The Isolation of Human-Erythrocyte Band-3 Polypeptide Labelled with a Photosensitive Hydrophobic Probe

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To investigate the intramembranous domains of the major band-3 polypeptide, human erythrocyte membranes were labelled with 1-azido-4-[¹²⁵I]iodobenzene. The anion-exchange protein has been isolated by a new procedure that decreases possible contamination by other integral membrane proteins of similar molecular weight. After chemical modification, the protein was subjected to proteolytic cleavage by several proteinases to yield a distinct pattern of peptides, a few of which contained radioactivity.

The major polypeptide in the band-3 region of SDS/polyacrylamide gels after electrophoresis of the membrane proteins from human-erythrocyte membranes is a major integral glycoprotein (mol.wt. 95000) which spans the bilayer (Fairbanks *et al.*, 1971; Bretscher, 1971; Staros & Richards, 1974). Since it functions as the principal translocator of anions across the erythrocyte membrane (Cabantchik & Rothstein, 1974; Ho & Guidotti, 1975) and constitutes up to 30% of the total membrane protein, this polypeptide has become a major focus of interest for expanding our knowledge of protein transport mediators. To fulfil this role the protein is thought to have an amphipathic organization, possessing two different areas of relatively hydrophilic character separated by a largely hydrophobic centre portion, constituting up to 20% of the molecule, that is responsible for the integration of the molecule in the phospholipid bilayer. Some appreciation of the disposition of the polypeptide backbone in the membrane has resulted from degradative studies *in situ* (Jenkins & Tanner, 1975, 1977; Steck *et al.*, 1976, 1978; Drickamer, 1976, 1977; Fukuda *et al.*, 1978) but more detailed knowledge of its structure can only come from the amino-acid sequence of the protein together with the identification of defined structural markers.

Such domains include those sections of peptide exposed to the hydrophobic milieu of the membrane, which represent the primary sites through which lipid-mediated regulatory effects must act. In order to ascertain the nature of these domains, and their location within the intact polypeptide chain, we have explored the possibility of labelling techniques, which can be used on the intact membrane rather than relying on reconstituted systems (Klip & Gitler,

Abbreviation used: SDS, sodium dodecyl sulphate.

1974; Klip *et al.*, 1976; Abu-Salah & Findlay, 1977; Karlsh *et al.*, 1977; Sigrist-Nelson *et al.*, 1977; Bercovici & Gitler, 1978; Kahane & Gitler, 1978; Bayley & Knowles, 1978*a,b*; Wells & Findlay, 1979*a,b*; Sator *et al.*, 1979). This paper reports the labelling of the anion-exchange protein of human erythrocytes with one such compound and its isolation by a new procedure that resolves the protein from other integral polypeptides of similar molecular weight.

Experimental

Materials

Human packed cells (A, Rh +ve) were supplied by the Regional Blood Transfusion Centre, Seacroft Hospital, Leeds, U.K. These were stored at 4°C and used before the expiry date. Radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Chymotrypsin and trypsin [1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamido butan-2-one ('TPCK')-treated] were obtained from Worthington Biochemicals, Croydon, Surrey, U.K. Sodium dodecyl sulphate was supplied by Sigma, and all other reagents (AnalaR grade) by BDH Chemicals, Poole, Dorset, U.K.

Purification of band-3 polypeptide

Erythrocyte membranes were prepared and labelled with 0.25 mM-1-azido-4-[¹²⁵I]iodobenzene (0.075 mCi/mol) as described earlier (Wells & Findlay, 1979*a*; Booth *et al.*, 1979). The band-3 polypeptide was purified from washed and dialysed membranes as described in Scheme 1.

Spectrin (bands 1, 2 and 5) and glyceraldehyde-3-phosphate dehydrogenase (band 6) were eluted from the membrane by 1 mM-EDTA, pH 8.0,

Scheme 1. Isolation of the major band-3 polypeptide

and 0.15 M-NaCl respectively. These membranes were then treated with 100 μ M-*o*-phenanthroline/50 μ M-Cu²⁺ to cross-link the major band-3 polypeptide; the reaction was stopped by washing in 1 mM-EDTA (Steck *et al.*, 1976). The membranes were then solubilized by the addition of SDS to 2–3% (w/v), with heating at 100°C for 2–3 min.

Any insoluble material was removed by centrifugation (80 000 g for 15 min at 20°C). The solubilized material was applied to a column of Sepharose 4B and eluted with 15 mM-Tris/H₂SO₄/0.1% (w/v) SDS, pH 8.0. The protein elution profile was monitored by SDS/polyacrylamide-gel electrophoresis on 5% gels of aliquots of selected fractions, both with and without the inclusion of 1% (v/v) 2-mercaptoethanol in the sample (Findlay, 1974). The fractions containing cross-linked band-3 were pooled to exclude any monomeric band-3 and

lower-molecular-weight material. The material was freeze-fried, dissolved in water and the pH was adjusted to 8.0. The disulphide bonds were reduced with 5 mM-dithiothreitol for 2 h and the protein was reappplied to the same Sepharose 4B column to remove any traces of high-molecular-weight material. Fractions were again pooled on the basis of SDS/polyacrylamide-gel electrophoresis [7.5% (w/v) in acrylamide] of fractions across the peak.

