄ and layered on a sucrose 'cushion' containing 43% sucrose, 25 mM-NaCl and 25 mM-Tris/HCl, pH 7.5, and centrifuged at 65000g_{av.} for 1 h in a swing-out

rotor. The resealed labelled 'ghosts' were washed with 0.15 M-NaCl and assayed for sealing by measuring the activity of glyceraldehyde 3-phosphate dehydrogenase in the absence and presence of Triton X-100 as described by Boxer *et al.* (1974). Thermolysin digestion of the resealed labelled 'ghosts' and subsequent steps were done as shown in Scheme 1 (preparation A) for labelled erythrocytes. (e) shows membranes from thermolysin-digested resealed ^{125}I -labelled 'ghosts'. (f) is a control from the same preparation as that shown in (e), but with the thermolysin-digestion step omitted. Separation was on a gel containing a gradient of 8–15% (w/v) acrylamide and a 4% acrylamide overlay by using the buffers described by Fairbanks *et al.* (1971). Abbreviations: 3, intact anion-transport protein; S, major sialoglycoprotein.

defined area migrating in front of and up to the fragment Th1 band. Fragment Th3 migrated as a broad band and was more clearly defined and better separated on gels containing a high acrylamide concentration (Fig. 3d). The same labelled bands were found in six separate experiments. The yields of the fragments were somewhat variable and fragment Th5 was only obtained in four of these experiments.

Fragments Th1–Th5 were isolated and redigested with thermolysin and the radioactive peptides separated on peptide 'maps' (preparation A, Scheme 1). The peptide 'map' of fragment Th1 (Fig. 4b) contained peptides 1, 2 and 3 and smaller amounts of peptides 4 and 5 than the labelled intact protein from which it was derived (Fig. 4a). Fragment Th3 contained peptides 4 and 5 only (Fig. 4c), whereas fragments Th4 and Th5 gave similar peptide 'maps', which contained peptides 1, 2 and 3 with traces of peptide 4 (Fig. 4d).

In the above experiment the protein was ^{125}I -labelled only in the extracellular region. To label the protein fragments in both intracellular and extracellular domains, membranes from thermolysin-digested erythrocytes that had been ^{125}I -labelled with lactoperoxidase were again ^{125}I -labelled with lactoperoxidase and the labelled peripheral proteins removed by 'stripping' with 0.1 M-NaOH (preparation B, Scheme 1). No new fragments were obtained compared with digests from erythrocytes that had been labelled at the extracellular surface only. The thermolysin peptide 'map' of fragment Th1 (Fig. 5a) from this experiment was similar to that of the intact protein (Fig. 2a), except that only traces of peptides 8, 9 and 10 were observed. Fragment Th3 contained peptides 4 and 5 and peptides 8, 9 and 10 (Fig. 5b). Fragments Th4 and Th5 contained peptide 14 in addition to peptides 1, 2 and 3 (Fig. 5c). Band Th2 appeared to be impure. Band Th2 contained a fragment rich in peptides 4 and 5 and peptides 8, 9 and 10.

Fig. 4. Peptide 'maps' of thermolysin fragments of anion-transport protein ^{125}I -labelled in the extracellular domain. Radioautographs are shown of peptide 'maps' of thermolysin digests of fragments purified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of samples derived as shown in Scheme 1 (preparation A). (a) Intact anion-transport protein from a separation similar to that shown in Fig. 3(b). (b) Fragment Th1 from a separation similar to that shown in Fig. 3(a). (c) Fragment Th3 from a separation similar to that shown in Fig. 3(d). (d) Fragment Th4 from a separation similar to that shown in Fig. 3(a). Abbreviations: C, chromatography dimension; E, electrophoresis (pH 3.5) dimension.

We reported previously that fragment Th1 contained the same set of lactoperoxidase-labelled sites as the intact anion-transport protein (Jenkins & Tanner, 1975, 1977a). In these previous experiments fragment Th1 was purified by electrophoresis in gels containing 8% acrylamide and these give much inferior resolution compared with the acrylamide-gradient gels used in the present study. In our present experiments, the labelled peptides 8, 9 and 10 were hardly detectable in fragment Th1 (compare Figs. 2a and 5a), whereas peptides 1, 4 and 5 were present in lower amounts than in the whole protein (compare Figs. 4a and 4b). The Th1 preparations in Figs. 5(a) and 4(b) were derived from the labelled intact proteins shown in Figs. 2(a) and 4(a) respectively. This suggested that peptides 4 and 5 and peptides 8, 9 and 10 observed previously in peptide 'maps' of fragment Th1 might be due to contaminating material, perhaps from the Th2 band, which was not resolved during electrophoresis.

