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The human erythrocyte anion-transport protein

Partial amino acid sequence, conformation and a possible molecular mechanism for anion exchange

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The *N*-terminal 72 residues of an integral membrane fragment, P5, of the human erythrocyte anion-transport protein, which is known to be directly involved in the anion-exchange process, was shown to have the following amino acid sequence:

Met-Val-Pro-Lys-Pro-Gln-Gly-Pro-Leu-Pro^{10}-Asn-Thr-Ala-Leu-Leu-Ser-Leu-Val-Leu-
Met^{20}-Ala-Gly-Thr-Phe-Phe-Phe-Ala-Met-Met-Leu^{30}-Arg-Lys-Phe-Lys-Asn-Ser-Ser-Tyr-
Phe-Pro^{40}-Gly-Lys-Leu-Arg-Arg-Val-Ile-Gly-Asp-Phe^{50}-Gly-Val-Pro-Ile-Ser-Ile-Leu-Ile-
Met-Val^{60}-Leu-Val-Asp-Phe-Phe-Ile-Gln-Asp-Thr-Tyr^{70}-Thr-Gln^{72}-

The structure of this fragment was analysed, with account being taken of the constraints that apply to the folding of integral membrane proteins and the topographical locations of various sites in the sequence. It was concluded that this sequence forms two transmembrane α-helices. These are probably part of a cluster of amphipathic transmembrane α-helices, which could comprise that part of the protein responsible for transport activity. The presently available evidence relating to the anion-exchange process was considered with the structural features noted in this study and a possible molecular mechanism is proposed. In this model the rearrangement of a network of intramembranous charged pairs mediates the translocation of an anion between anion-binding regions at each surface of the membrane, which are composed of clusters of positively charged amino acids. This model imposes a sequential exchange mechanism on the system. Supplementary material, including Tables and Figures describing the compositions of peptides determined by amino acid analysis and sequence studies, quantitative and qualitative data that provide a residue-by-residue justification for the sequence assignment and a description of modifications to and use of the solid-phase sequencer has been deposited as Supplementary Publication SUP 50123 (12 pages) with the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained as indicated in *Biochem. J.* (1983) **209**, 5.

The permeability of the erythrocyte to anions is mediated by the major integral glycoprotein of its membrane (band-3 protein; for a review see Knauf, 1979). This protein carries out the strict one-for-one exchange of anions across the membrane; kinetic studies have shown that this proceeds by a sequential ('ping-pong') mechanism (Gunn & Fröhlich, 1978, 1979). Previous structural studies have shown that the protein migrates on sodium dodecyl sulphate/polyacrylamide gels as a diffuse band with an approximate M_r of 95000. The broadness of the band is apparently due to heterogeneity in the carbohydrate content of the molecule and not to any structural variation in the polypeptide chain (Jenkins & Tanner, 1977*b*; Mueller *et al.*, 1979). The protein comprises an intracellular *N*-terminal domain of

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about 40000Da, to which the erythrocyte cytoskeleton, many glycolytic enzymes and haemoglobin are known to bind (reviewed by Gillies, 1982). This segment is not involved in anion exchange, since it can be removed without affecting transport activity (Grinstein *et al.*, 1978). The transport function is associated with the *C*-terminal part of the molecule, to which all the known inhibitors of anion exchange bind, and in which the polypeptide chain has been shown to traverse the membrane at least four times. The oligosaccharide chain is attached to the protein extracellularly close to its *C*-terminus (Williams *et al.*, 1979).

We have been studying the amino acid sequence of that part of the anion-transport protein that is known to be integral to the erythrocyte membrane. In particular we have examined a fragment, P5, that is produced by peptic digestion of erythrocyte 'ghosts' (Tanner *et al.*, 1979). This fragment binds specifically the anion-transport inhibitor phenyl isothiocyanate at pH7.3 and is known to be directly involved in the anion-exchange process (Kempf *et al.*, 1981). It also contains a site susceptible to lactoperoxidase-catalysed radioiodination that is located in an extracellular part of the anion-transport protein (Tanner *et al.*, 1979). We have determined the amino acid sequence of the *N*-terminal 72 residues of fragment P5. The likely conformations of this and related parts of the protein have been analysed in accordance with several criteria, particular account being taken of the special constraints that must apply to the folding of integral membrane proteins. This information is then considered with the other data that exist relating to the anion-exchange process, and a molecular mechanism is proposed that unifies the presently available evidence.

Experimental

Materials

All chemicals were of the AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. unless otherwise specified. Recently outdated human erythrocytes were obtained from South West Regional Blood Transfusion Centre, Southmead, Bristol, U.K. Na^{125}I and phenyl isothio[^{14}C]cyanate were obtained from Amersham International, Amersham, Bucks., U.K. Lactoperoxidase, 2-mercaptoethanol, CNBr and phenylmethanesulphonyl fluoride were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Phenyl isothiocyanate, methanol, benzene, trifluoroacetic acid, acetonitrile and ethyl acetate were Sequencer-grade reagents from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland. Triethylamine, dimethylformamide, *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane, *p*-phenylene di-isothiocyanate and amino acid phenylthiohydantoin derivative standards were Sequencer-grade reagents from Pierce and Warriner, Chester, U.K. Sephadex gel-filtration media were from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel gel-filtration media were from Bio-Rad Laboratories, Watford, Herts., U.K. Spectrapor dialysis tubing was from Spectrum Medical Laboratories, Los Angeles, CA, U.S.A. All solutions were prepared with deionized glass-distilled water.

