Labelling of the Intramembranous Region of the Major Sialoglycoprotein of Human Erythrocytes with a Photosensitive Hydrophobic Probe

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Human erythrocyte membranes were incubated with the photosensitive hydrophobic reagent 1-azido-4-iodo[³H]benzene and the mixture was irradiated. The major sialoglycoprotein was then isolated and the labelled polypeptide subjected to proteolytic dissection. Characterization of the purified tryptic and chymotryptic peptides show that the probe is covalently attached only to the transmembrane region of the protein. This labelling pattern is discussed in relation to the use of such reagents for the identification of segments of membrane proteins exposed to the hydrophobic milieu of the membrane.

Before any reliance can be placed on the ability of photosensitive hydrophobic probes to label regions of the polypeptide chain of membrane proteins exposed to the non-polar lipid environment it must be demonstrated with a well-understood system that such covalent attachment occurs and is limited to the intramembranous domains. Such a system exists with the major sialoglycoprotein (also called glycophorin and PAS-1) in the membrane of human erythrocytes. Its sequence has been published (Tomita & Marchesi, 1975) and the approximate disposition in the bilayer ascertained (Cotmore *et al.*, 1977). Continuing the previous studies from both here (Abu-Salah & Findlay, 1977; Wells & Findlay, 1979) and other laboratories (Klip & Gitler, 1974; Bercovici & Gitler, 1978; Gupta *et al.*, 1977; Bayley & Knowles, 1978*a,b*) on the efficacy of such probes, we report the use of the relatively simple reagent 1-azido-4-iodo[³H]benzene with the major sialoglycoprotein. The results indicate that compounds such as this have a future in unravelling the molecular anatomy of membrane proteins.

Experimental

Materials

Chymotrypsin and trypsin [Tos-Phe-CH₂Cl (TPCK)-treated] were purchased from Worthington Biochemical Corporation, Freehold, NJ, U.S.A. Sodium dodecyl sulphate and Tris (7–9 or Trizma grade) were obtained from Sigma, Poole, Dorset, U.K., and Sepharose 4B and Sephadex G150 were from Pharmacia, Uppsala, Sweden. All other chemicals came from British Drug Houses, Poole, Dorset, U.K., AnalaR grade being used when available.

Abbreviation used: SDS, sodium dodecyl sulphate.

Human packed cells (group A and Rh⁺) were supplied by the Regional Blood Transfusion Centre, Seacroft Hospital, Leeds, U.K.

Methods

Erythrocyte membranes. These were prepared and treated with 1-azido-4-iodo[³H]benzene as described in the preceding paper (Wells & Findlay, 1979). The labelled 'ghosts' (200 ml) were suspended in 5 vol. of lysing buffer (15 mM-Tris/H₂SO₄, pH 7.8) and then recovered by centrifugation (23 000*g* for 1 h). The pellet was resuspended in 10 vol. of 1 mM-EDTA at 30°C and the pH adjusted with 1 M-NaOH to 8.0. After 30 min the mixture was centrifuged (23 000*g* for 30 min) and the supernatant, which contained 'spectrin', discarded.

These spectrin-depleted membranes were then suspended to 200 ml in water and extracted with 1 litre of chloroform/methanol (2:1, v/v). The aqueous phase was retained, dialysed against water to remove methanol and freeze-dried. The fluffy white freeze-dried powder was dissolved in 100 ml of water and similarly extracted twice more with 5 vol. of chloroform/methanol (2:1, v/v). Low-molecular-weight water-soluble contaminants could be removed by gel filtration on Sephadex G150, the major sialoglycoprotein being eluted in the void volume.

Alternatively, to eliminate the possibility of 'trapped label', the treated membranes, still containing spectrin, were dissolved in 2% SDS/25 mM-Tris/H₂SO₄, pH 8.0, and subjected to gel filtration on Sepharose 4B as described in the preceding paper (Wells & Findlay, 1979). The main glycophorin-containing fractions, detected by the presence of sialic acid, were then pooled, care being taken to exclude lipid, dialysed against water (3 × 2 litres)

and subjected to the chloroform/methanol extraction procedure described above.

