SOLUBILIZATION OF HEPATIC BINDING SITES FOR ASIALO-GLYCOPROTEINS

Anatol G. Morell and I. Herbert Scheinberg

Division of Genetic Medicine, Department of Medicine,
Albert Einstein College of Medicine, Bronx, New York 10461

Received July 6,1972

SUMMARY

A lyophilized preparation of a particulate fraction of rabbit liver homogenate exhibits specific binding affinity for a desialylated glycoprotein comparable to that found in rat liver plasma membranes. Treatment of this preparation with Triton X-100 solubilizes the receptor sites which are capable of retaining their specific binding activity in solution.

INTRODUCTION

Several desialylated glycoproteins, when injected intravenously into rats and rabbits, are promptly transferred from the circulation into hepatic parenchymal cells where they are subsequently catabolized (1-7). Pricer and Ashwell have shown that several asialo-glycoproteins are specifically bound in vitro to plasma membranes of rat liver (8), and Van Lenten and Ashwell have studied these membranes with respect to the kinetics of binding and the specificity of proteins bound (9). The formation of a complex between these desialylated proteins and the plasma membrane of hepatocytes may be an essential intermediate stage in the transport of these proteins from plasma into the cell. We now describe procedures which, applied to the particulate fraction of rabbit liver, solubilize these binding sites and show that the specific association of desialylated glycoprotein and receptor can occur with the latter in solution.

MATERIALS AND METHODS

Male albino rabbits, weighing 2 to 2.5 kg, were killed by injecting 150 mg of Na pentobarbital into the marginal ear vein.

The liver was removed, washed, blotted dry, minced, and 2 - 3 g portions were homogenized at 4°C in 50 ml of 1.0 mM Na bicarbonate buffer, pH 7.5, containing 0.5 mM $CaCl_2$. Homogenization was effected by 5 strokes with a loose, and 10 strokes with a tight, pestle in a Dounce homogenizer. In a modification of the procedure which Ray (10) used to prepare rat liver membranes, the total homogenate from 10 g of liver was diluted to 1 liter with the bicarbonate - $CaCl_2$ buffer, strained through 8 layers of cheesecloth, and centrifuged at 1800 g for 30 min. The pellets of precipitate were twice resuspended in 1 liter of the buffer and recentrifuged at 1200 g for 15 min, suspended in 10 ml of the buffer and lyophilized. The dry material, amounting to 13 - 15 mg per g of fresh liver, was stored in a desiccator at 4°C. Its ability to bind desialylated glycoproteins was stable for several months and represented 75 - 85% of that found in the whole liver homogenate.

Orosomucoid (α_1 - acid glycoprotein) was isolated from pooled human serum by the procedure of Whitehead and Sammons (11) Asialo-orosomucoid was prepared by incubating a 1.0% solution of orosomucoid at 37°C in 0.1 M Na acetate buffer, pH 5.6, for 4 hr with 0.1 unit per ml of Clostridium perfringens neuraminidase (Worthington). Protein determinations, using bovine serum albumin as the standard, were made according to Lowry et al (12).

Proteins were labelled with carrier-free iodine-125 (New England Nuclear) by a Chloramine T method (13). The resulting specific activities ranged from 40 to 60 µCi per µg of protein, permitting dilution with the unlabelled proteins for most experiments. The content of iodine-125 was assayed in a Nuclear-Chicago well-type scintillation detector (DS-202) with an analyzer scaler (8725).

Crystalline trypsin was obtained from Behringer; soya bean trypsin inhibitor from Worthington; Triton X-100 from Rohm and Haas.

The following buffers were used as described in the text and legends: buffer A (0.05 M Tris-HCl, pH 7.5, containing

TABLE I

Binding of desialylated human orosomucoid by the particulate fraction of rabbit liver homogenate.

Labelled human glycoprotein*	Treatment of particulate fraction after suspension	Glycoprotein bound (ng)
^{125}I-asialo-orosomucoid	None	24.2
	Acidified (pH 5.6)	0.2
	EDTA (20 mM)	0.1
	Digested with neuraminidase**	1.7
^{125}I-orosomucoid	None	0.4

* Asialo-orosomucoid, 12,100 CPM per ng; orosomucoid, 5,100 CPM per ng.

** Tubes incubated for 2 hr at 37°C with 0.005 units of Clostridium perfringens neuraminidase prior to the addition of ^{125}I-asialo-orosomucoid.

1000 ng of ^{125}I-glycoprotein and 200 µg of lyophilized particulate fraction of liver, suspended in 0.5 ml of buffer A, were incubated, in triplicate, at 37°C in 4 ml glass tubes for 1 hr with constant shaking. The tubes were chilled in ice and, following the addition of 2.0 ml of the cold buffer, centrifuged at 2000 g for 5 min. The pellets were washed twice with the same buffer and the glycoprotein bound was calculated from their radioactivity.

0.10 M NaCl, 0.01 M $CaCl_2$ and 1.0 mg/ml of bovine albumin) and buffer B (0.025 M Tris-HCl, pH 7.5, containing 1.0 mM $CaCl_2$).