Labelling of the anion-transport protein

Lactoperoxidase-catalysed radioiodination of intact human erythrocytes was performed as described by Boxer *et al.* (1974).

Phenyl isothio[^{14}C]cyanate labelling of erythrocyte 'ghosts' was performed as described by Kempf *et al.* (1981).

Digestion of erythrocytes with trypsin

Intact erythrocytes were digested with trypsin under low-ionic-strength conditions (300mM-sucrose/5mM-sodium phosphate buffer, pH8.0) as described by Jenkins & Tanner (1977*a*).

Purification of the proteolytic fragment, P5, from the anion-transport protein

Haemoglobin-free erythrocyte 'ghosts' were prepared by the method of Dodge *et al.* (1963). The membrane integrated fragment P5, produced by peptic digestion of the anion-transport protein, was prepared and purified as described by Tanner *et al.* (1979), with the modifications given by Kempf *et al.* (1981) for purification on a scale suitable for amino acid sequencing studies. The freeze-dried peptide obtained contained variable amounts of detergent. The precise peptide content was determined by amino acid analysis.

Cleavage of fragment P5 with CNBr and purification of peptides

Freeze-dried peptide was dissolved in 80% (v/v) formic acid to a final concentration of 2–5mg/ml, and solid CNBr was added to a concentration of approx. 10M. The solution was incubated for 24h at room temperature in the dark and then dried under a stream of N_2.

Peptides were purified from this digest by gel filtration on a column (8mm × 1400mm) of Bio-Gel P10 (200–400 mesh) in 80% formic acid.

Amino acid composition

Amino acid analyses were performed with the aid of a Rank–Hilger Chromaspek amino acid analyser. Peptides were hydrolysed for 24h *in vacuo* at 105°C in 6M-HCl containing 0.1% phenol.

Solid-phase Edman degradation

Peptides for sequence analysis were covalently coupled to *N*-(2-aminoethyl)-3-aminopropyl-glass, which was made by incubating controlled-pore glass (200–400 mesh; 7.5 nm nominal pore diameter) with 4% *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane in anhydrous acetone at 45°C for 48 h (the silane solution changed once after 24 h). The silylated glass was washed thoroughly with acetone and methanol and dried *in vacuo*.

Before being coupled, all peptides were freeze-dried from 5% triethylamine to remove any free ammonia.

CNBr-cleavage peptides bearing a *C*-terminal homoserine lactone residue were coupled to *N*-(2-aminoethyl)-3-aminopropyl-glass as follows. Up to 10 nmol of peptide was incubated in 200 μl trifluoroacetic acid at 45°C for 1 h. This solution was dried down *in vacuo* over KOH and dissolved in 400 μl of dimethylformamide. Then 10 μl of triethylamine was added, and the pH was checked by spotting a sample (<1 μl) on to moist pH paper. If the solution was acidic, further 10 μl portions of triethylamine were added until it became alkaline (pH >8 indicated by the pH paper). Next 200 mg of *N*-(2-aminoethyl)-3-aminopropyl-glass was added, and the mixture was incubated at 45°C for 2 h. The glass, carrying the lactone-coupled peptide, was washed thoroughly with methanol and trifluoroacetic acid and dried *in vacuo*.

Other peptides were coupled via their lysine-residue side chains as follows. Up to 50 nmol of peptide was dissolved in 200 μl of trifluoroacetic acid and immediately dried down *in vacuo* over KOH. The peptide was then dissolved in 400 μl of dimethylformamide, and sufficient triethylamine was added in 10 μl portions to make the solution alkaline (as described above). A 2 mg portion of *p*-phenylene di-isothiocyanate was dissolved in the solution, which was incubated at 45°C for 20 min. Next 200 mg of *N*-(2-aminoethyl)-3-aminopropyl-glass was added, and the 45°C incubation was continued for 1 h. The glass, now carrying *p*-phenylene di-isothiocyanate-coupled peptide, was washed thoroughly with methanol and dried *in vacuo*.

Fragment P5 was also coupled through its carboxy groups by using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide as described by Previero *et al.* (1973) except that *N*-(2-aminoethyl)-3-aminopropyl-glass was used as the solid support.

Coupled peptides were sequenced with the aid of a modified Anachem-APS-2400 solid-phase peptide sequencer (see Supplement SUP 50123).

The amino acid thiazolinones obtained from the sequencer were dried down under a stream of N_2 and converted into their phenylthiohydantoin derivatives by incubation in 200 μl of 0.1 M-HCl at 80°C for 10 min. This solution was extracted twice with 600 μl of ethyl acetate. The ethyl acetate extract contained all the amino acid phenylthiohydantoin derivatives except for those of arginine and histidine, which remained in the aqueous phase.

Identification of amino acid phenylthiohydantoin derivatives

High-pressure liquid chromatography. All amino acid phenylthiohydantoin derivatives were identified quantitatively by reverse-phase high-pressure liquid chromatography with an Altex 110A pump, Anachem column-heating block, Pye Unicam LC3 u.v. detector, Bryans 27000 chart recorder and a Du Pont 25 cm × 4.6 mm column of Zorbax ODS. Elution was performed under isocratic conditions at 55°C with 32% acetonitrile/68% 0.02 M-sodium acetate buffer, pH 4.52, containing 1% acetonitrile, at 2 ml/min (Gates *et al.*, 1979).