Enzymic digestions. Purified freeze-dried glycophorin was dissolved in 100 mM-NH₄HCO₃ (8–10 mg of protein/ml). Three portions of pepsinase, each containing 1% (w/w) of the amount of protein present (excluding carbohydrate), were added at 3 h intervals and the digests maintained at 35°C. After the final addition, the chymotryptic digestion was allowed to proceed for a further 3 h and the tryptic digestion was continued overnight. These digests were immediately applied to Sephadex G-150 in 100 mM-NH₄HCO₃ as detailed in the respective legends to Figures.

Descending chromatography. Peptides from the Sephadex G-150 columns were freeze-dried, dissolved in 5% pyridine and applied as a band to Whatman 3MM chromatography paper. Separation was effected by descending chromatography for 18 h, with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) as solvent. Peptides were detected with 0.25% ninhydrin in acetone or Fluorescamine (0.1 mg/ml) in 3% triethylamine in acetone.

Amino acid analysis and the determination of peptide concentration were carried out on the Rank-Hilger Chromaspek instrument. Protein was assayed by the manual ninhydrin method of Hirs (1967). *N*-Terminal residues were identified by the dansyl chloride method, used, where mentioned, in conjunction with the Edman degradation procedure (Gray, 1967).

Sialic acid. This was determined by the method of Warren (1959).

Organic phosphate. This was determined by the

method of Bartlett (1959), after prior liberation as inorganic phosphate by digestion with 72% perchloric acid.

Results

Purification of protein

On extraction of spectrin-depleted 'ghosts' with chloroform/methanol, the major sialoglycoprotein was retained in the aqueous phase. The aggregated but soluble protein could be separated from any low-molecular-weight water-soluble material on Sephadex G-150 (Fig. 1). The purified protein gave a single band on SDS/polyacrylamide-gel disc electrophoresis (Fig. 2). No contaminating bands were visible at a loading of 120 µg (50 µg of protein material), and a single *N*-terminal amino acid, leucine, was obtained. The yield from 200 ml of 'ghosts' (1 g of protein) was 70 mg (corresponding to 30 mg of protein material). The specific radioactivity of the major sialoglycoprotein was found to be in the region of 3×10^4 d.p.m./mg of peptide or 1.4×10^4 d.p.m./mg total weight, which includes the carbohydrate side chains (Tables 2 and 4).

It has been reported that glycophorin binds tightly to certain phospholipids, notably phosphatidylserine (Van Zoelen *et al.*, 1977). Some macrolipids carrying the A, B and O antigens are also thought to co-purify with the protein on account of their solubility in water (Dejter *et al.*, 1978). In order to ensure that no label associated with the major sialoglycoprotein is contributed by labelled lipid or unbound photolysis by-products, a control experiment was included. Membranes labelled with 1-azido-4-iodo[³H]benzene were solubilized in SDS

Fig. 1. Gel filtration of chloroform/methanol-extracted glycophorin on Sephadex G-150 in 0.1 M-NH₄HCO₃. The column was 2.8 cm × 112 cm and the fraction volume was 7.0 ml. The fractions containing glycophorin were pooled as indicated by the horizontal bar.

Fig. 2. SDS/polyacrylamide-gel disc electrophoresis of purified human glyophorin

(a) The gel is 7.5% in acrylamide and stained with Coomassie Brilliant Blue. The loading is $120\mu\text{g}$ (inclusive of carbohydrate). (b) Scans of Coomassie Blue (----) and periodic acid/Schiff reagent-stained gels (—).

and subjected to gel filtration on Sepharose 4B. The fraction containing the sialoglycoprotein, detected by the presence of sialic acid, was pooled to exclude

lipid running in the SDS mixed micelle. After extensive dialysis (3×2 litres of water) to lower the SDS content, the protein was extracted by the chloroform/methanol procedure and gave a single band upon SDS/polyacrylamide-gel disc electrophoresis. The initial solubilization with SDS assisted the removal of tightly bound phospholipid and non-covalently associated probe. The specific radioactivity of the protein so prepared was, however, no lower than that prepared without the SDS delipidation step. The phosphate content of the major sialoglycoprotein prepared by either method was $0.2\text{--}0.25\mu\text{g}/\text{mg}$ of protein. This value is similar to the basal level reported by Van Zoelen *et al.* (1977), who did not detect the presence of any fatty acids, suggesting that this phosphate did not arise from phospholipid.

