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Characterization and partial sequence of di-iodosulphophenyl isothiocyanate-binding peptide from human erythrocyte anion-transport protein

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We investigated the presumed anion-binding domain of the anion-transport protein from human erythrocyte membranes, using 2,6-di-iodo-4-sulphophenyl isothiocyanate, an inhibitor of anion transport. The ^{125}I-labelled reagent binds covalently to the protein with a half-maximal inhibitory concentration of 86 μM. Treatment of unsealed erythrocyte 'ghosts' with chymotrypsin yielded a membrane-bound fragment (mol.wt. 14500 ± 1000) that contained all the protein-bound radioactivity. The binding of the inhibitor to this peptide gave a pattern very similar to that obtained for the effect of the compound on phosphate transport into erythrocytes. The peptide is therefore presumed to be intimately involved in the mediation of anion exchange. Cleavage of the 14500-mol.wt. transmembrane fragment with CNBr resulted in the production of two peptides with apparent molecular weights of 8800 and 4700. The 4700-mol.wt. peptide is the *N*-terminal portion of the 14500-mol.wt. peptide. The attachment site for 2,6-di-iodo-4-sulphophenyl isothiocyanate is situated near the *C*-terminal of the 8800-mol.wt. peptide. This locates the inhibitor-binding site near the chymotrypsin cleavage point at the extracellular surface of the membrane. A partial sequence (residues 1–38) of the 8800-mol.wt. peptide was obtained.

The anion-transport protein (band 3 protein; Steck, 1974) is the major integral membrane protein of human erythrocytes. It spans the lipid bilayer (Bretscher, 1971; Boxer *et al.*, 1974) and mediates the exchange of chloride, bicarbonate, phosphate and sulphate ions across the membrane (Lepke *et al.*, 1976; Ship *et al.*, 1977; Cabantchik *et al.*, 1978). The protein has an apparent molecular weight of 90000–100000 and is glycosylated (Findlay, 1974; Yu & Steck, 1975; Tanner *et al.*, 1976).

Investigations into the nature of the specific region(s) of the protein involved in anion transport have employed transport inhibitors that combine covalently with the protein. Inhibitors that have been used include the disulphonic stilbenes such as 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid (Cabantchik & Rothstein, 1974; Lepke *et al.*, 1976; Ship *et al.*, 1977; Cabantchik *et al.*, 1978; Jennings & Passow, 1979) and the arylsulphonates such as 4-sulphophenyl isothiocyanate (Ho & Guidotti, 1975). These reagents, when used in conjunction with proteolysis of the erythrocyte membrane (Steck *et al.*, 1976), have led to the location of the inhibitor binding site(s) in a 15000–19000-mol.wt. transmembrane fragment of the protein (Grinstein *et al.*, 1978; Williams *et al.*, 1979; Drickamer, 1980).

We have utilized a previously unexploited iodinated analogue of 4-sulphophenyl isothiocyanate, namely di-iodosulphophenyl isothiocyanate (DIOSPITC), to investigate the nature of this transmembrane region further. DIOSPITC is a more potent inhibitor of anion transport than its non-iodinated analogue and, used at low concentrations, is more specific in its reactivity. In the present paper we report the isolation of the labelled fragment and the location of the attachment site for DIOSPITC.

Abbreviations used: DIOSPITC, 2,6-di-iodo-sulphophenyl isothiocyanate; SDS, sodium dodecyl sulphate; AEAP-glass, aminoethylaminopropyl-glass.

Materials

Blood (A rh$^+$) was obtained from the Regional Blood Transfusion Centre (Leeds, U.K.). Chymotrypsin (di-isopropyl phosphorofluoridate-treated) and trypsin (1-chloro-4-phenyl-3-tosylamidobutan-2-one-treated) were supplied by Worthington

(Croydon, Surrey, U.K.). Thiophosgene was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). $Na^{125}I$ was supplied by The Radiochemical Centre (Amersham, Bucks., U.K.). Trifluoroacetic acid, *N*-methylmorpholine, phenyl isothiocyanate, methanol and *p*-phenylene di-isothiocyanate were sequencer grade from Rathburn (Walkerburn, Peeblesshire, Scotland, U.K.). *NN*-Dimethylformamide (Sequanal grade, Hypovial) was purchased from Pierce and Warriner (Chester, U.K.). Triethylamine from BDH Chemicals (Poole, Dorset, U.K.) was redistilled before use and stored under N_2. Controlled-pore AEAP-glass (pore size 7.5 nm) was purchased from LKB (Cambridge, U.K.). All other chemicals were supplied by BDH Chemicals and were the highest grades commercially available.

Methods

Preparation of DIOSPITC

2,6-Di-iodosulphanilic acid was synthesized by the published method (Helmkamp & Sears, 1970) and recrystallized three times from 0.1 M-$NaHCO_3$. The purity of the compound was confirmed by t.l.c. on Kieselgel $60F_{254}$ plates (Merck) with methanol/diethyl ether (1:4, v/v) as solvent. Only one spot was observed (R_F 0.28). No residual sulphanilic acid (R_F 0.07) was noted. The composition of the compound was confirmed by elemental analysis (I 59.5%). This material (1 mmol) was treated with thiophosgene (6 mmol) for 1 h at room temperature with vigorous stirring according to standard procedures (Johnstone & Crumpton, 1979). Excess of thiophosgene was removed by three extractions with equal volumes of ether, and the aqueous layer was freeze-dried. The product was examined by t.l.c. as above and yielded one component (R_F 0.52). With heavily loaded plates a trace of the starting material, di-iodosulphanilic acid, could be seen. The i.r. spectrum confirmed the presence of the isothiocyanate derivative. Since the degree of contamination was so low and the contaminant unreactive in this system, no further purification was necessary. Before use, it was dissolved in 0.1 M-sodium phosphate buffer, pH 8.0, and its concentration ascertained by using the absorbance maximum at 304 nm ($\varepsilon = 4.198 \times 10^3\ M^{-1} \cdot cm^{-1}$).