The lyophilized purified protein was carboxymethylated and subjected to digestion with trypsin (3%, w/w) or chymotrypsin (3%, w/w) in 0.1 M-NH₄HCO₃ for 3 h.

SDS/polyacrylamide disc-gel electrophoresis

Gels were either 7.5% (w/v) acrylamide + 0.1% (w/v) methylene bisacrylamide, or 5% (w/v) acrylamide + 0.06% (w/v) methylene bisacrylamide. The

buffer was 0.1M-boric acid/0.1M-sodium acetate/0.1% SDS adjusted to pH 8.5 with NaOH. Samples were dissolved in 2–3% (w/v) SDS with or without 1% 2-mercaptoethanol, then heated at 100°C for 2–3 min.

SDS/urea/polyacrylamide disc-gel electrophoresis

Gels contained 12.5% (w/v) acrylamide, 0.42% (w/v) methylene bisacrylamide, 0.1% (w/v) SDS, 8M-urea, 0.1M-phosphoric acid and 0.07% (v/v) NNN'N'-tetramethylethylenediamine. The pH was adjusted to 6.8 with Tris solution and 0.07% ammonium persulphate was added prior to casting the gels. For the range of molecular weights studied, 1/30th cross-linker was preferred to the original value (1/15th). The reservoir buffer was 0.1M-phosphoric acid/0.1% (w/v) SDS, adjusted to pH 6.8 with Tris solution. Freeze-dried peptides or proteins were solubilized in 2% (w/v) SDS/8M-urea/1% (v/v) 2-mercaptoethanol/0.1M-phosphoric acid, adjusted to pH 6.8 with Tris (Swank & Munkries, 1971).

Gels were fixed and stained for protein with 0.25% Coomassie Brilliant Blue R250 in methanol/water/acetic acid (45:45:10, by vol.) for 4h, and destained in water/methanol/acetic acid (53:40:7, by vol.).

Fig. 1. Labelling of membrane components with 1-azido-4-[¹²⁵I]iodobenzene

Human-erythrocyte membranes labelled with the probe were dissolved in 2% (w/v) SDS/1% 2-mercaptoethanol and applied to the gel (see the Experimental section). The ¹²⁵I content (bars) of 2mm-slices of a SDS/polyacrylamide gel (7.5% acrylamide) is superimposed on the absorbance profile at 530nm of Coomassie-Blue-stained material.

Results

Labelling of the protein components

The main radioactive bands in Fig. 1 were band 3 and the region on the gel between bands 4 and 5. The regions of bands 1 and 2 and band 7 also contained a little radioactivity, as did aggregated material present at the top of the gel. Bands 4.1, 4.2 and 5 contained much less radioactivity. The highest mobility radioactivity was due to labelled lipid and unbound material. Fig. 1 also indicates a component(s) migrating in the band 4–5 region of the gel, which was highly labelled but represents only a small

Fig. 2. SDS/polyacrylamide-gel electrophoresis of membranes treated with Cu²⁺/o-phenanthroline after removal of components 1, 2, 5 and 6

Gels are 5% acrylamide. (a) Untreated membranes; (b) membranes stripped of components 1, 2, 5 and 6; (c) stripped membranes, treated with Cu²⁺/o-phenanthroline, and electrophoresed in the absence of 2-mercaptoethanol.

proportion of the total protein. Purification of the protein components of the EDTA extract indicated that the actin-like component (band 5) contained no radioactivity and the spectrin fraction very little.

Labelling of the anion-transport protein

Purification. A new purification scheme was devised, based on the ability of the protein *in situ* to form intermolecular disulphide bonds in the pres-

Fig. 3. Fractionation of cross-linked membranes on Sepharose 4B

Cross-linked membranes (Fig. 2) were solubilized in SDS and applied to a column of Sepharose 4B (128 cm $\times$ 3.4 cm) eluted with 0.025 M-Tris/ H_2SO_4 /0.1% SDS, pH 8.0. The original protein content of the membrane was 450 mg. The fraction volume was 9.6 ml. The fractions indicated by the bar were pooled.

Fig. 4. Analysis by SDS/polyacrylamide-gel electrophoresis of the elution profile obtained after fractionation (Fig. 3) of cross-linked membranes on Sepharose 4B

Aliquots were taken from the fractions indicated (see Fig. 3) and analysed in the absence (a) and presence (b) of 1% 2-mercaptoethanol. The gels were 5% acrylamide.

ence of Cu^{2+} /*o*-phenanthroline, giving mainly dimers (Steck *et al.*, 1976). This permitted separation of these high-molecular-weight species from low-molecular-weight proteins by gel filtration. Purification was facilitated by the elution of bands 1, 2, 5 and 6 from the membrane before cross-linking. SDS/polyacrylamide-gel electrophoresis of membranes so treated are shown in Fig. 2. After cross-linking, only a small amount of protein still migrates in the band-3 region; how much consists of co-running minor components is uncertain. Addition of 1% 2-mercaptoethanol to (c) gave the pattern shown in gel (b).