The radioactivity in the labelled peptides 1–5 found in the thermolysin peptide 'maps' of the intact protein that had been ^{125}I -labelled in the extracellular region was measured and compared with the radioactivity in these peptides on peptide 'maps'

from fragments Th1, Th2 and Th4 derived from the same labelled-protein preparation (Table 1). Fragments Th1 and Th4 were very similar and gave substantial recovery of the labelled peptides 1, 2 and 3, but low recoveries of peptides 4 and 5. A substantial proportion of the peptide 5 in fragment Th1 is probably derived from contamination by the adjacent band Th2, which is very rich in this peptide. Although fragments Th1 and Th4 gave only about 30% of the amount of labelled peptide 1 obtained from the intact protein, the similar and substantial recovery of this peptide in fragments Th1 and Th4 and fragment ThT (discussed below) suggests that it really is derived from these fragments. Unpublished work (M. J. A. Tanner, D. G. Williams & D. Kyle) suggests that the labelled peptides 1 and 3 are related and may contain the same labelled tyrosine residue(s) of the intact protein. The relative proportions of peptides 1 and 3 obtained in peptide 'maps' is influenced by prior cleavage of the protein near site 3, and site 3 is close to the C-terminus of fragments Th1 and Th4 (see below). These results suggest that peptides 4 and 5 in the peptide 'maps' of fragment Th1 do result from contamination of material in the Th2 band.

Fig. 5. Peptide 'maps' of thermolysin fragments of anion-transport protein ^{125}I -labelled in both the extracellular and intracellular domains

Radioautographs of peptide 'maps' of thermolysin digests of fragments purified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The peptide 'maps' (a-c) were derived as shown in Scheme 1 (preparation B). (a) Fragment Th1, from a separation similar to that shown in Fig. 3(a). (b) Fragment Th3, from a separation similar to that shown in Fig. 3(d). (c) Fragment Th4 from a separation similar to that shown in Fig. 3(c). (d) Fragment Th6 from thermolysin-digested resealed ^{125}I -labelled 'ghosts'. The sample, from a separation similar to that shown in Fig. 3(f), was derived as described in the legend to Fig. 3(f). Fig. 2(a) shows the peptide 'map' of the intact anion-transport protein from the same preparation as that shown in (a). Abbreviations: C, chromatography dimension; E, electrophoresis (pH 3.5) dimension.

In the experiment using preparation B (Scheme 1), fragmentation was done on the intact erythrocytes and labelling was subsequently done on the 'ghosts' containing fragment Th1 prepared from the digested cells. However, the control (Fig. 2a) was prepared by labelling the intact protein in erythrocyte 'ghosts'. It was conceivable that fragment Th1 contained the amino acid sequences corresponding to sites 8, 9 and 10, but that the lack of labelling in sites 8, 9 and 10 of fragment Th1 (Fig. 5a) was due to a decreased susceptibility to labelling of fragment Th1 compared with the intact protein at these sites.

To clarify this point, the intact protein was labelled at both intracellular and extracellular domains before fragmentation. In this experiment leaky erythrocyte 'ghosts' were ^{125}I -labelled with lactoperoxidase and resealed as described by Passow (1969). The resealed labelled 'ghosts' were purified as described by Bodeman & Passow (1972), and subsequently digested with thermolysin. No activity of glyceraldehyde 3-phosphate dehydrogenase could

be detected in the resealed-'ghost' preparation unless Triton X-100 was present in the assay medium, confirming that the membranes were sealed and had the same orientation as the intact erythrocyte. Fig. 3(e) shows a radioautograph of the sodium dodecyl sulphate/polyacrylamide-gel electrophoretogram of the fragments obtained. In addition to fragments Th1-Th5, a low-molecular-weight fragment Th6, of apparent mol.wt. 16000, was resolved on this gel.

The radioactivity in peptides 8, 9, 10 and 11 was measured in the peptide 'maps' of fragment Th1 from this experiment and the intact labelled protein from which it was derived. When normalized to equal amounts of radioactivity in peptide 11, peptides 8, 9 and 10 of fragment Th1 contained 15, 14 and 10% respectively of the radioactivity in the same sites from the intact protein. The peptide 'map' of fragment Th2 obtained from this experiment was again found to be rich in sites 8, 9 and 10, suggesting that the presence of small amounts of these labelled peptides in the Th1 preparation was due to contamination.

tion by fragment Th2. These results further confirm that fragment Th1 does not contain peptides 8, 9 and 10. The peptide 'maps' of fragments Th3, Th4, Th5 contained the same labelled peptides as observed in the fragments from preparation B (Scheme 1) and shown in Figs. 5(a)–5(c). Peptide 'maps' of fragment Th6 (Fig. 5d) contained only peptides 4 and 5. Table 2 summarizes the labelled peptides found in peptide 'maps' of the fragments derived from extracellular thermolysin cleavage of the anion-transport protein.

Table 1. *The recovery of extracellularly labelled peptides in thermolysin fragments relative to the intact protein*

The fragments Th1, Th2 and Th4 were obtained by using the procedure for preparation A (Scheme 1). The intact protein was isolated as described by Jenkins & Tanner (1977a) except that electrophoresis was done on a gel containing a concentration gradient of acrylamide. All the samples were derived from the same batch of ^{125}I -labelled erythrocytes. The radioactivity in each of the labelled peptides in peptide 'maps' of the samples was estimated as described by Jenkins & Tanner (1975). For each peptide 'map' the radioactivity in the peptides was normalized so that the radioactivity in peptide 2 was 1.0. The recovery is the normalized radioactivity of a given labelled peptide in a particular fragment divided by the normalized radioactivity in the same labelled peptide in the peptide 'map' of the intact protein.