[^{125}I]DIOSPITC was prepared also after the method of Johnstone & Crumpton (1979). 2,6-Di-iodosulphanilic acid (250 μmol, 106 mg) prepared as above was dissolved in 1.2 ml of water by the addition of satd. Na_2CO_3. To this was added 2.5 mCi of $Na^{125}I$ and 0.4 ml of ICl mixture (comprising 1.8 mM-KI, 1.5 mM-KIO_3, 1.7 M-HCl, 1.7 M-NaCl). The reaction was allowed to proceed for 15 min at room temperature, after which was added 1 mmol of thiophosgene. The mixture was stirred for a further 1 h and the products were then treated as above.

Preparation of 14 500-mol.wt. fragment

Erythrocyte 'ghost' membranes were prepared essentially by the method of Dodge *et al.* (1963). Whole erythrocytes were washed with 0.1 M-sodium phosphate buffer, pH 8.0, lysed in 5 mM-sodium phosphate buffer, pH 8.0, and washed twice in the same buffer. A volume of 'ghosts' was then diluted 1:1 with 5 mM-sodium phosphate buffer, pH 8.0, containing chymotrypsin (200 μg/ml) and incubated at 23°C for 1 h. Phenylmethanesulphonyl fluoride (Sigma Chemical Co., Poole, Dorset, U.K.) was then added to the mixture to given a final concentration of 1 mM. The membranes were washed with 5 mM-sodium phosphate buffer, pH 8.0, treated with 50 mM-NaOH/5 mM-2-mercaptoethanol and centrifuged at 40000 *g* for 30 min. The resultant stripped pellet was washed once with 5 mM-sodium phosphate buffer, pH 8.0, and solubilized by boiling for 5 min in 50 mM-Tris/HCl buffer, pH 8.0, containing 10% (w/v) SDS, 1 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride and 5% (v/v) 2-mercaptoethanol (Kempf *et al.*, 1981). The resulting solution (13 ml) was applied to a column (2.0 cm × 161 cm) of Sephadex G-75 resin, developed with 10 mM-Tris/HCl buffer, pH 8.0, containing 1% (w/v) SDS and 1 mM-EDTA (Fig. 5). The column eluate was analysed by SDS/polyacrylamide-gel electrophoresis, and the pooled fractions containing the 14 500-mol.wt. peptide were freeze-dried.

CNBr-cleavage peptides from the 14 500-mol.wt. fragment

To remove SDS, the freeze-dried 14 500-mol.wt. peptide, obtained as above, was dissolved in 90% (v/v) formic acid/acetic acid/chloroform/methanol (1:1:2:1, by vol.) and applied (5.0 ml) to a column (2.4 cm × 45 cm) of Sephadex LH-60 resin, equilibrated with the same solvent. The eluate was monitored at 280 nm and the peptide-containing fractions were pooled (15 ml) and rotary-evaporated. The peptide (150–250 nmol) was redissolved in 90% (v/v) formic acid (3 ml), which was diluted with water to a final concentration of 80% (v/v) before the addition of CNBr (2 mg). The solution was flushed with N_2 and incubated with stirring for 24 h at room temperature in the dark. The reaction was terminated by rotary evaporation. The resultant peptide mixture was taken up in 0.3 ml of anhydrous trifluoroacetic acid, 0.8 ml of 90% (v/v) formic acid and 2.8 ml of 95% (v/v) ethanol, added sequentially, and the solution was applied to a column (1.6 cm × 86 cm) of Sephadex LH-60 resin, equilibrated with 90% (v/v) formic acid/95% (v/v) ethanol (3:7, v/v). The eluate was monitored at 280 nm and analysed by SDS/polyacrylamide-gel electrophoresis. Sam-

ples for electrophoresis were dried, in the presence of added SDS, under a stream of N_2. After evacuation for at least 24h, the samples were dissolved in gel sample buffer (see below), with added 1 M-Tris/HCl buffer, pH 8.0. Peptide pools were rotary-evaporated before being sequenced.

Coupling and sequencing of peptides

The peptides were coupled to AEAP-glass, the 4700-mol.wt. fragment via its *C*-terminal homoserine lactone residue (Horn & Laursen, 1973) and the 14500-mol.wt. and 8800-mol.wt. fragments by *p*-phenylene di-isothiocyanate activation (Laursen *et al.*, 1972). For both procedures, the peptides were pretreated with trifluoroacetic acid for 1h at room temperature, and then dried down.

Peptides were sequenced by using a Rank–Hilger APS 240 solid-phase sequencer with standard programmes. The buffer used for the reaction with phenyl isothiocyanate was 10% (v/v) *N*-methylmorpholine in methanol/aq. 50% (v/v) propan-1-ol (3:2, v/v), pH 8.5, with trifluoroacetic acid. Anilinothiazolinone derivatives of amino acids were converted into their phenylthiohydantoin derivatives with 20% (v/v) trifluoroacetic acid at 70°C for 10min.

Phenylthiohydantoin derivatives of amino acids were identified by high-pressure liquid chromatography with a Du Pont 8800 system with gradient elution and 5 μm Zorbax ODS resin by a procedure adapted from that of Zimmerman *et al.* (1977). This elution method did not unambiguously identity the phenylthiohydantoin derivatives of phenylalanine and of lysine, but these could be clearly separated by using isocratic elution (Lottspeich, 1980) or detected by back-hydrolysis of the amino acid derivative in 6 M-HCl/0.1% $SnCl_2$ at 150°C for 4h (Mendez & Lai, 1975).

Miscellaneous

Peptides were hydrolysed in 6 M-HCl (AristaR grade)/3% (w/v) phenol *in vacuo* at 110°C and analysed on a Rank–Hilger Chromaspek J180 amino acid analyser.

SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970), with 5–20% gradient slab gels and with a 5% stacking gel. The gel sample buffer was that described by Fairbanks *et al.* (1971). The 10%-SDS/urea/polyacrylamide gels were prepared and run as described by Swank & Munkres (1971). Gels were stained with PAGE Blue 83 (BDH Chemicals) and scanned with a Gilford 260 spectrophotometer.

