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Binding characteristics of reduced hepatic receptors for acetylated low-density lipoprotein and maleylated bovine serum albumin

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The binding characteristics of reduced hepatic membrane proteins for acetylated low-density lipoprotein (acetyl-LDL) and maleylated bovine serum albumin (Mal-BSA) have been examined. Two receptor activities were extracted from hepatic membranes in the presence of octyl β-D-glucoside and β-mercaptoethanol, and were separated by chromatography on Mal-BSA–Sepaharose 4B. The receptors were revealed by ligand blotting. The active binding proteins had apparent molecular masses of 35 and 15 kDa in SDS/polyacrylamide gels. Equilibrium studies with protein–phosphatidylcholine complexes indicated that the reduced 35 kDa protein expresses two binding sites for Mal-BSA and one for acetyl-LDL, whereas the 15 kDa protein–phosphatidylcholine complex binds ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL with a 4:1 stoichiometry. ^{131}I-Mal-BSA binding was linear with both proteins, with a K_d of 4.8 nM at the 35 kDa protein and a K_d of 5.6 nM at the 15 kDa protein. The 35 kDa protein displayed saturable binding of ^{131}I-acetyl-LDL with a K_d of 5 nM; the 15 kDa binding protein bound ^{131}I-acetyl-LDL with a K_d of 2.3 nM. A 85 kDa protein was obtained by Mal-BSA–Sepharose chromatography when the hepatic membranes had been solubilized with Triton X-100 in presence of GSH/GSSG. This protein displayed saturable ^{131}I-Mal-BSA binding with a K_d of 30 nM and ^{131}I-acetyl-LDL binding with a K_d of 6.5 nM. The ^{131}I-Mal-BSA binding capacity was four times higher than that of ^{131}I-acetyl-LDL. Competition studies with the 35 kDa, 15 kDa and 85 kDa proteins binding Mal-BSA, acetyl-LDL, formylated albumin and polyanionic competitors provide evidence for the existence of more than one class of binding sites at the reduced binding proteins.

INTRODUCTION

The rat liver expresses a membrane-associated receptor that binds both acetylated low-density lipoprotein (acetyl-LDL) and maleylated bovine serum albumin (Mal-BSA). Saturation of the hepatic uptake of these ligands can be demonstrated by the intravenous administration of increasing amounts of ^{131}I-labelled acetyl-LDL or ^{131}I-labelled Mal-BSA. The hepatic clearance capacity for ^{131}I-Mal-BSA is at least 15 times higher than that for ^{131}I-acetyl-LDL, and only 20% of the ^{131}I-Mal-BSA uptake is inhibited by an excess of acetyl-LDL. Thus the liver shows characteristic differences in the uptakes of ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA. However, polyanions such as polyinosinic acid, poly(vinyl sulphate) (PVS) and fucoidan completely inhibit ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL uptake in the liver (Goldstein *et al.*, 1979; Dresel *et al.*, 1985, 1987; Eskild *et al.*, 1987).

The biological role of the hepatic uptake of acetyl-LDL and Mal-BSA is still unclear. Among the organs studied (e.g. spleen, liver, muscle, kidney, heart, brain), the liver has the highest uptake capacity for intravenously administered acetyl-LDL and Mal-BSA (Nagelkerke *et al.*, 1983; Dresel *et al.*, 1985; Pitas *et al.*, 1985). The sinusoidal endothelial cells appear to be the cells in the liver that take up the most acetyl-LDL (Nagelkerke *et al.*, 1983; Blomhoff *et al.*, 1984; Pitas *et al.*, 1985). It has been suggested that hepatic receptors might represent a defence mechanism against the accumulation of modified proteins in the blood (Nagelkerke *et al.*, 1983). Comparable receptors detected on macrophages have been termed 'scavenger receptors' (Brown & Goldstein, 1983).

Binding sites for acetyl-LDL and Mal-BSA can be solubilized from rat liver. Under non-reducing conditions, high-molecular mass binding sites for both ligands (≥250 kDa) have been identified by ligand blotting. This species of high-molecular-mass receptor was solubilized and purified 1450-fold, and shown to possess a common binding site for ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA, but with a 20-fold higher binding capacity for ^{131}I-Mal-BSA than for acetyl-LDL (Dresel *et al.*, 1985, 1987). Recently, a protein of similar size (220 kDa) was purified from bovine lung membranes by affinity chromatography and immunoaffinity chromatography and showing to be an acetyl-LDL binding protein which is also present on sinusoidal liver cells and induced THP-1 macrophagocytic cells (Kodama *et al.*, 1988).