Peptide	Fragment	...	Recovery of radioactivity		
			Th1	Th2	Th4
1			0.32	0.28	0.24
2			1.0	1.0	1.0
3			1.17	1.07	1.17
4			0.046	0.50	0.043
5			0.18	3.06	0.014

Carboxypeptidase A treatment of the fragments

Treatment of ^{125}I -labelled undigested erythrocytes with carboxypeptidase A did not cause any change in the recovery of peptides 1–5 in the peptide 'maps' of the anion-transport protein. Carboxypeptidase A treatment of thermolysin-digested labelled erythrocytes (preparation C, Scheme 1) did not change the mobilities on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the labelled fragments. Peptide 'maps' were done on corresponding fragments derived from thermolysin-digested ^{125}I -labelled cells before and after carboxypeptidase A treatment. Table 3 shows the relative radioactivity

Table 3. *Effect of carboxypeptidase A treatment on the extracellular thermolysin fragments of the anion-transport protein*

The radioactivity in labelled peptides 1–5 was measured in peptide 'maps' of the corresponding fragments derived from thermolysin-treated erythrocytes and carboxypeptidase A-treated thermolysin-digested ^{125}I -labelled erythrocytes (Scheme 1, preparation C). The radioactivity of the peptides in each peptide map was normalized to equivalent amounts of peptide 2 for fragments Th1 and Th4, and peptide 4 for fragment Th3. The normalized radioactivity in the labelled peptides from the carboxypeptidase-treated fragment was divided by the normalized radioactivity in the same peptide from the corresponding fragment to yield the recovery of the labelled peptide. — indicates that the peptide was very weak or absent on the peptide 'map'.

Fragment	Peptide number	...	Recovery of peptide				
			1	2	3	4	5
Th1			2.0	1.0	0.12	—	—
Th3			—	—	—	1.0	1.0
Th4			2.3	1.0	0.09	—	—

Table 2. *Apparent molecular weight and content of labelled peptides in peptide 'maps' of the membrane-bound thermolysin fragments of the anion-transport protein*

Peptides 1–5 are derived from extracellular sites and peptides 6–14 are from sites located in the cytoplasmic region of the protein. + indicates the presence of the labelled peptide in peptide 'maps' of the fragment, whereas — indicates its absence. Molecular weights were determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by using the buffers described by Fairbanks *et al.* (1971) on gels containing a gradient of 8–15% (w/v) acrylamide.

Fragment	$10^{-4} \times$ Apparent mol.wt.	Labelled-peptide number ...	Presence or absence of labelled peptide in peptide 'map'													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Th1	60		+	+	+	—	—	+	+	—	—	—	+	+	+	+
Th3	42		—	—	—	+	+	—	—	+	+	+	—	—	—	—
Th4	24		+	+	+	—	—	—	—	—	—	—	—	—	—	+
Th5	22.5		+	+	+	—	—	—	—	—	—	—	—	—	—	+
Th6	16		—	—	—	+	+	—	—	—	—	—	—	—	—	—
ThT	21.5		+	+	+	—	—	—	—	—	—	—	—	—	—	—

Scheme 2. Preparation of fragment ThT

Fig. 6. Trypsin treatment of 'ghosts' from thermolysin-treated ¹²⁵I-labelled erythrocytes and peptide 'map' of fragment ThT

(a) and (b) show radioautographs of dried slab gels containing a gradient of 8–15% (w/v) acrylamide, and a 4% acrylamide overlay. Electrophoresis was done by using the buffers described by Fairbanks *et*

of peptides 1–5 in the peptide 'maps' of fragments Th1, Th3 and Th4 from carboxypeptidase A-treated thermolysin-treated cells. The peptide 3 in fragments Th1 and Th4 was almost totally removed by carboxypeptidase A treatment. There was also an increase in the amount of peptide 1 or a smaller decrease in the amounts of peptide 2 in these fragments. Peptides 4 and 5 of fragment Th3 were resistant to carboxy-

al. (1971). The digest sample (b) was obtained as shown in Scheme 2. The control sample (a) was derived from the same preparation except that trypsin treatment was substituted for thermolysin treatment of the erythrocytes and the trypsin treatment of the 'ghosts' was omitted. Abbreviation: 3, intact anion-transport protein. (c) Radioautograph of peptide 'map' of thermolysin digest of fragment ThT ¹²⁵I-labelled in both the extracellular and intracellular regions. The sample was derived as described in Scheme 2, except that after lysis of the thermolysin-treated labelled erythrocytes, the 'ghosts' were again ¹²⁵I-labelled by using lactoperoxidase. Fragment ThT was isolated from a separation similar to that shown in (b).

peptidase A treatment. These results suggest that fragments Th1 and Th4 share a common C-terminus, which yields peptide 3. This is consistent with our previous finding that the thermolysin cleavage that leads to fragment Th1 occurs around the site that yields peptide 3 (Jenkins & Tanner, 1975) and the presence of tyrosine as the C-terminal residue of fragment Th1 (Jenkins & Tanner, 1977b) and the comparable chymotrypsin fragment Chy-A (Drickamer, 1976).