Thin-layer chromatography. The ethyl acetate-extractable amino acid phenylthiohydantoin derivatives were identified qualitatively by t.l.c. on 5 cm × 7.5 cm silica-gel 60 F_{254} pre-coated HPTLC aluminium sheets with the following solvents: solvent 1, chloroform/methanol (99:1, v/v); solvent 2, chloroform/methanol (9:1, v/v) (Cohen-Solal & Barnard, 1973).

Back hydrolysis. Many assignments were confirmed by regeneration of the corresponding free amino acid for analysis. Amino acid phenylthiohydantoin derivatives were hydrolysed *in vacuo* in 6 M-HCl containing 0.1% $SnCl_2$ at 150°C for 4 h (Mendez & Lai, 1975).

Results and discussion

Strategy of amino acid sequence analysis

The fragment P5 was produced by peptic digestion of acid-stripped erythrocyte 'ghosts', which contained all the integral proteins of the erythrocyte membrane. The sequence given in the present paper contains two labelled sites that are known to originate from the anion-transport protein. One of these is a lysine residue labelled specifically by the anion-exchange inhibitor phenyl isothiocyanate (Kempf *et al.*, 1981). The other is a radioiodinated tyrosine residue that has previously been mapped in the anion-transport protein (Tanner *et al.*, 1979). The advantage of this strategy was that the membrane-integrated portions of the protein could be separated from the cell contents, peripheral proteins and even the extramembranous parts of integral proteins by washing and proteolysis procedures alone. This simplified the subsequent purification of peptides, a process that has often proved difficult when working with integral membrane proteins.

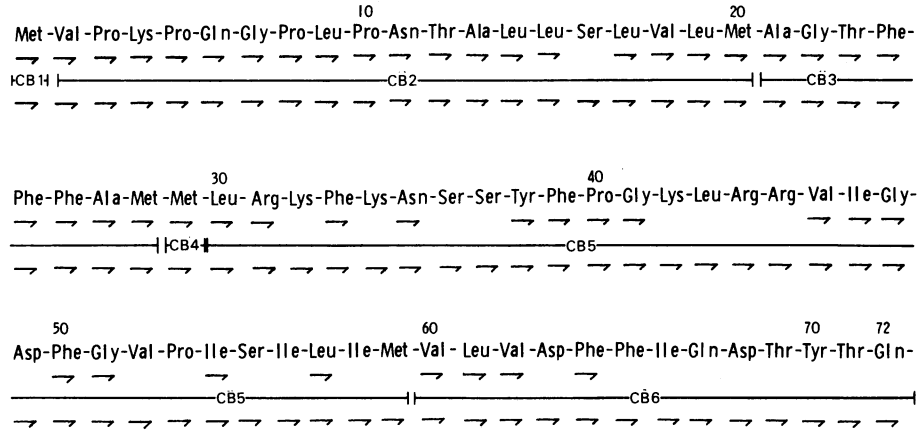

Fig. 1. *Construction of the amino acid sequence of the N-terminal 72 residues of fragment P5 from the human erythrocyte anion-transport protein*
→, Sequenced by automated solid-phase Edman degradation.

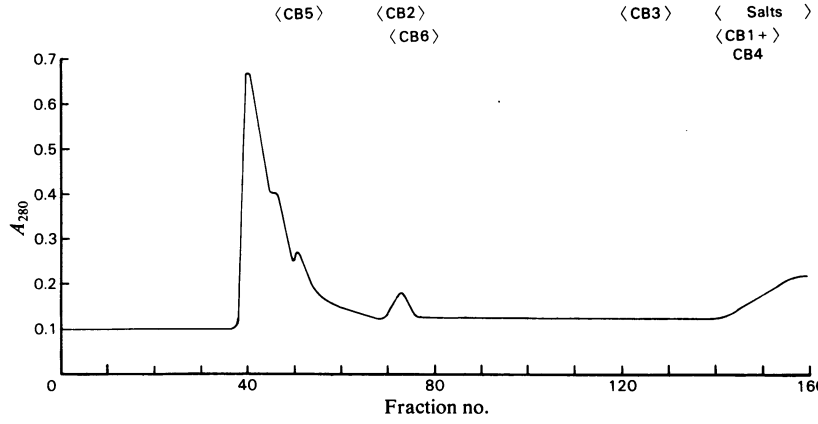

Fig. 2. *Purification of peptides from CNBr cleavage of fragment P5*
A 0.5 μmol portion of cleaved peptide was fractionated on a 1400 mm × 8 mm column of Bio-Gel P10 (200–400 mesh) in 80% formic acid; 500 μl fractions were collected.

The amino acid sequence, shown in Fig. 1, was derived by automated solid-phase sequencer analysis of fragment P5 and six of the peptides produced by chemical cleavage of fragment P5 with CNBr.

The use of the solid-phase Edman degradation facilitated the elucidation of long sequences from polypeptides that were relatively rich in hydrophobic amino acids. The programme used in the sequencer was one designed to give the minimum of overlap, so that the amino acid phenylthiohydantoin derivatives released could be clearly identified through many cycles in a simple high-pressure liquid-chromatographic system. The major factor decreasing the repetitive yield during sequencing (typically 89–93%; see Supplement SUP 50123) was a blocking reaction of unknown nature.