Abbreviations used: acetyl-LDL, acetylated low-density lipoprotein; Mal-BSA, maleylated bovine serum albumin; f-BSA, formylated bovine serum albumin; PMSF, phenylmethanesulphonyl fluoride; OG, octyl glucoside (*n*-octyl β-D-glucopyranoside); PVS, poly(vinyl sulphate); TNE buffer, 50 mM-Tris/HCl (pH 8)/150 mM-NaCl/0.1 mM-EDTA.

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Our initial strategy to purify the 250 kDa non-reduced receptor by affinity chromatography failed, and we were unable to recover more than 5–10% of the binding capacity of solubilized rat liver membrane proteins after affinity chromatography under non-reducing conditions at the 250 kDa receptor (Dresel *et al.*, 1984). We have therefore attempted to improve our conditions for purifying the scavenger receptors, this time under reducing conditions. With the simultaneous disappearance of ligand binding at the 250 kDa receptors, the appearance of reduced receptors for acetyl-LDL and Mal-BSA (85 kDa and 35 kDa) was observed. The possibility that the 85 kDa and 35 kDa proteins might be closely related to the 250 kDa non-reduced scavenger receptors has been raised (Ottnad *et al.*, 1988). In the present paper, we report the detailed binding characteristics of separated and reconstituted 85 kDa, 35 kDa and 15 kDa reduced acetyl-LDL and Mal-BSA binding proteins.

MATERIALS AND METHODS

Materials

$Na^{131}I$ (10–20 mCi/μg) was obtained from Amersham International (Braunschweig, Germany). Egg phosphatidylcholine, Triton X-100, BSA, polyinosinic acid, polyadenylic acid, and polycytidylic acid were from Serva (Heidelberg, Germany); PVS, *n*-octyl β-D-glucopyranoside (OG) and molecular mass standards were from Sigma (Munich, Germany); cellulose acetate filters (diam. 25 nm; pore size 0.45 μm; catalogue no. HULP 025 00) were from Millipore (Bedford, MA, U.S.A.); nitrocellulose paper was from Schleicher & Schüll (Göttingen, Germany). Electrophoresis reagents (acrylamide, methylenebisacrylamide, tetramethylethylenediamine, ammonium persulphate) were from BRL (Offenbach, Germany) and Bio-Rad (Munich, Germany); SDS was from Pierce (Rockford, IL, U.S.A.); CNBr-activated Sepharose CL 4B was from Pharmacia (Uppsala, Sweden); and a TSK G4000 h.p.l.c. column (length 30 cm) was from LKB (Broma, Sweden). Centricon ultrafiltration tubes were obtained from Amicon (Lexington, MA, U.S.A.). Phenylmethanesulphonyl fluoride (PMSF) from Boehringer (Mannheim, Germany) and Trasylol (100000 kallikrein inactivating units/10 ml) from Bayer (Leverkusen, Germany) were used as proteinase inhibitors.

Acetylation of LDL

Human LDL was prepared from the plasma of normal healthy blood donors by sequential ultracentrifugation (Havel *et al.*, 1955). Acetyl-LDL was prepared by treatment of LDL with acetic anhydride (Basu *et al.*, 1976). The lipoprotein concentration was expressed in terms of protein content, using BSA as a standard (Lowry *et al.*, 1951).

Maleylation of BSA and formylation of BSA

BSA was maleylated with maleic anhydride (Imber *et al.*, 1982), and maleylation was measured by using the method of Fields (1972). In our experiments, 50 out of 59 lysine residues of the albumin molecule were maleylated. f-BSA was prepared from BSA by formaldehyde treatment by the method of Horiuchi *et al.* (1985).

Iodination of acetyl-LDL and Mal-BSA

Radio-iodination of Mal-BSA and acetyl-LDL was performed with $Na^{131}I$ as described by Sinn *et al.* (1988).