RESULTS AND DISCUSSION

Binding properties of the receptors.

The binding properties of the crude receptors from the particulate fraction of rabbit liver homogenate are similar to those previously described for the rat liver plasma membranes (8,9), as illustrated in Table I. As is true of rat liver plasma membranes, binding by the particulate fraction derived from rabbit liver is specific for the asialo-derivative of orosomucoid and does not occur in the presence of an excess of EDTA,

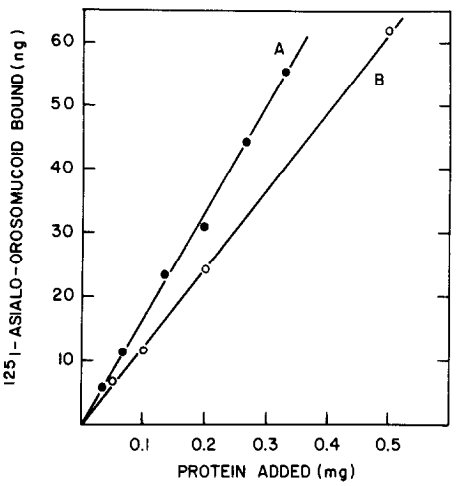

Fig. 1.

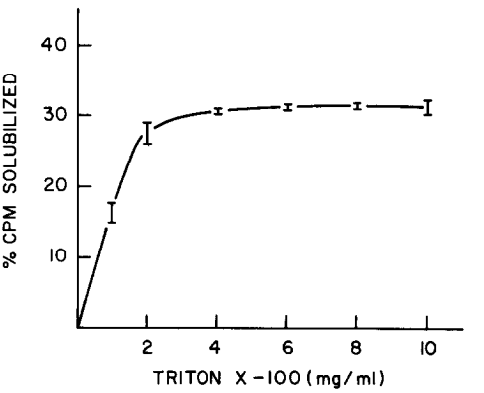

Fig. 2.

Fig 1 Binding of ^{125}I-asialo-orosomucoid as a function of receptor protein added. 1000 ng of ^{125}I-asialo-orosomucoid were incubated, in duplicate, with varying amounts of:

A Particulate fraction of rabbit liver homogenate. Conditions for incubation and recovery of the bound radioactivity as in the legend to Table I;

B Solubilized particulate fraction of rabbit liver homogenate in 0.50 ml of buffer A containing 0.5% Triton X-100. After 5 min incubation at 37°C, the solution was chilled in ice, precipitated with 0.50 ml of ice-cold acetone and centrifuged at 2000 g for 5 min. The pellet was washed three times with buffer A. The asialo-orosomucoid bound was calculated from the radioactivity of the pellet.

Fig 2 Solubilization of the rabbit liver particulate fraction charged with ^{125}I-asialo-orosomucoid by Triton X-100. 60 mg of the lyophilized particulate fraction of rabbit liver homogenate, containing 33.8 mg of protein, were suspended in 8 ml of buffer A. Following incubation for 1 hr at 37°C with 100 µg of ^{125}I-asialo-orosomucoid (1.3×10^7 CPM), the suspension was centrifuged at 2000 g, washed twice with 10 ml of buffer B, and suspended in 33.8 ml of this buffer. Determinations of the resulting radioactivity indicated that 183 ng of ^{125}I-asialo-orosomucoid were bound per mg of protein.

Aliquots (1.0 ml) of the suspension above and varying amounts of Triton X-100 were incubated at room temperature for 15 min, centrifuged at 100,000 g for 20 min and the radioactivity determined on aliquots of the clear supernatant.

at a pH below 5.6, or after exposure of the fraction to neuraminidase. The amount of asialo-orosomucoid bound is proportional to

the amount of particulate receptor added (Fig 1, curve A). Binding of asialo-orosomucoid appears to be essentially irreversible: bound radioactive asialo-orosomucoid can not be displaced by the non-radioactive asialo-protein, nor can the latter, once bound, be displaced by the radioactive asialo-protein.

Solubilization of the receptor - asialo-orosomucoid complex.

When the particulate receptors, previously complexed with ^{125}I-asialo-orosomucoid, were treated with the non-ionic detergent, Triton X-100, a substantial portion of the bound radioactive protein became soluble (Fig 2). Partial tryptic digestion of the insoluble radioactive complex, prior to further treatment with Triton X-100, resulted in further solubilization of the labelled complex:

To the suspension described in the legend to Fig 2 Triton X-100 was added to a final concentration of 0.5% (w/v). After incubation at room temperature for 15 min and centrifugation at 100,000 g for 20 min, the clear supernatant contained about one-third of the radioactivity of the suspension (Solution 1).

The insoluble pellet, remaining after decanting Solution 1 from 10 ml of the suspension, was resuspended in 10 ml of buffer B and digested with 0.18 mg of crystalline trypsin for 2 min at 37^{o}C. The digestion was stopped by the addition of 0.36 mg of soya bean trypsin inhibitor. Following centrifugation at 2000 g, the pellet was washed, resuspended in 10 ml of the same buffer, and again extracted with Triton X-100. Virtually all of the radioactivity remaining after the initial extraction with Triton X-100 was in solution (Solution 2).