After fractionation of the cross-linked protein by

gel filtration on Sepharose 4B (Fig. 3), the column eluate was monitored by SDS/polyacrylamide-gel electrophoresis (5% acrylamide) both in the presence and absence of 2-mercaptoethanol (Fig. 4). Fractions containing the high-molecular-weight forms of the band-3 protein were pooled and, after reduction, the protein was re-applied to the same Sepharose 4B column to remove any remaining high-molecular-weight contaminants (Fig. 5a). Analysis of the profile on SDS/polyacrylamide gels (7.5% acrylamide) is shown in Fig. 5b. The isolated protein gave a single band on SDS/polyacrylamide-gel electrophoresis (Fig. 6). After extensive dialysis, and carboxymethylation, the protein generally remained soluble, although occasionally a slight colloidal precipitate appeared.

Fig. 5. Gel filtration of the fractions pooled from Fig. 3, containing cross-linked band 3, re-applied to the same column after reduction with dithiothreitol

(a) Column elution profile; (b) analysis of the fractions, as numbered, by SDS/polyacrylamide disc gel electrophoresis (7.5% acrylamide). Fractions were pooled as indicated by the solid bar. The fraction volume was 15 ml.

Fig. 6. SDS/polyacrylamide disc gel electrophoresis of purified anion-transport protein

The gel was 7.5% acrylamide and was stained with Coomassie Brilliant Blue. The gel contained $30\mu\text{g}$ of protein.

Fig. 7. Proteolytic digestion of band 3 labelled with 1-azido-4-[125 I]iodobenzene. SDS/urea gels (12.5%) were run and cut into 2 mm slices for counting. (a) Digestion with trypsin; (b), digestion with chymotrypsin.

Proteolytic digestion. Treatment of carboxy-methylated protein with trypsin (Fig. 7a) gave rise reproducibly to a range of highly labelled aggregates and several other discrete fragments of which two (mol.wts. 13 000 and 5500) were of high specific activity. The major bands, of mol.wts. 25 000, 20 000, 10 000 and 6500, carried little radioactivity, demonstrating a differential labelling of the polypeptide chain.

Cleavage with chymotrypsin (Fig. 7b), as expected for a disaggregated protein, yielded a quite different pattern with broad, heterogeneous Coomassie-Blue-staining bands of approximate mol.wts. 6500 and 4000; minor bands were also obtained (mol.wts. 18 000 and 12 000). The 4000-mol.wt. region contained labelled material and the label occurring in the 6500-mol.wt. region was associated predominantly with the leading and trailing edges.

Uncleaved protein just penetrates this gel system with no other radioactive or Coomassie-Blue-staining bands being observed.

Discussion

Previous publications have documented the covalent attachment of hydrophobic probes to the intramembraneous regions of membrane components (Klip & Gitler, 1974; Chakrabarti & Khorana, 1975; Klip *et al.*, 1976; Abu-Salah & Findlay, 1977; Gupta *et al.*, 1977; Karlsh *et al.*, 1977; Sigrist-Nelson *et al.*, 1977; Bercovici & Gitler,

1978; Kahane & Gitler, 1978; Bayley & Knowles, 1978a,b; Wells & Findlay, 1979a,b; Sator *et al.*, 1979). The results of this and an earlier paper (Wells & Findlay, 1979a) indicate substantial labelling of components in the band-3 position. To eliminate the co-purification of other components in this region a new method of isolation has been developed, based on the ability of the protein to form interchain disulphide bonds in the presence of *o*-phenanthroline/CuSO₄ (Steck *et al.*, 1976). Any contaminant of the final preparation must now not only co-migrate with the anion-exchange protein in SDS but it must also exhibit similar cross-linking characteristics when in the membrane. Some of the possible contaminants may remain in the band-3 region of SDS/polyacrylamide gels after cross-linking.

Cleavage of the purified protein was studied in a series of digestion systems utilizing different proteinases, of which two are shown here. All of the digests gave rise to a distinct and reproducible set of cleavage products, with those proteinases specific for charged residues giving rise to larger peptides. Proteolysis was used as a means of verifying that the protein as isolated by this method was amenable to normal cleavage procedures and that the hydrophobic probe was covalently interacting in a non-uniform manner with the anion exchange protein rather than being distributed over the entire structure. It is likely that the labelled hydrophobic aggregates contain covalently-bound rather than trapped probe because of the method of purifying the protein and the absence of much free radioactive label after the digestion procedures.

Cleavage of the membrane protein *in situ* by Pronase has indicated that the highly labelled peptides remain membrane-bound (Wells & Findlay, 1979a). Stripping the membrane after digestion *in situ* with trypsin and chymotrypsin leaves behind a 17 000–20 000-mol.wt. fragment of band 3 that is assumed to be thoroughly integrated into the lipid bilayer (Steck *et al.*, 1976). This large peptide contains the binding site for 1-isothiocyanobenzene-4-sulphonic acid (Drickamer, 1976) and also binds the hydrophobic label. It is likely therefore that these labelled peptides represent cleavage products containing parts of this region of the protein, but until labelling and purification studies are complete, the evidence remains circumstantial.

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