Purification and characterization of peptides

The CNBr-cleavage peptides used to construct the sequence that has been determined were purified in a single gel-filtration step on Bio-Gel P10 (200–400 mesh) in 80% formic acid (Fig. 2). The elution positions of the peptides were identified by amino acid analysis of a portion of each fraction. Individual fractions that apparently contained pure peptides were then subjected to solid-phase sequencer analysis. By this means it was possible to identify and sequence fractions containing each peptide purified to homogeneity, even though peptides CB2 and CB6 were not fully resolved from each other, and peptide CB5 was a shoulder on the higher-M_r peak eluted from the column. A consequence of this strategy was that peptide yields after purification

were variable. This was not a serious drawback, however, because peptide purification in high and consistent yield was not used as a criterion for assigning peptide sequences to the anion-transport protein in this particular experimental strategy.

The high-M_r peak in Fig. 2 contains a peptide covering the *C*-terminal part of fragment P5, partial cleavage products and the undigested polypeptide. This *C*-terminal peptide is more difficult to purify, and its primary structure has yet to be completed and overlapped with the *N*-terminal 72 residues (C. J. Brock & M. J. A. Tanner, unpublished work).

Amino acid sequence

Methionine at residue 1 (peptide CB1) was identified directly from the *N*-terminal sequence of fragment P5, but in low yield, because the peptide was *p*-phenylene di-isothiocyanate-coupled, so that much of the *N*-terminal amino acid remained attached to the glass (see Supplement SUP 50123). This residue was also identified directly and in good yield from fragment P5 that was *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide-coupled before being sequenced, but the yields of amino acid phenylthiohydantoin derivatives deteriorated rapidly after Gly-5 in this experiment. This assignment could also be inferred from the known specificity of the CNBr cleavage reaction, which yielded peptide CB2. In addition, homoserine and homoserine lactone were isolated with the low-M_r material from the cleavage mixture (Fig. 2) and shown by sequence analysis to be present as free amino acids corresponding to peptide CB1 as well as peptide CB4.

Residues 2–20 were determined by sequencing peptide CB2 to completion, and were found to be entirely consistent with the data for this region from intact fragment P5 (Fig. 1).

Similarly peptide CB3 was sequenced to completion, and the assignments and peptide alignment were confirmed by the sequence of fragment P5, thus elucidating residues 21–28 (Fig. 1).

Methionine at residue 29 (peptide CB4) was identified directly during sequencing of intact fragment P5 (Fig. 1), and this was confirmed by a surprisingly high yield of homoserine phenylthiohydantoin derivative in cycle 9 of the peptide-CB3 sequence (see Supplement SUP 50123), due to incomplete cleavage of the Met–Met peptide bond by CNBr. The presence of free homoserine and homoserine lactone in the cleavage mixture as described above for residue 1 was also consistent with this assignment.

The peptide CB5 was sequenced to completion. Its position in the primary structure at residues 30–59 was determined, along with confirmation of many assignments, by the sequencing of intact fragment P5 (Fig. 1).

Residues 60–72 were identified from the partial sequence of peptide CB6 with the overlap (Val-60, Leu-61, Val-62 and Phe-64) coming from the *N*-terminal sequence of intact fragment P5 (Fig. 1).

Location of labelled sites

In order to prove unequivocally that the primary structure given in the present paper originated from the anion-transport protein, it was necessary to locate the labelled sites that have been mapped in fragment P5 and that show it to be a membrane-integrated portion of the protein that is directly involved in anion exchange.

The CNBr-cleavage peptides from the *N*-terminal 72 residues of fragment P5 were at various times purified from erythrocyte 'ghosts' that had been covalently labelled with the anion-exchange inhibitor phenyl isothio[^{14}C]cyanate (Kempf *et al.*, 1981) or from intact erythrocytes labelled by lactoperoxidase-catalysed radioiodination of tyrosine residues (Tanner *et al.*, 1979).

The only phenyl isothio[^{14}C]cyanate to be found in this sequence co-purified with fragment CB5, and when the labelled peptide was subjected to solid-phase Edman degradation the radioactivity was released in cycle 3 (Fig. 3), which corresponded to Lys-32 in the sequence of fragment P5.

The only radioiodinated tyrosine residue located in the sequence presented here co-purified with peptide CB6. When this peptide was sequenced the radioactivity was released in cycle 11 (Fig. 4), corresponding to Tyr-70 of fragment P5. Since erythrocytes are impermeable to lactoperoxidase, this site must be located extracellularly. This is entirely consistent with previous studies, which had shown that all the tyrosine residues in fragment P5 that can be radioiodinated are extracellular (Tanner *et al.*, 1979).