The addition of an equal volume of ice-cold acetone to either Solution 1 or 2 precipitated in excess of 80% of the solubilized radioactivity, which could not be redissolved by washing the pellet with buffer B. In contrast, washing with this buffer completely dissolved acetone-precipitated uncomplexed ^{125}I-asialo-orosomucoid.

Extraction of the acetone-precipitated complex with either 20 mM EDTA in 0.05 M Tris-HCl buffer, pH 7.5, or with 0.1 M Na

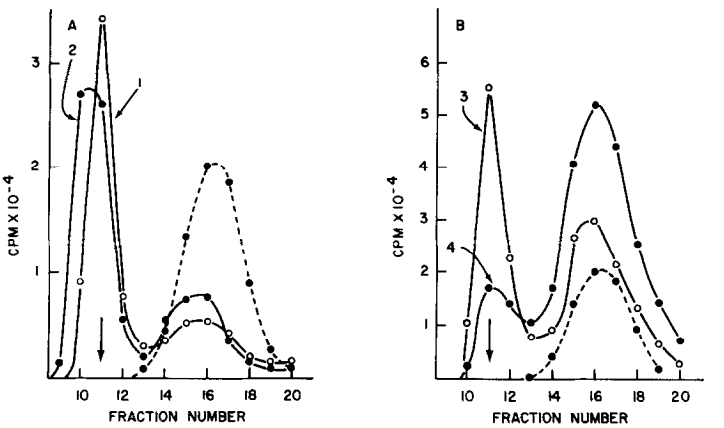

Fig 3 Chromatographic evidence for (A) solubilization of a receptor previously complexed with ^{125}I-asialo-orosomucoid and (B) binding of ^{125}I-asialo-orosomucoid by the solubilized receptor. Aliquots of Solutions 1 and 2 (Fig 3A) and 3 and 4 (Fig 3B) were placed on a 0.95 x 44 cm column of Sephadex G-100 equilibrated with buffer A containing 1 mg/ml of Triton X-100. 1.0 ml fractions were collected. The arrow indicates the void volume at which the peak of Blue Dextran 2000 (Pharmacia, M.W. approx. 2×10^6) appears. The dotted line represents elution of uncomplexed ^{125}I-asialo-orosomucoid.

acetate buffer, pH 5.6, removed over 95% of the radioactivity from the precipitate. Reincubation of the precipitate with ^{125}I-asialo-orosomucoid under the conditions described in the legend to Table I resulted in re-complexing labelled protein equivalent to 60% and 85% of the radioactivity released by the EDTA or the acetate buffer, respectively.

By gel filtration on Sephadex G-100, the ^{125}I-asialo-orosomucoid present in Solutions 1 and 2 was shown to be part of a high molecular weight complex. Fig 3A shows that the bulk of the radioactivity, of either solution, appeared in the void volume of the column, whereas unbound ^{125}I-asialo-orosomucoid (M.W. 40,000) was eluted only in later fractions.

Solubilization of the uncharged receptors and formation of the receptor - asialo-orosomucoid complex in solution.

The particulate fraction of rabbit liver homogenate, to

which no asialo-glycoprotein had been bound, could also be solubilized with Triton X-100 by the same procedure as that applied to the charged receptors. To two 10 ml aliquots of the resulting clear supernatant solution, each containing 38 mg of protein in buffer A containing 0.5% Triton X-100, 260 ng (16,500 CPM per ng) or 3280 ng (1,650 CPM per ng) of ^{125}I-asialo-orosomucoid were added, forming Solutions 3 and 4, respectively. Following incubation for 5 min at 37°C, an aliquot of each solution was chromatographed on a column of Sephadex G-100. Fig 3B shows that 45.8% of the radioactivity of Solution 3 and 13.7% of that in Solution 4 appeared in the void volume of the column, indicating it to be part of a high molecular weight complex.

The capacity of the solubilized, particulate fraction to bind ^{125}I-asialo-orosomucoid in solution was also demonstrable by adding acetone, to a final concentration of 50% (v/v), to Solutions 3 and 4: the buffer-washed precipitates contained 48.0 and 14.7%, respectively, of the original radioactivity.

For both particulate and solubilized receptors, the amount of desialylated protein bound is proportional to the amount of receptor present (Fig 1, curves A and B).

These results parallel recent work of Cuatrecasas (14,15) in which receptors for insulin present in liver and fat cell membranes have been solubilized by Triton X-100 with retention of their binding activity.

Solubilization of receptor sites may permit isolation and purification of hepatic binding sites for desialylated glycoproteins. This should facilitate a more detailed examination of the mechanism of binding and transport of these macromolecules across the plasma membranes of parenchymal liver cells.

ACKNOWLEDGMENTS

This work was supported by a grant from the United States Public Health Service AM-1059 from the National Institute of Arthritis and Metabolic Diseases.

We are indebted to Mrs. Julie Windsor for her excellent technical assistance.

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