Mal-BSA–Sepharose

Mal-BSA was covalently attached to CNBr-activated Sepharose CL 4B according to the manufacturer's instructions. Using a ratio of 30 mg of Mal-BSA/g of dry gel, the coupling efficiency was 98%. A column of 1.2 cm diam. containing 50 mg of immobilized Mal-BSA was used. A 5 ml portion of the activated Sepharose CL 4B was washed with 250 ml of 50 mM-Tris/HCl, pH 8, containing 1 M-NaCl, 0.1 mM-EDTA and 40 mM-OG or 1% Triton X-100.

Solubilization of membrane proteins from rat liver and fractionation by affinity chromatography on Mal-BSA–Sepharose 4B

Procedure A. Sliced rat liver tissue (40 g) was disrupted in 200 ml of TNE buffer [50 mM Tris/HCl (pH 8)/150 mM-NaCl/0.1 mM-EDTA] containing 0.2% β-mercaptoethanol, 23 μg of PMSF/ml and 0.1% Trasylol. The homogenate was centrifuged at 200 ***g*** for 5 min at 20 °C. Membranes were pelleted from the supernatant by high-speed ultracentrifugation at 100000 ***g*** for 2 h. The membrane pellet was solubilized in 200 ml of TNE buffer containing 40 mM-OG and 0.2% β-mercaptoethanol, and recentrifuged. The solubilized membrane proteins were applied to a Mal-BSA–Sepharose 4B column (100 ml of the solubilized material/5 ml of column volume) equilibrated with 50 ml of buffer A (50 mM-Tris/Cl (pH 8)/0.1 mM-EDTA/40 mM-OG/0.2% β-mercaptoethanol) containing 50 mM-NaCl. The column was washed with 100 ml of buffer A and subsequently eluted with 30 ml of buffer A containing 250 mM-NaCl and then 30 ml of buffer A containing 1 M-NaCl (flow rate 20 ml/h). Single fractions of 1.4 ml were collected. The fractionated proteins were assayed for ^{131}I-Mal-BSA binding activity by filter assay and ligand blotting. The protein content of the samples was determined by the method of Lowry *et al.* (1951) as modified by Schneider *et al.* (1980) with BSA as standard.

Procedure B. Sliced rat liver (50 g) was disrupted in 250 ml of TNE buffer without β-mercaptoethanol but supplemented with 5 mM-GSSG and 5 mM-GSH. The homogenate was centrifuged at 200 ***g*** for 5 min at 20 °C. Membranes were pelleted from the supernatant by ultracentrifugation at 100000 ***g*** for 2 h. The membrane pellet was solubilized in TNE buffer containing 5 mM-GSSG and 5 mM-GSH (instead of β-mercaptoethanol) and 1% Triton X-100. The solubilized membrane proteins (250 ml) were diluted to 0.5% Triton X-100 and subjected to a Mal-BSA–Sepharose 4B column (125 ml of solubilisate/5 ml of column volume) equilibrated with 50 ml of buffer B [50 mM-Tris/HCl (pH 8)/50 mM-NaCl/0.1 mM-EDTA/5 mM-GSSG/5 mM-GSH/0.1% Triton X-100]. The affinity column was washed with 100 ml of buffer B without GSSG. The column was then eluted with 30 ml of buffer containing 50 mM-Tris/HCl (pH 8)/250 mM-NaCl/0.1 mM-EDTA/5 mM-GSH/0.1% Triton X-100. Single fractions of 1.4 ml were collected. The fractions were assayed for ^{131}I-Mal-BSA binding activity by filter assay and ligand blotting. The protein content of the samples was determined by the method of Schneider *et al.* (1980) with BSA as standard.

Gel-permeation chromatography

For gel-permation chromatography we used a TSK

G4000 h.p.l.c. preformed column (300 mm × 7.5 mm). Before the protein fractions with the highest ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL binding activities obtained by Mal-BSA affinity chromatography were subjected to the TSK G4000 column, they were concentrated by centrifugation at 10000 *g* in Centricon 30 ultrafiltration tubes and subsequently passed through 0.45 μm millipore filters. The TSK G4000 column was pre-equilibrated in 125 mM-Tris/HCl (pH 6.8)/0.1% SDS/0.1% β-mercaptoethanol. The sample size for the column chromatography was 200 μl. Chromatography was performed at a flow rate of 0.5 ml/min with a constant pressure of 800 kPa. Fractions of 330 μl were collected.