Trypsin digestion of 'ghosts' derived from thermolysin-treated erythrocytes

Trypsin digestion at iso-osmotic ionic strengths of thermolysin-treated lactoperoxidase-labelled erythrocytes (Scheme 2) yielded a single major labelled membrane-bound fragment of the anion-transport protein (fragment ThT, Fig. 6b). This band was also the only major protein-staining band obtained under these conditions. A band of the same electrophoretic mobility was also obtained as the only major band when 'ghosts' from thermolysin-treated cells were digested with chymotrypsin at iso-osmotic ionic strengths. Peptide 'maps' of fragment ThT isolated after trypsin treatment of 'ghosts' from thermolysin-treated ^{125}I -labelled cells, or after trypsin treatment of ^{125}I -labelled 'ghosts' from thermolysin-treated cells contained the same labelled peptides (Fig. 6c). Thus even when the protein was labelled in both intra- and extra-cellular regions, only the extracellular peptides 1, 2 and 3 were obtained in fragment ThT. Fragment ThT had a slightly lower apparent molecular weight than fragment Th5 (Table 2) and gave a similar peptide 'map', except that labelled peptide 14 was absent from fragment ThT. Since the site yielding peptide 3 is located at the C-terminus of fragment Th1, fragment ThT must be derived from the C-terminal region of fragment Th1. The site giving rise to peptide 14 is therefore located in the N-terminal side of the trypsin cleavage leading to fragment ThT (Fig. 8a).

Fragments similar to fragment ThT, but with an estimated mol.wt. of 17000, have been obtained by chymotrypsin treatment of erythrocytes followed by trypsin treatment of 'ghosts' (Grinstein *et al.*, 1978) and by chymotrypsin treatment of erythrocyte 'ghosts' (Steck *et al.*, 1976, 1978). These fragments may be of a similar size to fragment ThT, and the different value for the apparent molecular weight that we obtain may result from the different acrylamide-gel system that we have used (as is also found with fragment T4s).

Fragments that bind DIDS

DIDS is a specific covalently bound inhibitor of erythrocyte anion transport that labels essentially only the anion-transport protein under conditions

that result in total abolition of erythrocyte anion transport (Cabantchik & Rothstein, 1974a; Passow *et al.*, 1977; Ship *et al.*, 1977). Erythrocytes were incubated with DIDS, washed with serum albumin solutions, and fragmentation was performed subsequently. The fragments that covalently bound DIDS were detected after gel electrophoresis by visualization of the fluorescence of bound DIDS in the unstained gel on illumination with u.v. light. DIDS fluorescence was found associated with fragments Th1 and ThT and the major trypsin-produced fragment, T1 (Jenkins & Tanner, 1977b). Although fragments Th4 and Th5 might be expected to bear DIDS fluorescence, none was detected in these bands, probably because of the low yields of these fragments that are obtained and the relative insensitivity of the detection method. These results confirm those of other workers who used radioactive DIDS or dihydro-DIDS on equivalent fragments (Cabantchik & Rothstein, 1974b; Lepke & Passow, 1976; Grinstein *et al.*, 1978) and suggest that the binding site for DIDS is in the C-terminal region of fragment Th1.

Lectin receptors on fragments of the anion-transport protein

The anion-transport protein contains receptors for concanavalin A and the lectins from *R. communis* and *P. vulgaris* (Findlay, 1974; Tanner & Anstee, 1976). The oligosaccharides binding these lectins are present in the tryptic fragment T1, which contains the C-terminal region of the protein (Jenkins & Tanner, 1977b). Fig. 7 shows the pattern of binding of radioactive concanavalin A to 'ghosts' from thermolysin-treated erythrocytes, and to trypsin-treated 'ghosts' from thermolysin-treated erythrocytes. The gels show some non-specific binding to the most intense protein-staining bands, but specific binding (which could be displaced by methyl α -mannoside) was obtained to a band corresponding to fragment Th3 in the 'ghosts' from thermolysin-treated erythrocytes (Fig. 7a). In addition, a broad area of concanavalin A binding of lower mobility than fragment Th1 was found in this sample. No corresponding protein-staining band was evident, and no binding in this region was found in 'ghosts' from untreated erythrocytes. When trypsin-treated ghosts from thermolysin-treated erythrocytes were examined (Fig. 7b) specific binding of concanavalin A in the region of fragment Th3 was again obtained, with some binding to fragment ThT that appeared to be non specific in nature, since it could not be displaced by methyl α -mannoside. Similar binding experiments with the lectin from *R. communis* gave results (not shown) that were less clear, since several membrane components other than the anion-transport protein bind this lectin. 'Ghosts' from thermolysin-treated erythrocytes showed binding in

Fig. 7. Binding of ^{125}I -labelled concanavalin A to fragments of the anion-transport protein

(a) shows membranes from thermolysin-treated trypsin-digested erythrocytes. Trypsin-treated erythrocytes were digested with thermolysin and washed twice with 0.2% bovine serum albumin in iso-osmotic sodium phosphate buffer, pH 7.4. The cells were washed four times with iso-osmotic sodium phosphate, pH 7.4, and lysed into hypo-osmotic sodium phosphate buffer, pH 7.4. The 'ghosts' were washed in hypo-osmotic sodium phosphate buffer, pH 7.4, until free of haemoglobin and depleted of bands 1, 2, 5 and 6, as described by Steck *et al.* (1976). (b) shows membranes from trypsin-treated 'ghosts' derived from thermolysin-treated trypsin-digested erythrocytes. Membranes prepared as described for (a) were digested with 1 mg of trypsin/ml at iso-osmotic ionic strength. After quenching the reaction with di-isopropyl phosphorofluoridate, the membranes were washed three times in hypo-osmotic sodium phosphate buffer, pH 7.4. Each sample was run in duplicate on a sodium dodecyl sulphate/polyacrylamide-slab gel containing a gradient of 5–15% (w/v) acrylamide with a 4% acrylamide overlay by using the buffers described by Fairbanks *et al.* (1971).