^{125}I radioactivity was counted with an Intertechnique γ-radiation counter.

Phosphate transport into whole erythrocytes was performed as in Mawby & Findlay (1978).

Results

Erythrocyte labelling with [^{125}I]DIOSPITC

The effect of DIOSPITC on [^{32}P]phosphate influx into whole erythrocytes is shown in Fig. 1. Its observed half-maximal inhibitory concentration was 86 μM, which is markedly less than that for the non-iodinated analogue 4-sulphophenyl isothiocyanate, previously used as an affinity label (Ho & Guidotti, 1975; Drickamer, 1977). DIOSPITC therefore represents a more potent inhibitor of anion transport, and one that can be more conveniently made radioactive.

The pattern of labelling obtained by using [^{125}I]DIOSPITC on whole erythrocytes is shown in Fig. 2. Fig. 2(*a*) shows the profile of radioactivity obtained when erythrocyte membrane preparations were fractionated on SDS/polyacrylamide gels. All the radioactivity was coincident with band 3 protein, the anion-transport protein, with no detectable label associated with the other major transmembrane proteins, the sialoglycoproteins (e.g. PAS 1 in Fig. 2*a*).

The pattern of labelling obtained with 'ghosts' treated with chymotrypsin and stripped of extrinsic membrane proteins/peptides before electrophoresis is given in Fig. 2(*b*). The major radioactive band, Chb, has an apparent molecular weight on 5–20%-gradient gels of 19000. The other major band, Cha, is very diffuse, presumably because it contains all of the carbohydrate of the native anion-transport protein (Drickamer, 1980).

The amount of radioactivity associated with the Chb band when intact erythrocytes were treated with increasing concentrations of [^{125}I]DIOSPITC is shown in Fig. 3. The concentration of inhibitor

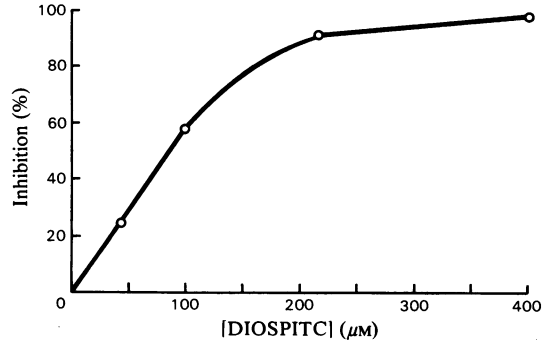


Fig. 1. *Effect of DIOSPITC on phosphate transport*
[^{32}P]Phosphate uptake into whole erythrocytes was measured at the concentrations of DIOSPITC shown and the percentage inhibition was calculated by comparison of the slopes of the semi-logarithmic plots of uptake versus time. For full experimental details see the text.

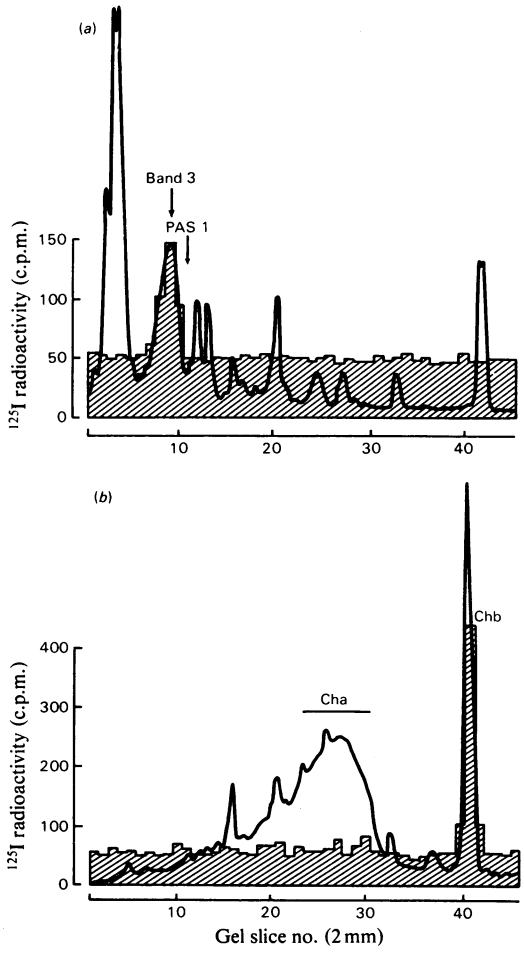


Fig. 2. *SDS/polyacrylamide-gel electrophoresis of DIOSPITC-labelled erythrocyte membranes*
Erythrocytes (50% suspension) were treated with 135 μM-[^{125}I]DIOSPITC (1.5×10^{10} c.p.m./mmol) for 1 h at 30°C. Electrophoresis was performed with 5–20%-gradient slab gels and staining was with PAGE Blue 83. Radioactivity (▨) was determined in 2 mm slices of the corresponding gel tracks. (*a*) Whole 'ghosts' from labelled erythrocytes; (*b*) chymotrypsin- and NaOH-treated membranes from labelled erythrocytes. For full experimental details see the text.

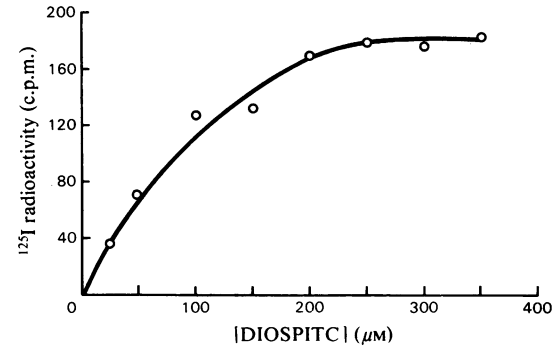


Fig. 3. [^{125}I]*DIOSPITC labelling of the 14500-mol.wt. peptide as a function of reagent concentration*
Erythrocytes (30% suspension) were incubated with the concentrations of reagent shown (1.25×10^{10} c.p.m./mmol). Electrophoresis of the proteinase- and NaOH-treated membranes was performed as indicated for Fig. 2. Radioactivity in the 14500-mol.wt. fragment was determined from 2 mm gel slices and normalized to the content of PAGE Blue 83 stain. For full experimental details see the text.

that gave half-maximal incorporation of label into Chb fragment was 75 μM, in reasonable agreement with the half-maximal inhibitory concentration when phosphate uptake was monitored.