It was already known that the anion-transport protein can be cleaved extracellularly with trypsin under low-ionic-strength conditions (Jenkins & Tanner, 1977*a*). Therefore fragment P5 was purified from erythrocytes that had been predigested with trypsin under these conditions. Sequence analysis of this material showed that partial digestion at the Lys-4–Pro-5 bond had occurred, indicating that these residues are located extracellularly

Structure of the anion-transport protein

The regions of any polypeptide chain that are embedded in the hydrophobic parts of the lipid bilayer must have all of their hydrogen-bonding requirements satisfied internally. Therefore in the cases of proteins, such as the anion-transport protein, that have a substantial intrinsic domain they will contain segments of amphipathic transmembrane secondary structure. These will be linked by more hydrophilic regions subject to fewer conformational constraints, but that might form

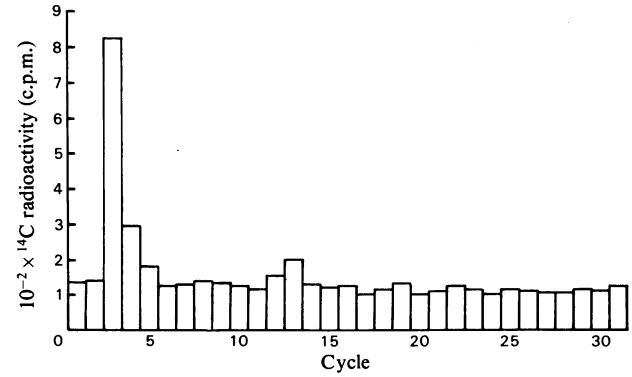

Fig. 3. *Release of ^{14}C during the sequencing of peptide CB5 prepared from phenyl isothio[^{14}C]cyanate-labelled erythrocytes*
See the text for experimental details.

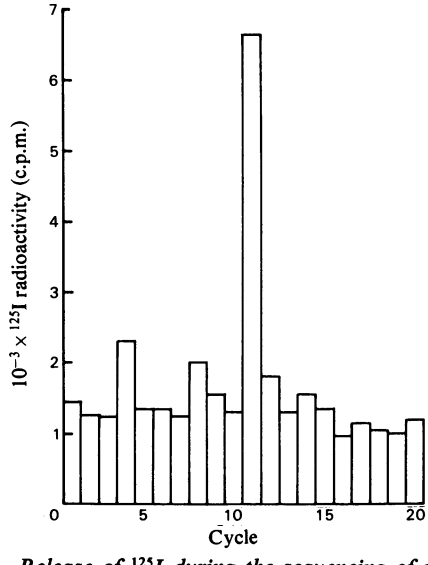

Fig. 4. *Release of ^{125}I during the sequencing of peptide CB6 prepared from radioiodinated erythrocytes*
See the text for experimental details.

bends where they are too short to fold into major extrinsic structures.

The sequence of the *N*-terminal 72 residues of fragment P5 was therefore examined for the potential to adopt a conformation that is consistent with the constraints that apply to integral membrane proteins. We first looked for sites exposed at the surface, and that could form the bends that might be expected to flank the regions of transmembrane secondary structure. The following points were noted. (i) Met-1 is a peptic cleavage site and must therefore be at one surface or the other. (ii) Lys-4–Pro-5 is an extracellular tryptic cleavage site and must therefore be exposed at the extracellular surface. (iii) Tyr-70 is an extracellular lactoperoxidase-catalysed radioiodination site and must therefore be at the extracellular surface also, whereas Tyr-38 is not iodinated and must be located elsewhere. (iv) Asn-35 is not glycosylated, even though it is part of an Asn-Xaa-Ser sequence that is associated with *N*-linked glycosylation of polypeptide chains when exposed extracellularly. It is unlikely therefore that Asn-35 has an extracellular location. (v) The regions Pro-3–Thr-12, Arg-31–Arg-45 and Gln-67–Gln-72 are particularly rich in hydrophilic amino acids (indicative of location in an aqueous environment) and proline and glycine residues (indicative of bends). (vi) The regions Pro-3–Pro-8 and Ser-36–Gly-41 were predicted to form bends bounding an α-helix (Pro-10–Lys-34) when analysed by McLachlan's (1977) statistical inference method.

The entire sequence was then examined for the characteristic features of amphipathic transmembrane secondary structures. The polypeptide antibiotic gramicidin A creates a channel for cations in biological membranes by adopting a β-helical conformation (Urry, 1982). However, D-amino acids are required for such a structure, and so the anion-transport protein could only adopt such a conformation if glycine residues were appropriately distributed throughout its sequence. The distribution of glycine residues in fragment P5 was found to be inconsistent with the formation of any β-helical structure.

The β-pleated sheet barrel is a structure that occurs in a number of proteins (Richardson *et al.*, 1975). As yet no integral membrane protein has been shown to have such a conformation, but it could be

envisaged. The membrane-traversing strands would have all of their backbone hydrogen-bonding requirements satisfied by neighbouring sheets. There would be the additional constraint, however, that any hydrophilic amino acid side chains would have to point towards the centre of the barrel, where they could interact with each other and would be isolated from the hydrophobic milieu. The distribution of hydrophilic amino acids in fragment P5 is not consistent with the presence of such amphipathic β-sheets.

