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BINDING OF LITHIUM DIIODOSALICYLATE TO GLYCOPHORIN

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Summary

Glycophorin was purified from human erythrocyte ghosts by the lithium diiodosalicylate -phenol procedure utilizing ^{125}I-labeled lithium diiodosalicylate. The glycophorin preparation was found to contain 8.9 ± 2.1 mol lithium diiodosalicylate per mol glycophorin. This bound lithium diiodosalicylate cannot be removed by extensive washings with a variety of polar organic solvents nor by treatment with the detergent, sodium deoxycholate. Further, the hydrophobic peptide produced from glycophorin by trypsin digestion contained 3.4 mol lithium diiodosalicylate per mol peptide.

Perhaps the best-characterized integral membrane protein is the major sialoglycoprotein, glycophorin (MN-glycoprotein), from human erythrocyte membranes [1—6]. Because of its defined molecular topography relative to the erythrocyte membrane, glycophorin is a popular protein for membrane reconstitution studies [7—10]. The glycophorin used in most studies was isolated by the lithium diiodosalicylate (LIS)-phenol procedure described originally by Marchesi and Andrews [11]. In the present communication we report that glycophorin preparations made by the LIS-phenol procedure contain almost 10 mol of LIS per mol of the sialoglycoprotein.

Preparation of ^{125}I-diiodosalicylic acid. ^{125}I-labeled 3,5-diiodosalicylic acid (DIS) was prepared by a slight modification of the chloramine-T iodination procedure [12]. The reaction mixture contained 1 mCi Na^{125}I in 34 μl 0.02 M NaOH, 40 μl 0.5 M phosphate buffer, pH 7.4, 0.5 nmol salicylic acid, and 3.5 μg chloramine-T. After the addition of chloramine-T, the mixture was stirred vigorously for 3 min. At that time 100 nmol NaI and another 3.5

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μg chloramine-T, each in 5 μl of the phosphate buffer, were added and the reaction mixture was again stirred vigorously for 3 min. This step insured that no monoiodosalicylic acid remained. To quench the iodination reaction, 50 μg sodium metabisulfite in 100 μl of the phosphate buffer was added with stirring followed by the addition of 1 ml of the phosphate buffer. The reaction mixture was applied to an Amberlite XAD-2 (Rohm and Haas) column and washed with 0.05 M phosphate buffer. 1-ml fractions were collected until the DIS was well washed. The column was then eluted with methanol to recover the DIS. After 70 fractions of 1 ml had been eluted, 1 mg unlabeled DIS in 1 ml MeOH was passed over the column to elute any ^{125}I-labeled DIS remaining.

Determination of purity of ^{125}I-diiodosalicylic acid. Aliquots from three fractions at different locations in the elution profile of [^{125}I] DIS were subjected to thin layer chromatography after being mixed with much larger amounts of unlabeled DIS. 500—600 μg DIS in 10 μl methanol was mixed with less than 1 μl of fractions corresponding to volumes of 41, 48 and 59 ml and spotted on 5 × 20 cm thin-layer plates. All plates were developed for 1 h in chloroform/cyclohexane/acetic acid, (5:4:1, v/v). After drying at room temperature, each plate was sprayed with 0.05% $KMnO_4$ in H_2O to locate the DIS. Upon location of the spot, sections corresponding to the width of the spot (0.75 cm) by 0.5 cm were scraped from the plate into gamma tubes and counted for 30 min in a Searle 1197 gamma counter.

Preparation of lithium ^{125}I-diiodosalicylate (LIS). Unlabeled DIS was twice recrystallized from hot methanol. The lithium salt was produced by the addition of an excess of lithium hydroxide and the resulting LIS was recrystallized from hot water. Methanol was evaporated from the required [^{125}I]-DIS in a large beaker before dry LIS was added. The appropriate amount of 25 mM Tris·HCl was added to bring the LIS concentration to 0.3 M. Gamma counting of aliquots verified that all the ^{125}I-labeled material was in solution.