It is probable that the Chb fragment is similar to that reported by others (Steck *et al.*, 1976; Grinstein *et al.*, 1978), for its apparent molecular weight varied, depending on the gel system used. Fig. 4(*b*) shows Chb fragment run on SDS/polyacrylamide gradient gels and Fig. 4(*d*) purified Chb fragment on the 10%-SDS/urea/polyacrylamide-gel system. The apparent molecular weight from the gradient gel was 19000, and that from the urea gel was 14500. On the assumption that SDS/urea/polyacrylamide gels give a more accurate estimate for the molecular weight of smaller peptides, Chb fragment is assumed to have a molecular weight closer to 14500.

Purification of 14500-mol.wt. peptide and its CNBr-cleavage products

The 14500-mol.wt. fragment was generated as detailed in the Methods section. Solubilized membranes were applied to Sephadex G-75 resin and the eluate was monitored at 280 nm (Fig. 5). The peaks were analysed by electrophoresis on SDS/polyacrylamide gradient gels (Fig. 5, inset), and the fractions were pooled as shown to obtain the pure 14500-mol.wt. fragment. The peptide was then rendered free of SDS by chromatography on Sephadex LH-60 resin in organic solvents before treatment with CNBr. The resulting peptide mixture was fractionated on Sephadex LH-60 resin with 90% formic acid/95% ethanol (3:7, v/v) and the eluate was monitored at 280 nm (Fig. 6). The eluate was also analysed for ninhydrin-positive material. The pattern obtained was very similar to the A_{280} profile. Pooled fractions from the column were analysed by electrophoresis on SDS/polyacrylamide gradient gels (Fig. 6, inset). To obtain more accurate estimates of the molecular weights of CNBr-cleavage peptides, samples were also electrophoresed on 10%-SDS/urea/polyacrylamide gels (Fig. 6, inset). The average apparent molecular weights obtained

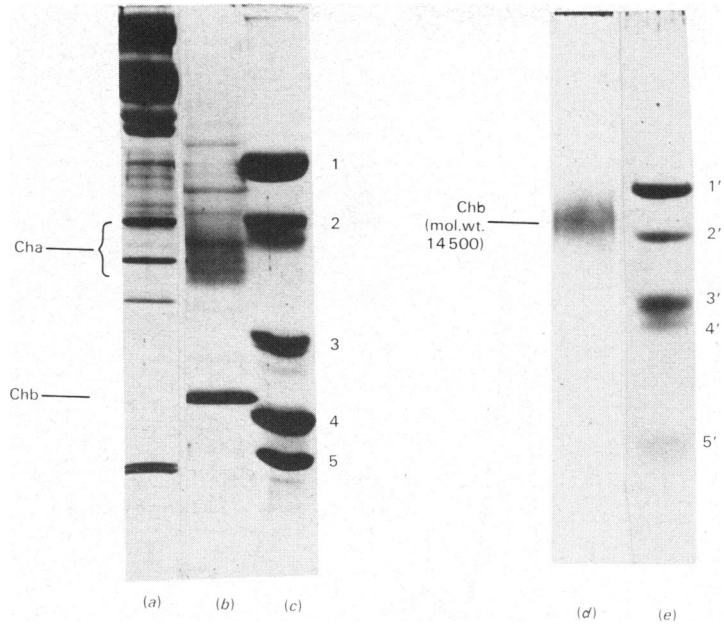


Fig. 4. *SDS/polyacrylamide-gel electrophoresis of chymotrypsin- and NaOH-treated membranes*
(*a*)–(*c*) 5–20%-gradient polyacrylamide gels, with (*a*) whole erythrocyte 'ghosts', (*b*) chymotrypsin-treated and NaOH-stripped erythrocyte 'ghosts' and (*c*) molecular-weight markers [1 (bovine serum albumin, mol.wt. 66000), 2 (egg albumin, mol.wt. 45000), 3 (trypsinogen, mol.wt. 24000), 4 (β-lactoglobulin, mol.wt. 18400) and 5 (lysozyme, mol.wt. 14300); (*d*) and (*e*) 10%-SDS/urea/polyacrylamide gel, with (*d*) purified fragment Chb and (*e*) molecular-weight markers [CNBr-cleavage peptides from myoglobin, 1′ (mol.wt. 17000), 2′ (mol.wt. 14400), 3′ (mol.wt. 8200), 4′ (mol.wt. 6200) and 5′ (mol.wt. 2500)]. For full experimental details see the text.

for peptide CNBr I (pool c) and peptide CNBr II (pool d) were 8810 and 4700 respectively. Small amounts of peptide material of higher (11000) and lower (3500) molecular weights were also seen in the digests, but their yields were variable, and we have evidence that this may represent cleavage at a sensitive bond elsewhere in the 14500-mol.wt. peptide. The 8800-mol.wt. peptide stained very poorly with PAGE Blue 83, also noted by others (Ramjeesingh *et al.*, 1980), which may be due to its higher relative content of hydrophobic amino acids. The peptide was sometimes seen as oligomers on SDS/polyacrylamide-gel electrophoretograms and was prone to aggregate in the organic solvents used in the separate procedures. The amino acid compositions of the 14500-mol.wt., 8800-mol.wt. and 4700-mol.wt. peptides are given in Table 1.