A transmembrane α-helix has all of the hydrogen-bonding requirements of its polypeptide backbone satisfied by groups on adjacent turns of the helix. Consequently this is the most usual conformation for the membrane-intercalated portions of proteins, such as glycophorin, which traverse the membrane only once (Schulte & Marchesi, 1979). A more substantial integral structure is formed by a cluster of transmembrane α-helices, such as that found in bacteriorhodopsin (Henderson & Unwin, 1975). Such a structure has certain distinct characteristics. Uncharged, but hydrophilic, side chains either intercalate with the side chains of adjacent helices ('knobs-into-holes packing'; Dunker & Jones, 1979) or they point towards the centre of the cluster. Charged amino acids either can occur close to the surface, where they will contact the polar phase, or may face the centre of the molecule and participate in intramembranous salt bridges (Engelman *et al.*, 1980). A great deal of energy is required to place charges, either singly or in pairs, into a medium of low dielectric constant (Edmonds, 1981), and so such salt bridges are likely to be surrounded by hydrophilic amino acid side chains. Thus in a protein such as bacteriorhodopsin, which bears such features, and does not have a water-filled channel running through its core (Engelman *et al.*, 1980), the environment of such charged amino acids will have a dielectric constant intermediate between that of the lipid bilayer and the aqueous phase.

When the sequence from the anion-transport protein was examined for the potential to form transmembrane α-helices, a concordant picture emerged (Fig. 5). The regions Pro-10–Lys-34 and Lys-42–Ile-66 could form α-helices similar in length to those of bacteriorhodopsin (Engelman *et al.*, 1980) and other intergral membrane proteins (Warren, 1981). The side chains of Asn-11, Arg-31, Lys-32, Lys-34, Lys-42, Arg-44 and Arg-45 would be close enough to the surface to contact the polar phase. The hydrophilic residues, which may be remote from the surface, would stack above each other to form a narrow strip on one face of the first helix (Thr-12, Ser-16 and Thr-23), and would range around half the circumference of the second (Asp-49, Ser-55 and Asp-63). These transmembrane segments would be bounded extracellularly by Met-1

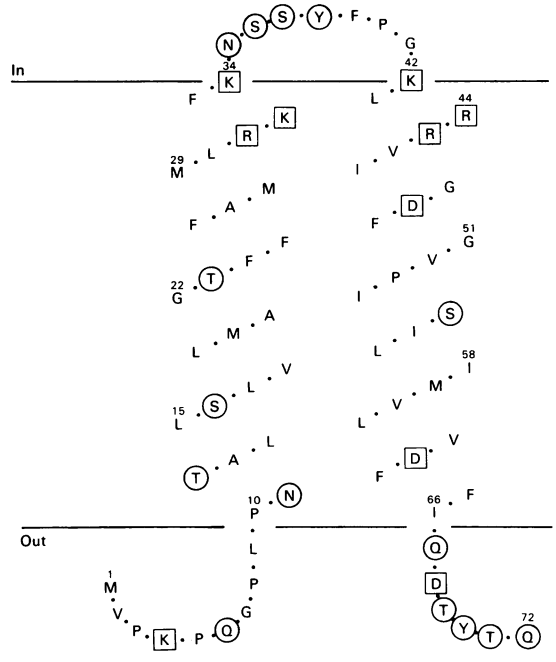

Fig. 5. *Proposed topology of fragment P5*
The *N*-terminal sequence of fragment P5 is presented with the use of the one-letter code for amino acids: A, alanine; D, aspartic acid; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine. α-Helical segments (3.5 residues per turn) are illustrated as though the sequence had been written out on the surface of a cylinder wrapped around the helix axis and laid flat. Amino acids with charged side chains are boxed and those with other hydrophilic side chains (Ser, Thr, Asn, Gln, Tyr) are circled.

to Leu-9 and Gln-67 to Gln-72 and linked intracellularly by Asn-35 to Gly-41, in accord with points (i)–(vi) described above. Thus it is clear that the *N*-terminal 72-residue portion of fragment P5 has the potential to form two transmembrane α-helices, which are structurally homologous with those of other integral membrane proteins. It is most likely therefore that the basic structure of the membrane-bound domain of the anion-transport protein is a cluster of transmembrane α-helices. Isolated strands of β-structure cannot occur, because of their hydrogen-bonding requirements, and it has already been noted that α-helices pack together efficiently in membranes (Dunker & Jones, 1979).

Some other noteworthy features emerge from this structural model. Arg-45 is so placed that, in addition to being able to reach the membrane surface, its side chain could re-orient to form a salt

bridge with Asp-49 on the more polar side of the second helix, and in the body of the protein. This helical segment, like those of some other proteins (Dunker, 1982), contains a proline residue (Pro-53). This might create an electron-rich site in the protein (Dunker, 1982), which could help to facilitate anion translocation. If the two helices shown in Fig. 5 are packed next to each other in the protein's tertiary structure (which seems likely, since the linking sequence Asn-35 to Gly-41 is so short), then Lys-32 would lie among a cluster of positively charged amino acids at the intracellular surface of the membrane. This is precisely the type of environment that would lead to a lowering of the pK of its ε-amino group thus making it reactive towards phenyl isothiocyanate.

Anion propagation model for the anion-transport protein

A body of evidence is emerging concerning the regions of the anion-transport protein to which anions bind before their translocation. Substrate binding (activation energy <17kJ/mol) can be distinguished kinetically from translocation of the anion (activation energy 84–125kJ/mol) (Brahm, 1977). Inhibitor-binding studies have shown that the extracellular substrate-binding site involves a cluster of at least five positively charged amino acid side chains (Passow *et al.*, 1980). Furthermore our present study has suggested that located intracellularly there is a cluster of six basic residues in fragment P5, which is itself known to be directly involved in transport activity. That the two positively charged clusters are separate entities that remain exposed to their respective surfaces is necessary on structural grounds, because all the known functional sites on the protein are asymmetrically distributed with respect to the membrane (Rothstein *et al.*, 1978), and on kinetic grounds, because anion exchange is too fast for a gross conformational change to take place (Knauf, 1979). The observation that substrate inhibition of transport occurs at high concentrations of anions (e.g. >150mM-Cl^-) (Cass & Dalmark, 1973) also supports the idea that the sites are composed of lysine and arginine residues. The affinity of such an anion-transport site is likely to be affected by swamping the surrounding basic amino acids with counterions.