Digestion with trypsin

Preliminary fractionation of the tryptic peptides was accomplished by gel filtration on Sephadex G-150 (Fig. 3). The profile could be conveniently divided into four pools, A–D. Pool A contains 88% of the recovered radioactivity and pool B 5%. The only other significant amount of radioactivity (7%) is retarded beyond the column volume and probably represents a small amount of unbound by-product of the probe. Glycopeptides, identified by the presence of sialic acid, were located in pool B, in the leading edge of pool C and in the higher-molecular-weight component of pool D. *N*-Terminal analysis showed pool A and pool B each to contain only one *N*-terminal residue, whereas pools C and D each contained three. Pools C and D were further purified by descending paper chromatography and eluted from the paper to yield pure peptides. *N*-Terminal and amino acid analyses of all these peptides allowed unequivocal identification of their position in the published sequence (Tomita & Marchesi, 1975). These analyses are given in Table 1, and the alignment of the peptides within the polypeptide chain is demonstrated in Fig. 4. Pool A contained high-molecular-weight aggregates of the hydrophobic peptide T4 (residues 62–97). Pool B contained the *N*-terminal glycopeptide T1 (1–31); the leading shoulder of this pool seems to be a partial cleavage product, T1+T2 (1–39). Pool C contained the glycopeptide T3 (40–61) on its leading edge, and the C-terminal peptides T6 (101–131) and T7 (102–131), which differ only by an *N*-terminal lysine. These three peptides were separated on paper with R_F values 0, 0.1 and 0.15 respectively. The higher-molecular-weight component of pool D was a glycopeptide, T2 (32–39), R_F 0.05 on paper chromatography, with T5 (98–100), R_F 0.40, and free lysine, R_F 0.17, as the components of the column volume peak.

The specific radioactivities of the tryptic peptides and the whole protein are compared in Table 2.

Fig. 3. Fractionation of tryptic peptides from human glycoporphin by gel filtration on Sephadex G-150 in 0.1 M-NH₄HCO₃. A tryptic digest of 110mg of human glycoporphin (total weight) was applied directly to the column (2.8cm × 112cm) and 7ml fractions were collected. The fractions were pooled as indicated (A-D). ●, A₂₂₀; △, A₅₄₉, sialic acid (0.15ml samples); ▲, radioactivity (0.1 ml samples).

These specific radioactivities are expressed in terms of d.p.m./mg of protein and d.p.m./mg total weight, inclusive of carbohydrate. The values are consistent with the distribution of radioactivity throughout the Sephadex G-150 profile. The hydrophobic peptide has a specific radioactivity 60 times greater than that of the *N*-terminal peptide (T1). The distribution of radioactivity throughout the length of the polypeptide chain is demonstrated in Fig. 4, the label being quite dramatically localized within the hydrophobic transmembrane peptide T4.

Cleavage with chymotrypsin

The distribution of radioactivity obtained from the tryptic digest was confirmed by the labelling pattern of the chymotryptic peptides. Initial separation was again achieved by gel filtration with Sephadex G-150 (Fig. 5). The vast majority of the radioactivity was

Fig. 4. Distribution of the ³H label among the tryptic peptides

In (a) the unbroken line represents the specific radioactivity expressed as d.p.m./mg (total weight) and the broken line shows d.p.m./mg (peptide weight). The alignment of the tryptic peptides is given in (b). CHO, Carbohydrate residues present.

Table 1. *Composition of tryptic peptides from human glyophorin*

Numbers in parentheses are those expected from the sequence given by Tomita & Marchesi (1975). Values less than 0.1 of a residue have been omitted. No corrections were made for destruction of certain amino acids during hydrolysis. AcNeu, *N*-acetylneuraminic acid.