the region corresponding to fragment Th3. Trypsin-treated 'ghosts' from thermolysin-treated cells showed similar binding in this region, but no binding to fragment ThT was obtained. These results suggest that fragment Th3 contains the receptors for concanavalin A and the *R. communis* lectin, and that these receptors are not present in fragment ThT. The latter observation is not unexpected since fragment ThT is derived from fragment Th1, which does not contain the lectin receptors (Jenkins & Tanner, 1977b). Neither in the present study, nor in previous ones, were any lectin receptors detected in the region corresponding to fragment Th1, although analyses of the isolated fragment showed the presence of carbohydrates in fragment Th1 (Jenkins & Tanner, 1977b). The possibility remains that this carbohydrate reflected the presence of tightly bound contaminating glycolipids in the preparations, as suggested by Drickamer (1978).

Alignment of the fragments on the amino acid sequence of the protein

Fig. 8(a) shows the location of the fragments on the linear amino acid sequence of the protein and the partial order of the sites that are ^{125}I -labelled with lactoperoxidase. It is assumed in Fig. 8(a), for the purpose of discussion, that each of the major radio-active peptides found in peptide 'maps' of the intact protein corresponds to a different ^{125}I -labelled tyrosine site in the protein. This is not necessarily the case since, although exhaustive thermolysin digestion was used to prepare the peptides, a single labelled site could yield two or more overlapping peptides. The labelled sites in the protein are assigned the numbers of the corresponding labelled peptides.

Fragments Th1 and Th3 contain the sum of the

The duplicate tracks were separated, fixed, and incubated with ^{125}I -labelled concanavalin A (1 mg containing 10^8 c.p.m. of ^{125}I) as described by Tanner & Anstee (1976). One track was incubated in the absence of inhibitor, whereas the other track was incubated in the presence of 0.3 M-methyl α -mannoside, 0.3 M-methyl α -mannoside being present in the washing solution. The washed gels were stained with the Coomassie Blue, dried and radio-autographed. The radioautographs and dried protein-stained gels (immersed in chloroform) were scanned at 550 nm. The bar in the absorbance scale of each Figure represents an absorbance increment of 1.0 for the radioautograph scans, and an absorbance increment of 0.5 for the protein-stain scans. In both Figures the scans are of: (i) protein-stained gel; (ii) radioautograph of gel incubated with ^{125}I -labelled concanavalin A without inhibitor; (iii) radioautograph of gel incubated with ^{125}I -labelled concanavalin A in the presence of methyl α -mannoside.

Fig. 8. Alignment of the fragments and topography of the anion-transport protein

(a) Alignment of the fragments on the amino acid sequence. The numbers refer to the ^{125}I -labelled sites, which yield the corresponding labelled peptides on peptide 'maps' of thermolysin (Th) and trypsin (T) digests of the protein. The broken lines indicate alternative positions for certain sites and fragments. The thickened portions of the line for the intact protein represent extracellular regions, and the upper-case letters show the positions of the loops referred to in (b). The suffix s is used to indicate water-soluble fragments. (b) Schematic diagram of the folding of the polypeptide chain in the membrane. The broken lines show alternative positions for the labelled sites in the C-terminus of the molecule and alternative possibilities for the topography of the protein in this region. The diagram shows the major (solid arrow) and minor (broken arrow) sites of cleavage by thermolysin (Th) and of trypsin (T), and the regions that bind DIDS and lectins. The detailed evidence for this model is given in the text and is briefly summarized here. The N-terminus of the molecule is contained in the large fragment T2s, which is located in the cytoplasmic domain of the protein. Since the N-termini of fragments Th4 and Th5 result from minor extracellular cleavages by thermolysin (at loop A) the polypeptide traverses the membrane between the C-terminus of fragment T2s and the N-terminus of fragment Th4. The N-terminus of fragment ThT arises from cleavage by trypsin at the cytoplasmic loop B, but the C-terminus of this fragment is produced by the major extracellular thermolysin cleavage at loop C. The fragment Th6 results in a minor thermolysin cleavage in loop E.