Gel electrophoresis and ligand blotting

Portions of 100 μl of purified membrane protein fractions containing ^{131}I-Mal-BSA binding activity were applied to Sephadex G-25 in small syringes pre-equilibrated in sample buffer. Columns were eluted by centrifugation (1000 *g* for 3 min). The eluates were subjected to SDS/polyacrylamide-slab-gel electrophoresis performed in the buffer system of Laemmli (1970). The resulting gels were equilibrated in transfer buffer containing 20 mM-Tris/HCl (pH 8.3), 150 mM-glycine and 20% (v/v) methanol. The proteins were electroblotted on to nitrocellulose membranes (12 h, 0.45 A) by using a Bio-Rad Transblot unit (Bio-Rad, Munich, Germany) at 4 °C. Non-specific binding was blocked by incubation with 5% BSA in 50 mM-Tris/HCl (pH 8)/90 mM-NaCl/2 mM-EDTA. The nitrocellulose strips were incubated for 30 min with 58.8 pmol of ^{131}I-Mal-BSA/ml, then washed for 2 h and autoradiographed on Kodak XR-5 films. Gels were calibrated with the following molecular mass standards: α_2-macroglobulin, 180 kDa; β-galactosidase, 116 kDa; fructose-6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarate hydratase, 48.5 kDa; lactate dehydrogenase, 36.5 kDa; triose-phosphate isomerase, 26.5 kDa.

Assay for binding of ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA to membrane proteins

Binding of ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA to the solubilized membrane proteins was determined by the membrane filter assay of Schneider *et al.* (1980). Aliquots of the eluted proteins were diluted to submicellar detergent concentration, complexed with egg phosphatidylcholine and precipitated by 33% acetone as described previously (Via *et al.*, 1985). After suspension in 10 mM-Tris/HCl, pH 8, the protein–liposome complexes were incubated with ^{131}I-Mal-BSA (42–90 c.p.m./fmol) or ^{131}I-acetyl-LDL (330–450 c.p.m./fmol) in the absence or presence of unlabelled inhibitors. Unbound radioligands were separated from bound radioligands on cellulose acetate filters. To correct for losses of protein not reconstituted into liposomes and not retained by the filter, control experiments were performed with ^{131}I-labelled solubilized membrane proteins (50 c.p.m./ng of protein) and non-radioactive ligands. Binding data were calculated as means from double determinations. The binding data were used for construction of linear plots of the ratio of receptor-bound radioligand (Scatchard, 1949). The equilibrium dissociation constant, K_d, was

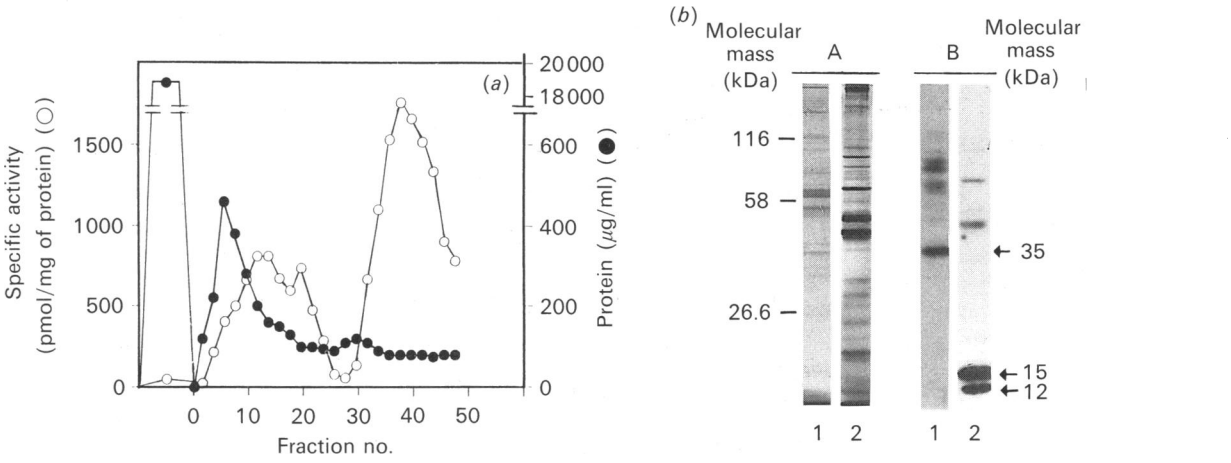

Fig. 1. Purification of reduced ^{131}I-Mal-BSA binding proteins by Mal-BSA affinity chromatography and revelation of ^{131}I-Mal-BSA binding