Peptide ...	T1	T2	T3	T4	T5	T6	T7
Asp	1.7 (2)	1.0 (1)	— (0)	0.7 (0)	— (0)	5.2 (5)	5.1 (5)
Thr	6.5 (7)	1.9 (2)	2.2 (2)	1.8 (2)	— (0)	1.8 (2)	1.9 (2)
Ser	6.8 (8)	— (0)	1.9 (2)	2.2 (2)	— (0)	5.3 (6)	5.9 (6)
Glu	2.2 (1/2)	0.2 (0)	5.9 (6)	2.9 (3)	— (0)	3.7 (4)	4.2 (4)
Pro	1.0 (0)	0.6 (1)	2.3 (2)	1.5 (0/1)	— (0)	6.5 (6)	5.0 (6)
Gly	0.7 (0/1)	0.2 (0)	1.0 (1)	3.6 (4/5)	— (0)	— (0)	0.7 (0)
Ala	1.9 (1)	2.3 (2)	1.3 (1)	2.5 (2/3)	— (0)	— (0)	1.3 (0)
Val	2.4 (2)	— (0)	3.1 (3)	3.7 (3/4)	— (0)	3.0 (3)	2.8 (3)
Met	0.8 (1)	— (0)	— (0)	0.8 (1)	— (0)	— (0)	— (0)
Ile	1.6 (1)	— (0)	1.3 (1)	5.7 (6/7)	1.0 (1)	0.8 (1)	1.0 (1)
Leu	1.6 (1)	— (0)	0.3 (0)	4.7 (4)	0.6 (1)	2.0 (2)	2.3 (2)
Tyr	1.2 (1)	0.7 (1)	0.9 (1)	1.1 (1)	— (0)	— (0)	— (0)
Phe	— (0)	— (0)	— (0)	2.0 (2)	— (0)	— (0)	— (0)
His*	2.2 (2)	0.2 (0)	1.0 (1)	1.5 (1/2)	— (0)	0.4 (0)	— (0)
Lys	1.8 (2)	0.2 (0)	— (0)	0.8 (0)	1.0 (1)	2.1 (2)	0.7 (1)
Arg	0.8 (1)	1.1 (1)	2.2 (2)	1.8 (2)	— (0)	— (0)	— (0)
<i>N</i> -Terminal	Leu	Asp	Ala	Val	Leu	Lys	Ser
AcNeu	+	+	+	Low	—	—	—
Residues	1–31	32–39	40–61	62–97	98–100	101–131	102–131

* Residual amino sugars also chromatograph in this region.

Table 2. *Specific radioactivities of the tryptic peptides derived from human glyophorin (PAS-1) labelled with 1-azido-4-iodo-[³H]benzene*

Specific radioactivities are expressed in terms of d.p.m./mg (peptide), which excludes carbohydrate, and d.p.m./mg (total), which accounts for the carbohydrate in calculating the total weight of the peptide. Values for the carbohydrate of individual peptides were taken from Tomita & Marchesi (1975). CHO, Carbohydrate residues present.

Peptide	Residues	³ H radioactivity		
		(d.p.m./nmol)	[d.p.m./mg (peptide)]	[d.p.m./mg (total)]
PAS-1	1–131 (CHO)	416	30458	14724
T1	1–31 (CHO)	20.6	5777	1466
T2	32–39 (CHO)	0.6	671	376
T3	40–61 (CHO)	1.9	733	323
T4	62–97	355.9	87115	87115
T5	98–100	1.3	4000	4000
T6	101–131	1.1	335	335
T7	102–131	1.4	421	421

again present in the void volume peak. *N*-Terminal analysis of this pool gave three main residues: alanine, as would be expected from the sequence of the protein, together with roughly similar amounts of serine and glycine. This result is consistent with complete cleavage by chymotrypsin at leucine residue 65, together with partial cleavages at the phenylalanine residues 68 and 78 (Fig. 6). As corroborative evidence, the amino acid composition of the material indicated a decrease in the amounts of certain amino acids (most noticeably glycine, isoleucine, phenylalanine and histidine) when com-

pared with the theoretical composition of the whole transmembrane peptide from residues 65–93 (Tomita & Marchesi, 1975). Up to 8% of the total amount of sialic acid present in the digest is also found in this peak, which, if as seems likely represents undigested protein, would account for much of the arginine and lysine present in the amino acid analysis.