^{125}I -labelled sites in the molecule. Fragment Th1 extends from the N-terminus of the molecule and has tyrosine as a C-terminal residue (Drickamer, 1976; Jenkins & Tanner, 1977b) and this tyrosine residue probably gives rise to the labelled peptide 3 on the peptide 'maps'. Fragment Th3 contains the remaining ^{125}I -labelled sites that are present in the intact molecule and not present in fragment Th1. Thus fragment Th3 overlaps fragment T1, the major product of intracellular cleavage of the protein by trypsin. Fragment T1 probably extends from site 14 to the C-terminus of the molecule and like fragment Th3 contains sites 4 and 5 and sites 8, 9 and 10 which are not present in fragment Th1 (Jenkins & Tanner, 1975, 1977a). As would be expected, the oligosaccharide receptors for concanavalin A and the *R. communis* lectin are present in both fragments Th3 and T1, but are not present in fragment Th1 (Jenkins & Tanner, 1977b). The minor fragment Th6 contains only the extracellular sites 4 and 5 and is clearly a subfragment of fragment Th3. The appearance of this fragment and the observation that labelled peptides containing sites 4 and 5 are released from erythrocytes on thermolysin treatment (Jenkins & Tanner, 1975) probably accounts for the low and variable yield of fragment Th3 and the equivalent fragments produced by other proteinases (Steck *et al.*, 1976). Fragment T6s, the very small fragment that is solubilized by extensive trypsin treatment of erythrocyte 'ghosts' and contains only the intracellular sites 8, 9 and 10, is also derived from the sequence in fragment Th3. These sites 8, 9 and 10 are present in the C-terminal portion of the molecule. The relative order of the two groups of sites 4 and 5 and sites 8, 9 and 10 cannot yet be defined. Fragment Th3 corresponds to the labile fragment of apparent mol.wt. about 40000, which is obtained in addition to fragment Th1 on treatment of erythrocytes with Pronase (Cabantchik & Rothstein, 1974b) and chymotrypsin (Steck *et al.*, 1976, 1978). The polypeptide in this fragment from the C-terminus of the molecule is clearly accessible from both sides of the membrane, since it contains both intracellular and extracellular labelled sites.

Fragments Th4, Th5 and ThT are all derived from the same region of the protein, that is the C-terminal region of fragment Th1 and share the C-terminus of fragment Th1. Fragments Th4 and Th5 differ from

If fragment Th6 is the C-terminal peptide then the cleavage occurs on the N-terminal side of sites 4 and 5 and sites 8, 9 and 10 are on the cytoplasmic loop D (shown by the solid line). Alternatively the cleavage may occur on the C-terminal side of sites 4 and 5, in which case sites 8, 9 and 10 are present on a cytoplasmic 'tail' at the C-terminus of the protein (shown as a broken line).

fragment ThT in that they are products of extracellular proteolysis and they both contain site 14, which is located in the cytoplasmic domain of the protein. Fragment ThT does not contain site 14 and the trypsin cleavage leading to the *N*-terminus of fragment ThT occurs in the intracellular domain of the protein. Fragment T1, the product of trypsin cleavage of the intact protein from the cytoplasmic side of the membrane, also lacks site 14 (Jenkins & Tanner, 1975, 1977a). It is likely that the trypsin cleavages leading to the *N*-termini of both fragments ThT and T1 occur at the same point in the cytoplasmic domain on the *C*-terminal side of site 14.

The alignment of Fig. 8(a) suggests that trypsin digestion of leaky membranes should yield a small membrane-bound fragment derived from the region between the *C*-terminus of fragment T2s and the *N*-terminus of fragment T1. Because of uncertainty of the exact molecular weight of fragment Th1 (estimates of the apparent molecular weight of this fragment range from 55 000 to 70 000; see Cabantchik *et al.*, 1978) it is difficult to predict the size of this fragment. It is likely to be a relatively small hydrophobic fragment that would make it difficult to detect and may not be labelled if site 14 is solubilized from the membrane. No fragment containing site 14 was identified in the trypsin-solubilized material from the anion-transport protein. However, the electrophoretic mobility of labelled peptide 14 suggests that the labelled tyrosine residue of site 14 is in a rather basic peptide. It is possible that trypsin cleavage occurs within the sequence in peptide 14, giving rise to a new labelled peptide with a different mobility on peptide 'maps'.

In previous work we obtained a band of apparent mol.wt. 24 000 (fragment T2) on trypsin digestion of the anion-transport protein in leaky erythrocyte 'ghosts' (Jenkins & Tanner, 1977a). (This band should not be confused with the soluble fragment designated T2s in the present study.) The T2 band contained the extracellular sites 1, 2, 3, 4 and 5, as well as the intracellular sites 6, 7, 11, 12 and 13, and was assumed to be a homogeneous polypeptide derived from the *N*-terminus of the protein, which contained the portion of the polypeptide that was not present in the major tryptic fragment T1. In this previous work the fragments were separated by electrophoresis on gels of uniform acrylamide concentration. In the present work we have used gradient-gel separations that give a greatly improved resolution of low-molecular-weight material. A band containing the same labelled site as fragment T2 was obtained in two separate experiments, but in very low yield. One explanation for these results is that the band T2 contained more than one polypeptide. It could contain the soluble fragment T3s (which has mol.wt. 23 000) co-migrating on gel electrophoresis with a minor fragment containing the extracellular labelled

site. Unsealed erythrocyte 'ghosts' tend to form sealed right-side-out vesicles on proteolysis (Avruch *et al.*, 1973; Steck *et al.*, 1976) and traces of the fragment T3s may have become entrapped within the membrane. It has been clearly established that a region of approximate mol.wt. 40 000 containing the *N*-terminus of the protein is totally in the aqueous domain at the cytoplasmic side of the membrane (Steck *et al.*, 1976, 1978; Drickamer, 1978; Fukuda *et al.*, 1978); thus if band T2 contained a single membrane-bound polypeptide this peptide could not extend to the *N*-terminus of the protein. However, the observation that the extracellular sites 2 and 3 present in band T2 have different characteristics of iodination with lactoperoxidase from the sites 2 and 3 present in fragment T1 remains unexplained (Jenkins & Tanner, 1977a).