Preparation of glycophorin and hydrophobic peptide (T(is)). Membrane disruption and glycophorin isolation were carried out by a slight modification of the LIS-phenol procedure described originally by Marchesi and Andrews [11]; the passage of glycophorin over a phosphocellulose column, originally designed to remove contaminating peptides, is routinely omitted by several laboratories, including our own, as an ineffective step. At each stage where the glycophorin was lyophilized, it was weighed and the amount of [^{125}I] LIS associated was determined by counting the lyophilized protein on a Searle 1197 gamma counter. A half-life of 60 days was used to calculate the decay constant for the [^{125}I] LIS. In order to calculate a ratio of LIS to glycophorin, it was assumed that all of the lyophilized dry weight at each step was pure glycophorin; this assumption is actually true only for the glycophorin preparation after the final ethanol wash. The glycophorin and T(is) preparations were subjected to sodium dodecyl sulfate electrophoresis in 5% and 12.5% polyacrylamide gels, respectively, and no differences from previous preparations [4] were noted when the gels were stained for protein and carbohydrate [13]. T(is) was prepared by trypsin-digestion of the [^{125}I] LIS-containing glycophorin [3]. T(is) concentrations were determined by amino acid analysis on a Beckman 121-M amino acid analyzer with computing integrator.

Amberlite easily separated free ^{125}I from [^{125}I] DIS with the latter being eluted by methanol as shown in Fig. 1. 15% of the total radioactivity eluted from the column is associated with the DIS peak.

Thin-layer chromatography (Fig. 2) showed conclusively that the material eluting from the Amberlite XAD-2 column in methanol was [^{125}I] DIS. This radiolabeled material co-migrated with unlabeled DIS (Eastman Organic Chemicals) in a chloroform/cyclohexane/acetic acid buffer system in a position quite distinct from that of salicylic acid.

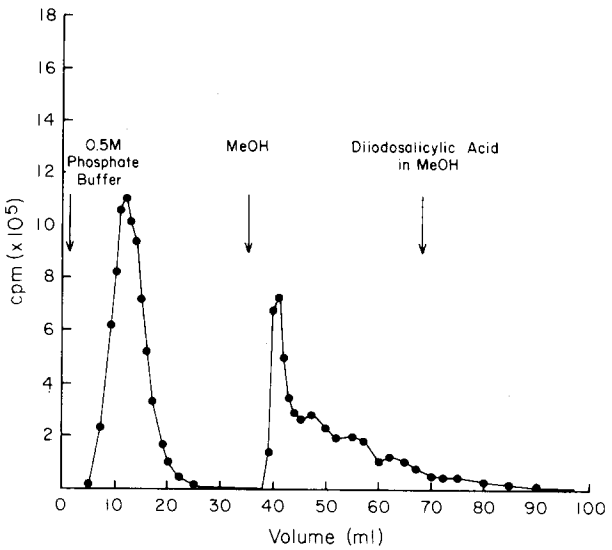

Fig. 1. Elution of [^{125}I] diiodosalicylic acid from an Amberlite XAD-2 column with methanol. 5-μl portions of each fraction were counted in a Searle 1197 gamma counter. Free ^{125}I eluted in the first 20 ml while [^{125}I] diiosalicylic acid eluted between 40 and 80 ml. 15% of the total radioactivity was associated with the diiodosalicylic acid.

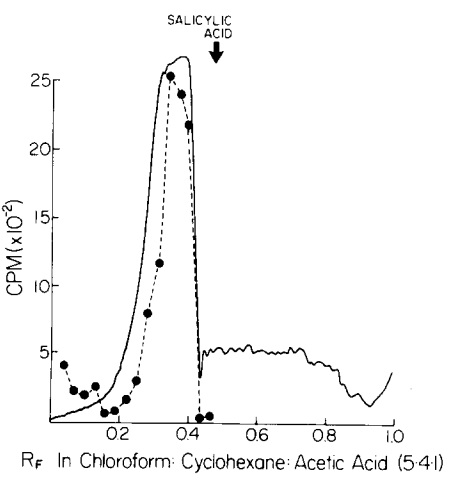

Fig. 2. Thin-layer chromatography of diiodosalicylic acid containing a trace amount of [^{125}I] diiodosalicylic acid. Chromatography was carried out as described in the text. ——, Densitometer scan of thin-layer chromatography plate showing position of DIS; •----•, cpm (X 10^{-2}) of thin-layer chromatography plate scrapings. The position of salicylic acid is indicated by the arrow.