The purified 14500-mol.wt. fragment from cells labelled with [^{125}I]DIOSPITC was treated with CNBr and the peptides were fractionated as above. From the profile obtained (Fig. 7) it can be seen that all the radioactivity was co-eluted with the void-volume material and with the 8800-mol.wt. fragment. The void-volume material was seen on SDS/polyacrylamide-gel electrophoresis to be uncleaved 14500-mol.wt. peptide, together with some aggregated material that did not enter the gel. Cleavage of all the 14500-mol.wt. material was never obtained even at very high CNBr concentrations. When samples from the pooled fraction shown were mixed and subjected to SDS/polyacrylamide-gel electrophoresis (Fig. 7, inset), the pattern of radioactivity in the gel slices confirmed that the label was located in the 8800-mol.wt. fragment rather than in the 4700-mol.wt. fragment. No other labelled material was obtained. Generally, about 30% of the total radioactivity (i.e. peptide) remained unaggregated on gel filtration. In contrast, the 14500-mol.wt. and the 4700-mol.wt. peptides did not aggregate markedly in the organic solvent systems.

Partial amino acid sequences of the 4700-mol.wt. and 8800-mol.wt. peptides

The 4700-mol.wt. peptide was coupled to AEAP-glass, and the sequence was obtained by automated solid-phase procedures as shown in Fig. 8(*a*). The yield obtained from Leu-3 to Leu-5 was 81% (corresponding to a repetitive yield of 93%), whereas that obtained between Asp-4 and the aspartic acid detected at residue 6 was only 33% (repetitive yield of 68%). This large decrease in recovery is due, we believe, to the presence of an Asx–Gly bond. The

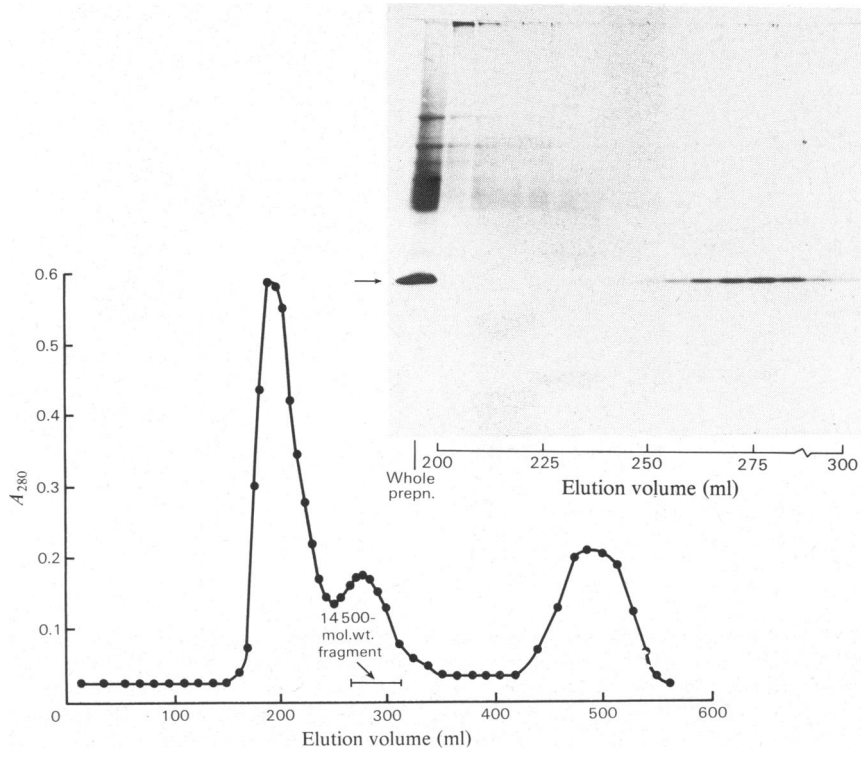


Fig. 5. *Gel filtration on a column (2 cm × 160 cm) of Sephadex G-75 of the chymotrypsin- and NaOH-treated erythrocyte membranes*

Membranes were dissolved with boiling in 10% SDS before being subjected to the gel filtration. Inset: samples from the eluate were subjected to electrophoresis on 5–20%-gradient polyacrylamide gels. The fractions were pooled as shown to obtain pure 14500-mol.wt. fragment. For full experimental details see the text.

juxtaposition of Asx-6 and Gly-7 may result in the formation of a cyclic imide in strong acid, such as is encountered during cleavage with CNBr. The alkaline conditions that occur during the Edman degradation process can cause the ring to open, giving a mixture of α- and β-aspartic acid. Since only the α-form is susceptible to Edman degradation, this leads to a fall in the yield (Konigsberg, 1967; Bornstein & Balian, 1977).

The presence of a homoserine residue in the 4700-mol.wt. peptide identifies this peptide as the *N*-terminal region of the 14500-mol.wt. fragment. This conclusion was confirmed in two ways. The sequence of the corresponding 4700-mol.wt. peptide from the 14500-mol.wt. fragment, produced by chymotrypsin cleavage on the outside of the membrane together with trypsin cleavage on the inside (Fig. 8*b*), is identical with that shown in Fig. 8(*a*) except for the loss of the *N*-terminal lysine residue. Secondly, sequence analysis of the whole 14500-mol.wt. fragment also gave an identical *N*-terminal structure.

The 8800-mol.wt. peptide, which contains the DIOSPITC-binding site, possessed no homoserine and failed to couple to AEAP-glass with use of the relevant coupling regime. It is therefore presumed to be the *C*-terminal portion of the 14500-mol.wt. fragment. As well as containing the binding site for DIOSPITC, it also contains the residue(s) that binds the non-iodinated analogue 4-[^{35}S]sulphophenyl isothiocyanate. The peptide was activated with *p*-phenylene di-isothiocyanate and coupled as described in the Methods section. The sequence obtained for the first 38 residues is shown in Fig. 8(*c*). The sequence analysis was performed on three different preparations, and there was no evidence of any peptide impurity (for yields see Figs. 9–11). The radioactively labelled residue was not recovered up to this point. The structure is very hydrophobic, containing only three charged residues, glutamic acid in each case, at positions 4, 37 and 38, and no basic amino acids. This absence of lysine and arginine residues from the unlabelled peptide sequence suggests that the DIOSPITC-binding site is to be found near the *C*-terminal portion of the peptide. During automated sequencing of radio-