The order of the labelled sites on the protein sequence (Fig. 8a), together with their known location in the extracellular or intracellular portion of the protein, show that from the *N*-terminus, which is located on the cytoplasmic side of the membrane, the polypeptide penetrates the membrane to the extracellular surface and returns to the cytoplasmic side of the membrane at some point in the *C*-terminal region of the protein. It is not known whether the *C*-terminus of the molecule is located at the intracellular or extracellular side of the membrane. This structure is similar to the models proposed by Steck *et al.* (1978) and Grinstein *et al.* (1978), except that we have been able to show that a section in the *C*-terminal third of the polypeptide is also part of the cytoplasmic domain of the protein.

A more detailed model for the topography of the protein can be derived by including the locations of the sites of proteolytic cleavage that give rise to the termini of the fragments (Fig. 8b). A domain extending approx. 40 000 daltons from the *N*-terminus of the protein is present at the cytoplasmic surface of the membrane (Steck *et al.*, 1978; Fukuda *et al.*, 1978) and gives rise to fragment T2s. Since the *N*-termini of fragments Th4 and Th5 result from extracellular cleavage, the polypeptide chain must penetrate the membrane from the intracellular to the extracellular surface at the *C*-terminal side of fragment T2s. The polypeptide chain then forms loop A (Fig. 8b) and returns to the cytoplasmic side of the membrane since the *N*-terminus of fragment ThT results from cleavage at this side of the membrane and fragment ThT lacks the intracellular site 14, whereas fragments Th4 and Th5 both contain this site. Since fragments Th1, Th4, Th5 and ThT share a common *C*-terminus derived from extracellular cleavage around site 3, the chain again traverses the membrane to the extracellular surface forming loop B. Fragment Th3 (and fragment T1; see Fig. 8a) contain both the extracellular sites 4 and 5 and the intracellular sites 8, 9 and 10. Thus the region of the

protein containing fragment Th3 must also traverse the membrane from the extracellular to the cytoplasmic side of the membrane on the C-terminal side of site 3 to form loop C. The membrane-bound fragment Th6 contains only the extracellular sites 4 and 5, and is also derived from this region of the molecule by extracellular cleavage. The absence of the intracellular sites 8, 9 and 10 from this fragment requires that the polypeptide chain makes a further extracellular loop (E) out of the membrane near its C-terminal end. There are two possible structures for this region of the molecule depending on the position of the groups of sites 4 and 5 and sites 8, 9 and 10. In one (shown by the solid line in Fig. 8*b*) sites 8, 9 and 10 are on the intracellular loop D, whereas sites 4 and 5 are on the C-terminal extracellular loop E. In this case the C-terminus of the protein may be imbedded in the membrane and does not necessarily penetrate through the membrane to the cytoplasmic site. In the alternative model (depicted by a broken line in Fig. 8*b*) sites 8, 9 and 10 are present on an intracellular tail at the C-terminus of the molecule. Here the polypeptide need not necessarily penetrate the membrane to form loop D, but this segment of the protein could simply be imbedded in the membrane. In this case the extracellular sites 4 and 5 could either be on loop C (adjacent to site 3) or loop E, depending on the position of the cleavage leading to fragment Th6. The presence of binding sites for concanavalin A and the lectin from *R. communis* in fragment Th3 confirms the location of these receptors in the C-terminal region of the protein, in an extracellular loop (C or E) C-terminal to the point of cleavage leading to fragment Th1. Drickamer (1977) suggested that the polypeptide may cross the membrane four times. His results with a different group of labels and chemical fragmentation of the isolated protein are consistent with the presence of loops A, B and C in the protein (Fig. 8*b*). Drickamer (1977) calculated the distribution of hydrophobic amino acids in different regions of the anion-transport protein and the regions of high-hydrophobic amino acid content correspond in position to the membrane-traversing regions that we have found.

The detailed model in Fig. 8*b* is based on assumptions about the sites of proteolytic cleavage. Whereas the major sites of proteolytic cleavage (leading to fragments Th1 and T2s) are well characterized (see reviews by Cabantchik *et al.*, 1978; Steck, 1978), the sites yielding the minor fragments are less certain. The conclusion that the N-termini of fragments Th4 and Th5 and the termini of fragment Th6 result from extracellular cleavage is based on the occurrence of these fragments on thermolysin digestion of erythrocytes and resealed 'ghosts' and it is difficult to obtain confirmatory evidence that this is indeed the case. Since these are minor fragments

it is conceivable that a small proportion of the erythrocytes became leaky during the digestion and the proteinase acted in part at the intracellular surface. However, the extensive washing of the cells at low centrifugal forces would be expected to remove the lysed cells, which require much higher centrifugal forces for sedimentation. In addition, unpublished experiments have shown that thermolysin treatment of erythrocyte 'ghosts' yields a fragment with the same gel-electrophoretic mobility and labelled peptide 'map' as fragment ThT and no fragments with a mobility corresponding to fragments Th4 or Th6 were obtained. Similarly if the region contained in the glycosylated fragment Th3 contained only a single traverse of the polypeptide from the extracellular region near site 3 to the cytoplasmic side of the membrane and fragment Th6 were derived from intracellular cleavage, one would expect fragment Th6 to migrate as a diffuse band rather than the well defined band shown in Fig. 3(*e*). In this case, fragment Th6 would be expected to extend from the C-terminal side of site 3 and contain the glycosylated region of the protein. This glycosylation appears to be responsible for the diffuse bands given by the intact protein and fragments T1 and Th3 (Jenkins & Tanner, 1977*b*). The observed result is consistent with the presence of the extracellular loop E in Fig. 8(*b*).