The specific activity of [^{125}I]LIS was calculated on the basis of the radioactivity and amount of LIS used to disrupt the red cell membranes. This specific activity, combined with the lyophilyzed dry weight of glycophorin, was used to calculate the LIS/glycophorin ratios at the various stages of purification, as shown in Table I. This specific activity, combined with the T(is) concentration calculated by amino acid analysis, was used to calculate the LIS/T(is) ratio in Table I.

The phenol-water partitioning of the LIS membrane extraction removes a large fraction of the LIS, presumably by preferential partitioning of LIS into phenol compared to water. There is a further decrease in the amount of bound LIS following extensive ethanol washings. However, an amount of bound LIS (8.9 ± 2.1 mol LIS per mol glycophorin) is soon achieved which cannot be appreciably diminished by further ethanol washings. Washings with methanol, acidic methanol, and acidic chloroform/methanol (2:1, v/v) also fail to appreciably decrease the level of LIS bound to the glycoprotein (Table I). Dissolution of the glycoprotein into 1 ml of a 0.75% solution of sodium deoxycholate and removal of the detergent by gel filtration on a Sephadex G-50 column also failed to appreciably affect the level of bound LIS (Table I).

The hydrophobic peptide from glycophorin, T(is), also contained significant amounts of LIS (3.4 mol LIS/mol T(is)) although less than that bound to the intact glycophorin. Dry weight analysis of the T(is) preparation suggests that 63% of the dry weight is accounted for by the T(is) peptide and 22% is accounted for by LIS, leaving approximately 14% unaccounted for. The unidentified portion of the dry weight is probably a mixture of lipids (in our hands predominantly glycolipids (unpublished observations)) and pigmented compounds such as heme or heme-like substances. The presence of heme or some other pigmented substance is suggested by the brownish coloration of

TABLE I

BINDING OF LIS TO GLYCOPHORIN DURING PREPARATION

Following the phenol-water partitioning step, the glycophorin preparation was divided into four fractions and each fraction subjected to one of four washing procedures. For each washing procedure, the glycophorin was suspended in washing media at approximately 1 mg/ml and stirred for 1 h at 40°C. The suspension was then centrifuged for 30 min at 6500 × *g* and 4°C. The pellet was resuspended in washing media and the procedure repeated for a total of three washes. Approximately half of the glycophorin preparation subjected to ethanol wash was dissolved in 1 ml of an 0.75% solution of sodium deoxycholate and the sample applied to an 0.9 × 40 cm column of Sephadex G-50 equilibrated with distilled water. Under these conditions free and micellar sodium deoxycholate is essentially completely separated from glycophorin (Segrest, Wilkinson and Sheng, manuscript submitted for publication). The glycophorin peak, measured by absorbance at 280 nm, was recovered and LIS retention determined.

Stage of purification	LIS/glycophorin (molar ratio) ratio
LIS disruption of membranes	2390
Phenol-water partition	64
Phenol-water partition + dialysis	44
Ethanol wash	8.9 ± 2.1
Methanol wash	14.2
Acidified methanol wash	9.5
Acidified chloroform-methanol wash (2:1, v/v)	6.0
Sodium deoxycholate-Gel filtration	8.0
T(is) peptide*	3.4**

*Made from ethanol-washed material.
**Mol LIS/mol T(is) peptide.

both the glycophorin and the T(is) produced by the LIS-phenol procedure. This brownish coloration can be reduced by more extensive washing of the erythrocyte membrane ghosts prior to glycophorin extraction but can never be entirely eliminated if LIS-phenol is used as the method of glycophorin isolation.

We conclude that glycophorin purified by the LIS-phenol procedure contains small but significant quantities of LIS. However, we, as well as certain other laboratories, omit the phosphocellulose step as originally described [11] While we feel it unlikely that a negatively charged phosphocellulose column would remove negatively charged diiodosalicyte ions bound to glycophorin, we cannot rule out this possibility or the possibility that some other ion exchange method will free glycophorin of contaminating LIS.

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