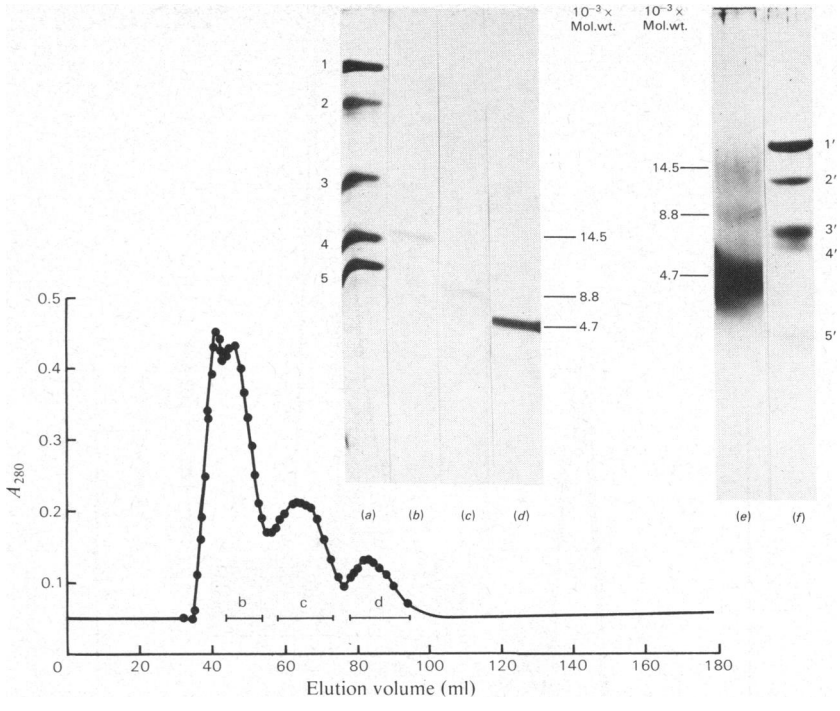


Fig. 6. *Fractionation of the CNBr-cleavage peptides of the 14500-mol.wt. fragment on a column (1.6cm × 86cm) of Sephadex LH-60*

Inset: samples from the eluate were subjected to gel electrophoresis. (*a*)–(*d*) 5–20%-gradient polyacrylamide gels, with (*a*) molecular-weight markers as in Fig. 4, (*b*) pool b (14500-mol.wt. fragment), (*c*) pool c (8800-mol.wt. fragment) and (*d*) pool d (4700-mol.wt. fragment); (*e*)–(*f*) 10%-SDS/urea/polyacrylamide gels, with (*e*) whole CNBr digest (250 μg of protein applied) and (*f*) molecular-weight markers as in Fig. 4. For full experimental details see the text.

active peptide, there was a steady loss at each round of approx. 0.5% of the radioactivity originally coupled. This correlated well with an overall loss of 24% in the radioactivity remaining on the glass at the end of the run. No peak of radioactivity was seen in up to 47 cycles.

Discussion

From the proteolytic procedures used and the molecular weight obtained it is reasonable to suggest that the *N*-terminus of the peptide is situated on the inside of the membrane, the *C*-terminus on the outside (Steck *et al.*, 1976, 1978; Drickamer, 1978), and there are three intervening transmembrane segments. These features are readily consistent with the more general models for the intact anion-transport protein that have been developed (Steck *et al.*, 1978; Williams *et al.*, 1979; Drickamer, 1980). Although there are also similarities with the structure put forward by Ramjeesingh *et al.* (1980) for their 15000-mol.wt. fragment, there are differences in some of the more detailed aspects. They found that cleavage of the fragment with CNBr produced three peptides, the middle one of which contained the attachment site for 4,4′-di-isothiocyanatostilbene-2,2′-disulphonic acid. In our hands, treatment with CNBr produced only two peptides in high yield. We locate the DIOSPITC (and 4-sulphophenyl isothiocyanate)-attachment site on the larger (8800 mol.wt.) of these, near to the *C*-terminal of the whole fragment.

The different interpretations may arise from the conditions used for the treatment with CNBr. We used a 50–100-fold molar excess of CNBr for 24h, whereas Ramjeesingh *et al.* (1980) used a 2500–5000-fold molar excess for up to 64h. Under these latter conditions, tryptophan residues may be susceptible to oxidative cleavage (Dell *et al.*, 1974). It could therefore be that the third fragment produced by Ramjeesingh *et al.* (1980) arose from cleavage at a tryptophan residue or at a particularly resistant methionine-containing bond (i.e. Met–Thr or Met–Ser). This resulting peptide could then represent the *C*-terminal portion of our 8800-mol.wt. peptide, since there are no basic residues in the sequence of the *N*-terminal portion that would act as a receptor for 4,4′-di-isothiocyanatostilbene-2,2′-disulphonic acid. If this is so, then the 4,4′-di-isothiocyanato-