Two points should be made about the model shown in Fig. 8(*b*). First, the model shown is the simplest one that accommodates the experimental results reported in the present paper. Since the techniques we have used can yield only a minimum estimate of the number of traverses of the polypeptide chain across the membrane, further experimental results may require a model with additional folds of the polypeptide chain in certain regions. Secondly, the structure shown is highly diagrammatic to illustrate the general topography of the protein. It is not intended to represent the three-dimensional structure of the protein. It is likely that the membrane-traversing segments of the protein are organized to form an ordered structure perhaps of the type found in the bacterial proton pump, bacteriorhodopsin (Henderson & Unwin, 1975), which contains seven membrane-traversing segments. This type of structure could be responsible for some of the transport properties of the protein by forming a channel through the membrane.

We thank Diana Barker, Sally Birch and Simon Peake for their assistance at certain stages of this work. R. E. J. and D. G. W. were recipients of Science Research Council Studentships. The work was supported by a grant from the Wellcome Trust.

References

- Avruch, J., Price, H. D., Martin, D. B. & Carter, J. R. (1973) *Biochim. Biophys. Acta* **291**, 494–505

- Bender, W. W., Garan, H. & Berg, H. C. (1971) *J. Mol. Biol.* **58**, 783–797
- Bodeman, H. & Passow, H. (1972) *J. Membr. Biol.* **8**, 1–26
- Boxer, D. H., Jenkins, R. E. & Tanner, M. J. A. (1974) *Biochem. J.* **137**, 531–534
- Bretscher, M. S. (1971) *J. Mol. Biol.* **59**, 351–357
- Cabantchik, Z. I. & Rothstein, A. (1974a) *J. Membr. Biol.* **15**, 207–226
- Cabantchik, Z. I. & Rothstein, A. (1974b) *J. Membr. Biol.* **15**, 227–248
- Cabantchik, Z. I., Knauf, P. A. & Rothstein, A. (1978) *Biochim. Biophys. Acta* **515**, 239–302
- Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130
- Drickamer, L. K. (1976) *J. Biol. Chem.* **251**, 5115–5123
- Drickamer, L. K. (1977) *J. Biol. Chem.* **252**, 6909–6917
- Drickamer, L. K. (1978) *J. Biol. Chem.* **253**, 7242–7248
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617
- Findlay, J. B. C. (1974) *J. Biol. Chem.* **249**, 4398–4403
- Fukuda, M., Eshdat, Y., Tarone, G. & Marchesi, V. T. (1978) *J. Biol. Chem.* **253**, 2419–2428
- Grinstein, S., Ship, S. & Rothstein, A. (1978) *Biochim. Biophys. Acta* **507**, 294–304
- Henderson, R. & Unwin, P. N. T. (1975) *Nature (London)* **257**, 28–32
- Ho, M. K. & Guidotti, G. (1975) *J. Biol. Chem.* **250**, 675–683
- Jenkins, R. E. & Tanner, M. J. A. (1975) *Biochem. J.* **147**, 393–399
- Jenkins, R. E. & Tanner, M. J. A. (1977a) *Biochem. J.* **161**, 131–138
- Jenkins, R. E. & Tanner, M. J. A. (1977b) *Biochem. J.* **161**, 139–147
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–682
- Lepke, S. & Passow, H. (1976) *Biochim. Biophys. Acta* **455**, 353–370
- Lepke, S., Fasold, H., Pring, M. & Passow, H. (1976) *J. Membr. Biol.* **29**, 147–177
- Passow, H. (1969) in *Laboratory Techniques in Membrane Biophysics* (Passow, H. & Stampfli, R., eds.), pp. 21–27, Springer-Verlag, Berlin
- Passow, H., Fasold, H., Lepke, S., Pring, M. & Schuhmann, B. (1977) in *Membrane Toxicity* (Miller, M. W. & Shamoo, A. E., eds.), pp. 353–379, Plenum Press, New York
- Ship, S., Shami, Y., Breuer, W. & Rothstein, A. (1977) *J. Membr. Biol.* **33**, 311–323
- Steck, T. L. (1974) *J. Cell. Biol.* **62**, 1–19
- Steck, T. L. (1978) *J. Supramol. Struct.* **8**, 311–324
- Steck, T. L., Ramos, B. & Strapazon, E. (1976) *Biochemistry* **15**, 1154–1161
- Steck, T. L., Koziarz, J. J., Singh, M. K., Reddy, G. & Kohler, H. (1978) *Biochemistry* **17**, 1216–1222
- Tanner, M. J. A. & Anstee, D. J. (1976) *Biochem. J.* **153**, 265–270
- Tanner, M. J. A., Jenkins, R. E., Anstee, D. J. & Clamp, J. R. (1976) *Biochem. J.* **155**, 701–703
- Yu, J. & Steck, T. L. (1975a) *J. Biol. Chem.* **250**, 9170–9175
- Yu, J. & Steck, T. L. (1975b) *J. Biol. Chem.* **250**, 9176–9184