(*a*) Mal-BSA–Sepharose 4B column chromatography. A 100 ml portion of membrane protein (20 mg/ml) solubilized with 40 mM-OG in 50 mM-Tris/HCl (pH 8)/150 mM-NaCl/0.1 mM-EDTA/0.2% β-mercaptoethanol was applied to a Mal-BSA–Sepharose CL 4B column containing 1.7 g of Mal-BSA–Sepharose. The column was washed with 50 mM-Tris/HCl (pH 8)/50 mM-NaCl/0.1 mM-EDTA/0.2% β-mercaptoethanol/40 mM-OG. The column was eluted with 250 mM-NaCl (fraction nos. 1–28) and then by 1 M-NaCl (fraction nos. 31–48) as described in the Materials and methods section. Single fractions of 1.4 ml were collected. ^{131}I-Mal-BSA binding to each fraction was determined by filtration assay. (*b*) Binding of ^{131}I-Mal-BSA by specific binding proteins from different fractions. Aliquots of fraction nos. 5–10 from the 250 mM-NaCl step (1), and of fraction nos. 35–40 from the 1 M-NaCl step (2) after electrophoretic separation in 11% polyacrylamide slab gels containing 0.1% SDS and 1% β-mercaptoethanol are shown. Group A, proteins stained by silver; group B, binding of ^{131}I-Mal-BSA. Transblots were incubated with 58.8 pmol of ^{131}I-Mal-BSA/ml (48 c.p.m./fmol) as described in the Materials and methods section, and autoradiographed. Molecular mass standards are indicated on the left and the molecular masses of the major ^{131}I-Mal-BSA binding proteins are shown on the right.

given by the ratio of $B_{max.}$ and the y-intercept. The number of receptor-bound ligands at saturation, $B_{max.}$, was given by the x-intercept of the linear plot.

RESULTS

Isolation of 35 kDa and 15 kDa proteins exhibiting high-affinity binding for ^{131}I-Mal-BSA

In previous studies, Mal-BSA and acetyl-LDL binding activity was readily extracted from rat liver membranes by non-ionic detergents. Under non-reducing conditions, a high-affinity binding protein with a molecular mass $\geqslant$250 kDa was detected (Dresel *et al.*, 1985, 1987). In the presence of β-mercaptoethanol as reducing agent, binding activity was detected mainly at a 35 kDa protein, and a minor activity was detected at 85 kDa (Ottnad *et al.*, 1988). Purification of the reduced 35 kDa Mal-BSA/acetyl-LDL binding protein of rat liver membranes was therefore carried out with buffers containing non-ionic detergents in the presence of β-mercaptoethanol. The soluble fractions were assayed for their protein content. Binding activity was assayed, after reconstitution of the soluble protein with phosphatidylcholine, by the filter assay and by ligand blotting.

In a typical experiment, liver membranes from 40 g of rat liver tissue were extracted (4 °C, 20 min) with 40 mM-OG in TNE buffer containing proteinase inhibitors and 0.1 % β-mercaptoethanol. The solubilized protein in the high-speed supernatant (1000–1200 mg of protein; specific ^{131}I-Mal-BSA binding activity 0.25 pmol/mg) was subsequently subjected to affinity chromatography on a Mal-BSA–Sepharose CL 4B column. Only 1 % of the applied protein was retained by the column. ^{131}I-Mal-BSA binding activity was eluted in the presence of 40 mM-OG by a stepwise increase of ionic strength, to 250 mM-NaCl and 1 M-NaCl.