The transmembrane segment derived from digestion with chymotrypsin is therefore on average shorter than the hydrophobic tryptic peptide (T4), having the first three and in some cases also the first seven and 17 residues missing, as well as being shorter

Fig. 5. Fractionation of chymotryptic peptides from human glycoporphin by gel filtration on Sephadex G-150 in 0.1 M- NH_4HCO_3

A chymotryptic digest of 22 mg of human glycoporphin (total weight) was applied directly to the column (1.8 cm $\times$ 92 cm) and 3 ml fractions were collected. $\bullet$, A_{220} ; Δ , A_{549} ; sialic acid (0.25 ml portions); $\blacktriangle$, radioactivity (0.15 ml portions).

Fig. 6. Sequence of the hydrophobic region of glycoporphin A (after Tomita & Marchesi, 1975)
Cleavage points are denoted by arrows: T, trypsin; C, chymotrypsin.

at the C-terminal end. As a result the total amount of radioactivity associated with this peptide is a little less than with peptide T4 (325 d.p.m./nmol compared with 356 d.p.m./nmol). The specific radioactivity, on the other hand, in terms of $^3\text{H}/\text{mg}$ is nearly 50% higher than for the peptide derived from digestion with trypsin, indicating perhaps that the entry point into the membrane is some way in from position 62. Indeed, manual sequence analysis of this latter peptide gave the first five residues as Val-Glx-Leu-Ala-His, with at most 0.26% of the radioactivity being released into the butyl acetate phase along with the amino acid derivative in any one round.

Rather than attempting to isolate all the rest of the chymotryptic peptides, those representative of the N- and C-terminal domains of the protein were selected for further identification and analysis. Fraction 40 (Fig. 5) contains a high-molecular-weight sialoglycopeptide, which, from its N-terminal leucine and amino acid composition, represents the whole N-terminal region C1, residues 1-64 (Table 3). As with the respective tryptic peptide, its specific radioactivity is very low (Table 4). The region 34-52 (C2) is present in fraction 54. The pool of fractions 60-65 (C4) gave N-terminal lysine but no sialic acid. When chromatographed on Sephadex G-75, one major peak was obtained. From its amino acid

Table 3. *Composition of selected chymotryptic peptides from human glycophorin*

Numbers in parentheses are those expected from the sequence given by Tomita & Marchesi (1975). No correction was made for destruction during acid hydrolysis. AcNeu, *N*-acetylneuraminic acid.

Peptide	... C1	C2	C3	C4
Asp	2.8 (3)	0.4 (0)	0.4 (0)	3.2
Thr	8.8 (11)	1.9 (2)	1.9 (2)	2.6
Ser	7.8 (10)	1.8 (2)	1.6 (2)	2.7
Glu	8.3 (8/9)	2.5 (2)	1.7 (2)	2.8
Pro	2.6 (3)	1.2 (1)	0.6 (0/1)	2.8
Gly	2.6 (1/2)	1.0 (1)	1.8 (3/4)	0.9
Ala	4.4 (4)	2.8 (3)	2.1 (2/3)	—
Val	5.8 (6)	2.5 (3)	2.0 (2/3)	2.5
Met	0.9 (1)	— (0)	0.7 (1)	—
Ile	2.7 (2)	0.7 (1)	2.8 (5/6)	1.0
Leu	2.8 (2)	0.6 (0)	2.4 (3)	3.0
Tyr	3.5 (3)	0.4 (1)	0.4 (1)	—
Phe	0.7 (0)	— (0)	0.8 (2)	—
His*	4.0 (3)	0.9 (1)	0.8 (1/2)	0.6
Lys	2.0 (2)	0.5 (0)	0.9 (0)	3.3
Arg	3.3 (4)	2.2 (2)	1.0 (0)	—
<i>N</i> -Terminal	Leu	Ala	Ala	Lys
AcNeu	+	+	Low	—
Residues	1–64	34–52	65–93	C-Terminal

* Contains residual amounts of amino sugars.

Table 4. *Specific radioactivities of selected chymotryptic peptides from human glycophorin (PAS-1) labelled with 1-azido-4-iodo[³H]benzene*

Specific radioactivities are expressed as in Table 2.