The rate of Cl^- exchange is constant from pH7 to pH11, indicating that univalent anions bind to the guanidine group of an arginine residue, since it is the only amino acid with a sufficiently high pK to remain positively charged over this pH range (Funder & Wieth, 1976). Bivalent anions (SO_4^{2-}, HPO_4^{2-}) that act as substrates are co-transported with a proton (Gunn, 1978), which is probably derived from the ε-amino group of a nearby lysine residue, itself distinguished by an unusually low pK. It has already been mentioned that Lys-32 of fragment P5 exhibits a lowered pK and that this is entirely consistent with the general environment of the anion-binding regions.

The next step was to examine the mechanism of anion translocation in the light of the available evidence from both structural and functional studies. It seems unlikely that there is an aqueous channel running through the anion-transport protein. Those proteins that create large water-filled pores, typified by the porins of bacterial outer membranes, show poor substrate selectivity and can only mediate the free diffusion of solutes (Nikaido, 1982). Gramicidin A creates a cation-specific water-filled channel, only about 0.4nm in diameter. In this case the ion is translocated freed from most of its hydration shell, but transport remains uncoupled from any other process (Urry, 1982). By contrast, in bacteriorhodopsin the pumping of protons is tightly coupled to the protein's photocycle, and water is completely excluded from the core of the molecule (Henderson & Unwin, 1975). A similar situation is likely to prevail in the anion-transport protein, where the permeability barrier is absolutely maintained except for the strict sequential exchange of anions. Further support for this view comes from the substrate-specificity of the anion-transport system. Cl^- and HCO_3^-, the physiological substrates, are exchanged far more rapidly than are larger anions. Aubert & Motais (1975) noted that the limiting size for molecules bearing only polar groups is less than 0.4nm, whereas much larger molecules can be accommodated provided that the additional moieties are hydrophobic. The sensitivity of the system to increases in substrate size, and the positive effect of hydrophobic interactions is clearly more consistent with a bacteriorhodopsin-like protein core filled with amino acid side chains than with a water-filled channel lined with polar groups.

The observation that the side chain of Arg-45 of fragment P5 could be either exposed intracellularly among the cluster of basic amino acids at that surface or involved in a charged pair with Asp-49 in the body of the protein suggested to us the following simple mechanism for anion translocation. An anion might be freed of at least some of its hydration shell by interacting with the cluster of positive charges at the surface and thus bind to Arg-45 at the inside of the membrane (Fig. 6*a*). The formation of the charged pair would promote the re-orientation of the side chain of Arg-45 so that it moves with the anion into the core of the α-helical cluster to form a complex with Asp-49. It is assumed that Asp-49 would already be involved in a charged pair with another basic amino acid (Fig. 6*b*). The anion may then catalyse the exchange of the two pairs, so that it can move further through the membrane in concert

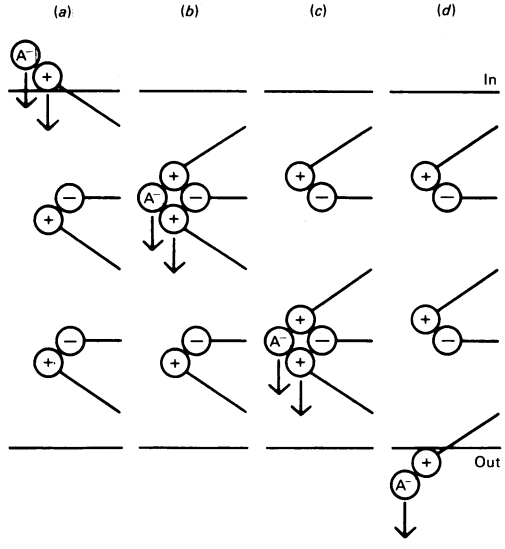

Fig. 6. *Anion-propagation model*

A schematic representation of the mechanism by which an anion (Ⓐ⁻) might be propagated through the erythrocyte membrane, catalysing the rearrangement of charged pairs of acidic (⊖—) and basic (⊕—) amino acid side chains as it passes. Any of the transmembrane α-helices, which are thought to form the intrinsic structure of the protein, may contribute residues to the system, provided that they are correctly disposed to make the requisite interactions. Precise details of the conformational changes that take place during transport and the number of intramembranous salt bridges are not known, though the Figure shows the smallest network that could traverse the hydrophobic part of the membrane. Essential features of the model not shown are the clusters of positively charged residues at each surface of the membrane, which comprise the anion-binding sites, and the hydrophilic residues surrounding the intramembranous charges, which raise the local dielectric constant.

with this second positively charged side chain. It could then interact with other intramembranous charged pairs in a similar manner (Fig. 6*c*), until it emerges at the outside of the membrane, bringing a previously buried arginine side chain out with it (Fig. 6*d*). Arg-45 would now be buried inside the protein and charge-paired with Asp-49, whereas the extracellular anion-binding residue would be exposed. This and the arrangement of the other intramembranous charged pairs would ensure that, next, an anion would be transported back from the outside to the inside. In this way a strict sequential exchange mechanism could be imposed on the anion-transport system.