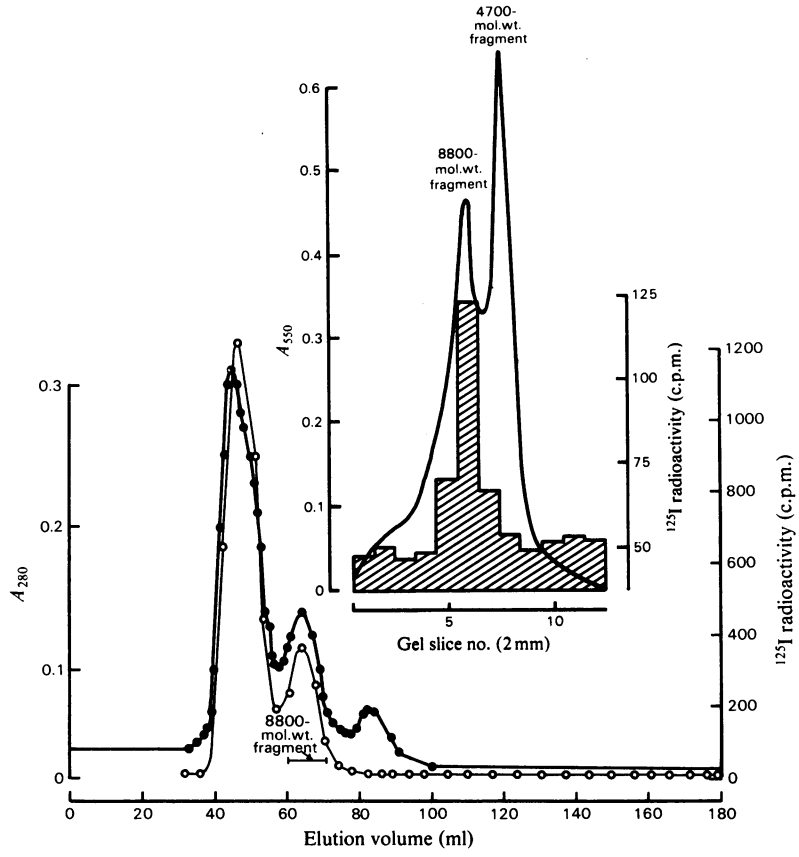


Fig. 7. *Fractionation on Sephadex LH-60 of the CNBr-cleavage fragments from the 14500-mol.wt. fragment labelled with [^{125}I]DIOSPITC*

Erythrocytes (30% suspension) were treated with 150 μM-[^{125}I]DIOSPITC (5.6×10^9 c.p.m./mmol). Samples of the 8800-mol.wt. and 4700-mol.wt. peptides were mixed and subjected to electrophoresis in a 5–20%-gradient polyacrylamide gel. ●, A_{280}; ○, ^{125}I radioactivity. The inset shows the PAGE Blue 83-staining profile of that part of the gel (the rest was clear) containing the two peptides, superimposed on the radioactivity content (▨) of 2 mm slices. For full experimental details see the text.

(*a*) Lys-Gly-Leu-Asp-Leu-^{5}[Asp/Asn]-Gly-Gly-Pro-^{10}Asp-

(*b*) Gly-Leu-Asp-Leu-

(*c*) X -Val-Ser-Glu-Leu-Leu-Ile-Ser-Thr-^{10}Ala-Val-Gln-Gly-Ile-Leu-Phe-Ala-Leu-Leu-
^{20}Gly-Ala-Gln-Pro-Leu-Leu-Val-Val-Gly-Phe-^{30}Ser-Gly-Pro-Leu-Leu-Val-Phe-Glu-Glu-

Fig. 8. *Amino acid sequences of the N-terminal portions of the 4700-mol.wt. and 8800-mol.wt. peptides*

(*a*) Sequence of the 4700-mol.wt. fragment produced by treatment of membranes with chymotrypsin. A 150 nmol sample of peptide was coupled (78% average coupling yield) for automated sequencing. (*b*) Sequence of the 4700-mol.wt. fragment resulting from treatment of the membranes with chymotrypsin following by trypsin (180 nmol of peptide was coupled, with a coupling yield of 83.6%). (*c*) *N*-Terminal sequence of the 8800-mol.wt. peptide (90 nmol coupled, coupling yield 33.2%); 93.6 nmol of ^{125}I-labelled 8800-mol.wt. peptide was coupled, with an efficiency of 43.6%. For full experimental details see the text.

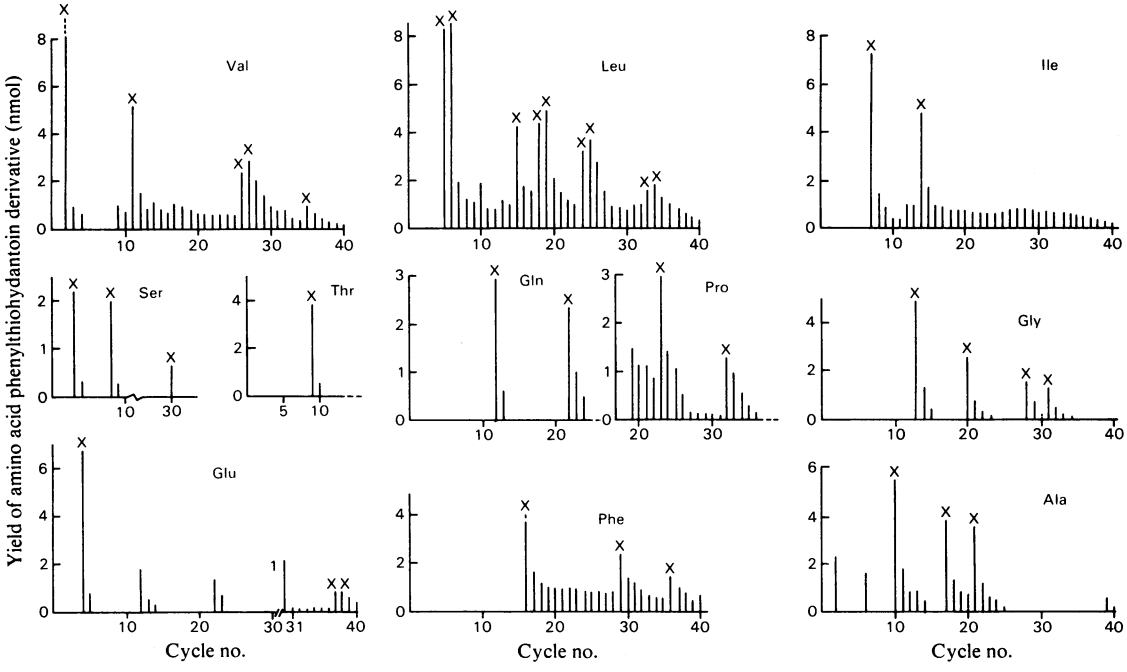
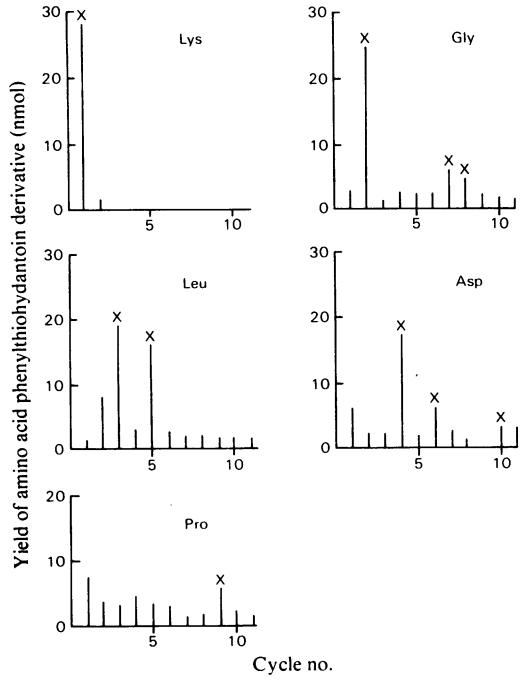