Peptide	Residues	³ H radioactivity		
		(d.p.m./nmol)	[d.p.m./mg (peptide)]	[d.p.m./mg (total)]
PAS-1	1–131 (CHO)	410.6	30070	14536
C1	1–61 (CHO)	31.1	4390	1250
C2	34–52 (CHO)	10.3	4300	1520
C3	65–98	325.0	133820	133820
C4	C-Terminal	—	820	820

composition this peptide is derived from the C-terminal domain of the major sialoglycoprotein. Again its specific radioactivity was very low (Table 4). Slightly more radioactivity appeared in the column volume fractions than was the case with the tryptic digest. This may be explained by the small peptides removed from the *N*-terminal region of the transmembrane peptide as a result of incomplete digestion with chymotrypsin.

Discussion

In the preceding paper, we investigated the potential of 1-azido-4-iodo[³H]benzene as a hydrophobic probe. The logical extension of this evaluation

was to study the distribution of the covalently attached derivatives in a well-characterized membrane protein. As indicated by both the tryptic and chymotryptic digests of the major sialoglycoprotein from human erythrocyte membranes, the reagent is seen to be highly localized in that region of the polypeptide previously inferred to traverse the lipid bilayer (Tomita & Marchesi, 1975). Three pieces of evidence lead us to believe that the association of the probe with the transmembrane peptide is covalent.

First, control experiments in which pre-photolysed 1-azido-4-iodo[³H]benzene was introduced into the membrane, and the protein subsequently isolated, indicated a specific radioactivity less than 2% of that obtained when membrane and reagent were first

equilibrated and then irradiated. Secondly, dissolution of labelled membrane in SDS, followed by gel filtration on Sepharose 4B and subsequent isolation of glycophorin with chloroform/methanol, yielded material of the same specific radioactivity as that obtained without the intervention of the SDS/filtration step. It is assumed that disaggregation of the native protein by SDS would have allowed the liberation of any unbound reagent, resulting in a markedly lower specific radioactivity. Thirdly, on coupling the radioactive transmembrane peptide to insoluble glass supports in the presence of 1% SDS, no excess label was eluted from the support, indicating concurrent immobilization of label on attachment of the peptide to the glass. For much the same reasons, taken together with the phosphate determinations, it is also unlikely that any significant amount of labelled lipid is present.

On the basis of the observation that reduced glutathione significantly decreased the labelling of artificial egg lecithin vesicles by phenylnitrene, Bayley & Knowles (1978a) have suggested that all hydrophobic nitrenes, produced by irradiation, will diffuse and attach to hydrophilic residues located at the aqueous surface of the bilayer. If so, then residues such as His-66 (67) and Arg-96 (97) should contain the bulk of the radioactivity found in the transmembrane peptide. Manual sequencing indicates, however, that at most 0.3% of the radioactivity in the peptide is released in conjunction with the removal of the histidine residues located at positions 66 and 67 of the protein. Further evidence against labelling at these two hydrophilic amino acids also comes from the version of the transmembrane peptide derived by digestion with chymotrypsin. In this instance, the highly labelled peptide contains significantly smaller amounts of histidine and arginine but no concomitant loss of radioactivity. Only further information on the sequence of the peptide will identify the exact sites of attachment of the probe. The evidence, so far, however, points strongly to the feasibility of using photo-sensitive hydrophobic reagents such as 1-azido-4-iodo[³H]benzene as markers of exposed intramembranous domains.

If the tight specificity for exposed non-polar regions seen with the major sialoglycoprotein is exhibited by other membrane-associated proteins, then the lower amount of radioactivity associated with the high-molecular-weight components of spectrin, even after SDS treatment (Wells & Findlay, 1979), may be due to the labelling of small regions of the molecule that penetrate the hydrophobic domains of the bilayer. Evidence for such interaction with lipid has previously been reported by Momers *et al.* (1977).

One final point concerns the identity of the major

sialoglycoprotein isolated in these studies. Furthmayr *et al.* (1975) reported the presence of a similar protein, glycophorin B, which accounted for 25% of their total major sialoglycoprotein fraction and which differed somewhat in its primary structure. In our preparation, no unidentified proteins or peptides were obtained in quantities that would indicate the presence of such a proportion of glycophorin B. Similar observations have been reported by Blumenfeld & Adamany (1978). The blood used in these experiments came from several donors and was not characterized for M and N blood types. However, only leucine was found as the N-terminal residue of the purified protein, suggesting the absence of the M-type version of glycophorin A.

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