The fragment P5 does not contain all the elements (e.g. intramembranous charged pairs) essential for anion transport, and so the amino acid sequences of the remaining transmembrane segments will be needed to characterize the system completely. For instance, a 35000Da glycopeptide (which includes the region of P5) contains the external anion-binding arginine residue (Bjerrum *et al.*, 1983), and a 17000Da membrane-traversing fragment that binds bulky transport inhibitors such as disulphonic stilbenes and arenesulphonates (Mawby & Findlay, 1982) may contain other essential elements of the system. Further study of such regions will facilitate the location of such elements and the verification of the structural model and molecular mechanism proposed in the present paper.

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References

Aubert, L. & Motais, R. (1975) *J. Physiol. (London)* **246**, 159–179

Bjerrum, P. J., Wieth, J. O. & Borders, C. L., Jr. (1983) *J. Gen. Physiol.* **81**, 453–484

Boxer, D. H., Jenkins, R. E. & Tanner, M. J. A. (1974) *Biochem. J.* **137**, 531–534

Brahm, J. (1977) *J. Gen. Physiol.* **70**, 283–306

Cass, A. & Dalmark, M. (1973) *Nature (London) New Biol.* **244**, 47–49

Cohen-Solal, M. & Barnard, J. L. (1973) *J. Chromatogr.* **80**, 140–143

Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130

Dunker, A. K. (1982) *J. Theor. Biol.* **97**, 95–127

Dunker, A. K. & Jones, T. C. (1979) *Membr. Biochem.* **2**, 1–16

Edmonds, D. T. (1981) *Trends Biochem. Sci.* **6**, 92–94

Engelman, D. M., Henderson, R., McLachlan, A. D. & Wallace, B. A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2023–2027

Funder, J. & Wieth, J. O. (1976) *J. Physiol. (London)* **262**, 679–698

Gates, F. T., III, Coligan, J. E. & Kindt, T. J. (1979) *Biochemistry* **18**, 2267–2272

Gillies, R. J. (1982) *Trends Biochem. Sci.* **7**, 41–42

Grinstein, S., Ship, S. & Rothstein, A. (1978) *Biochim. Biophys. Acta* **507**, 294–304

Gunn, R. B. (1978) in *Membrane Transport Processes* (Hoffmann, J. F., ed.), pp. 61–77, Raven Press, New York

Gunn, R. B. & Fröhlich, O. (1978) *J. Supramol. Struct. Suppl.* **2**, 213

Gunn, R. B. & Fröhlich, O. (1979) *J. Gen. Physiol.* **74**, 351–374

Henderson, R. & Unwin, P. N. T. (1975) *Nature (London)* **257**, 28–32

Jenkins, R. E. & Tanner, M. J. A. (1977*a*) *Biochem. J.* **161**, 131–138

Jenkins, R. E. & Tanner, M. J. A. (1977*b*) *Biochem. J.* **161**, 139–147

Kempf, C., Brock, C., Sigrist, H., Tanner, M. J. A. & Zahler, P. (1981) *Biochim. Biophys. Acta* **641**, 88–98
Knauf, P. A. (1979) *Curr. Top. Membr. Transp.* **12**, 249–363
Mawby, W. J. & Findlay, J. B. C. (1982) *Biochem. J.* **205**, 465–475
McLachlan, A. D. (1977) *Int. J. Quantum Chem.* **12**, 371–385
Mendez, E. & Lai, C. Y. (1975) *Anal. Biochem.* **68**, 47–53
Mueller, T. J., Li, Y. T. & Morrison, M. (1979) *J. Biol. Chem.* **254**, 8103–8106
Nikaido, H. (1982) *Membr. Transp.* **2**, 265–270
Passow, H., Kampmann, L., Fasold, H., Jennings, M. & Lepke, S. (1980) in *Membrane Transport in Erythrocytes* (Lassen, U. V., Ussing, H. H. & Wieth, J. O. eds.), pp. 345–372, Munksgaard, Copenhagen
Previero, A., Derancourt, J., Coletti-Previero, M.-A. & Laursen, R. A. (1973) *FEBS Lett.* **33**, 135–138
Richardson, J. S., Thomas, K. A., Byron, H. R. & Richardson, D. C. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1349–1353
Rothstein, A., Grinstein, S., Ship, S. & Knauf, P. A. (1978) *Trends Biochem. Sci.* **3**, 126–128
Schulte, T. H. & Marchesi, V. T. (1979) *Biochemistry* **18**, 275–280
Tanner, M. J. A., Williams, D. G. & Kyle, D. (1979) *Biochem. J.* **183**, 417–427
Urry, D. W. (1982) *Membr. Transp.* **2**, 285–294
Warren, G. (1981) in *Membrane Structure* (Finean, J. B. & Mitchell, R. H., eds.), pp. 215–257, Elsevier/North-Holland, Amsterdam
Williams, D. G., Jenkins, R. E. & Tanner, M. J. A. (1979) *Biochem. J.* **181**, 477–493