Fig. 9. *Automated sequence analysis of 29 nmol of the 8800-mol.wt. CNBr-cleavage peptide from the 14 500-mol.wt. fragment of the anion-transport protein*

The abscissa gives the cycle number of the Edman degradation, and the ordinate the yield (in nmol) of the amino acid phenylthiohydantoin derivative as denoted by a vertical line. × designates the assignment of the particular amino acid to the corresponding position in the sequence. A 40% portion of each cycle was analysed by high-pressure liquid chromatography. For full experimental details see the text.



stilbene-2,2′-disulphonic acid attachment site would seem to be in the same region of the polypeptide as that for DIOSPITC and 4-sulphophenyl isothiocyanate.

If the transmembrane sections take up a helical structure such as suggested for bacteriorhodopsin (Henderson & Unwin, 1975), several hydrophilic residues are likely to occur in intramembranous regions. This also appears to be the case for bacteriorhodopsin (Engelman *et al.*, 1980), and it is tempting to speculate a role for such residues in the stabilization of protein conformation or in its catalytic action.

The nature and position of the residues controlling anion movement, however, will only be fully

Fig. 10. *Automated sequence analysis on 112 nmol of the 4700-mol.wt. CNBr-cleavage peptide from the 14 500-mol.wt. fragment of the anion-transport protein produced by digestion of membranes with chymotrypsin*

Axes and designations are as indicated in Fig. 9 legend. A 40% portion of each cycle was analysed by high-pressure liquid chromatography. For full experimental details see the text.

Table 1. *Amino acid compositions of the 14500-mol.wt., 8800-mol.wt. and 4700-mol.wt. peptides*

The data are based on at least five separate determinations (hydrolysis time 30h) for the 8800-mol.wt. and 4700-mol.wt. peptides and three separate analyses (each of 24, 48 and 96h) for the 14500-mol.wt. fragment. Tryptophan was not determined. Cysteine was detected as the unmodified amino acid.

Amino acid	Amino acid composition (mol. of residue/mol of peptide) 14500-mol.wt. peptide	8800-mol.wt. peptide	4700-mol.wt. peptide
Asx	7.16	2.73	3.64
Thr	5.9	3.31	2.19
Ser	6.73	4.54	1.97
Glx	12.43	7.65	3.52
Pro	3.68	3.08	2.15
Gly	10.43	5.80	4.22
Ala	6.73	3.57	2.7
Val	6.67	5.00	1.93
Met	0.39	—	0.26*
Ile	7.7	5.61	2.24
Leu	15.23	9.62	5.25
Tyr	3.9	2.27	1.69
Phe	9.86	7.26	2.69
His	3†	1.97	0.93
Lys	3.21	2†	1†
Arg	3.86	2.22	2.19
Cys	+	+	—

* Detected as homoserine and homoserine lactone.
† Normalized to these residues.

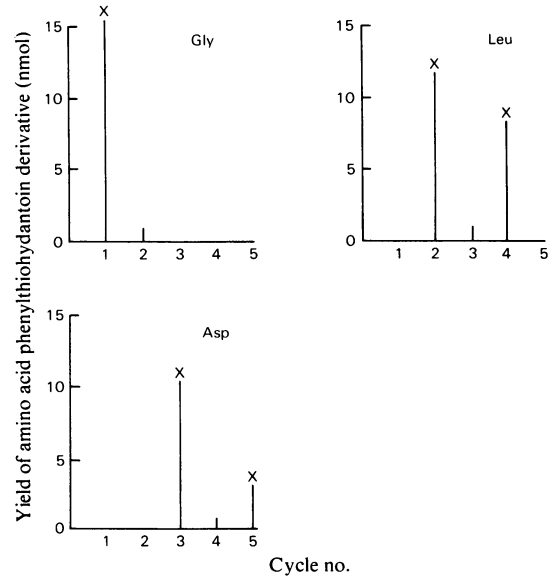


Fig. 11. *Automated sequence analysis of 113nmol of the 4700-mol.wt. CNBr-cleavage peptide from the 14500-mol.wt. fragment of the anion-transport protein produced by tryptic and chymotryptic digestion of membranes*

Axes and designations are as indicated in Fig. 9 legend. A 40% portion of each cycle was analysed by high-pressure liquid chromatography. For full experimental details see the text.

appreciated when the sequences of all the transmembrane regions are elucidated. From kinetic studies on the influence of pH on chloride transport (Funder & Wieth, 1976; Brahm, 1977) and on the effects of arginine-specific reagents (Zaki, 1981), the guanidinium group of an arginine residue has been implicated in the process. Most of the inhibitors are thought to attach to the protein via a lysine residue. From the amino acid compositions, sequence data and putative disposition of the polypeptide chain reported in the present paper, it is possible to deduce that most of the arginine and lysine residues contained in the 14500-mol.wt. fragment are to be found nearer to the extracellular surface of the protein. It is not yet known how they relate to the mechanisms for the binding and exchange of anions.

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