The eluate in the 250 mM-NaCl fraction contained 50–60 % of the binding activity; the rest (40–50 %) of the activity was eluted at 1 M-NaCl (Fig. 1). The specific binding activity was 820 pmol of ^{131}I-Mal-BSA/mg of protein in the 250 mM-NaCl eluate and 1850 pmol of ^{131}I-Mal-BSA/mg of protein in the 1 M-NaCl fraction. A silver stain of a polyacrylamide gel containing the 250 mM-NaCl eluate and a corresponding ^{131}I-Mal-BSA ligand blot demonstrate the presence of a 35 kDa binding protein in addition to other contaminating proteins (Fig. 1*b*, lane 1). The yield of the 35 kDa protein varied to some extent between different preparations. An average of 2 mg of total protein was recovered in the 250 mM-NaCl eluate from 400 mg of protein solubilized from total rat liver tissue. Some binding could be also detected at 85 kDa. To separate this minor 85 kDa binding protein from the 35 kDa protein, the 250 mM-eluate was concentrated and fractionated on a TSK G4000 h.p.l.c. column. Ligand blots with ^{131}I-Mal-BSA demonstrate the effective separation of the 35 kDa binding activity from the 85 kDa binding activity on the TSK G4000 column with recovery of 85 % of the applied ^{131}I-Mal-BSA binding activity (Fig. 2).

The 1 M-NaCl solution also eluted ^{131}I-Mal-BSA binding activity, but the peak contained much less protein. Analysis of the eluate by polyacrylamide-gel electrophoresis and silver staining showed two major proteins at 12–15 kDa as well as numerous bands of higher molecular mass. ^{131}I-Mal-BSA binding activity was present in the two protein bands at 12 kDa and 15 kDa (Fig. 1, lane 2). The 12 kDa component was not seen in all of our experiments. The 1 M-NaCl eluate was

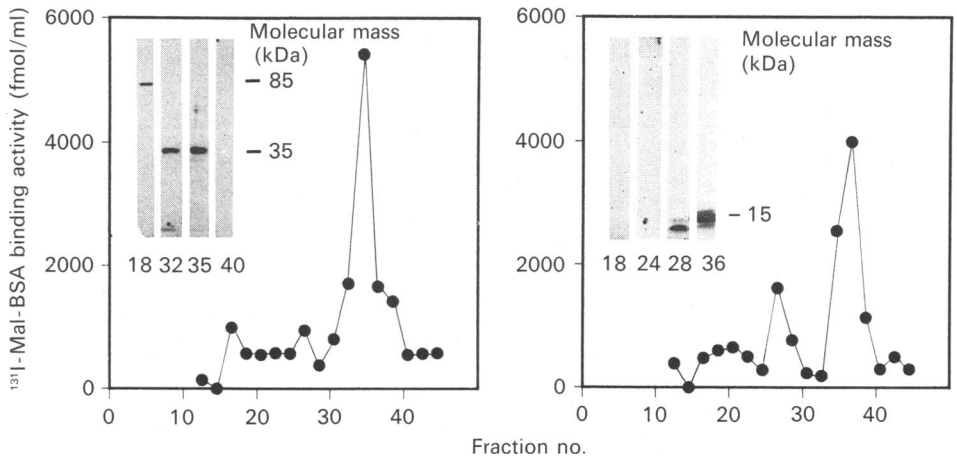

Fig. 2. Gel-permeation chromatography of the 250 mM- and 1 M-NaCl fractions and revelation of the ^{131}I-Mal-BSA binding sites

(*a*) ^{131}I-Mal-BSA binding activity eluted by the 250 mM-NaCl salt step in Fig. 1 was concentrated (12.8-fold) by centrifugation in ultrafiltration tubes. A 200 μl portion of the concentrated solution was subjected to gel-permeation chromatography on a TSK G4000 column equilibrated in 125 mM-Tris/HCl (pH 6.8)/0.1 % β-mercaptoethanol/0.1 % SDS. The flow rate of the column was 0.5 ml/min at a constant pressure of 800 kPa. ^{131}I-Mal-BSA binding activity was determined by the filtration assay. Inset: revelation of ^{131}I-Mal-BSA binding to transblots of the separated proteins. Fractions nos. 18, 32, 35 and 40 of the TSK G4000 chromatograms (Laemmli buffer + 1 % β-mercaptoethanol) were separated in a 11 % polyacrylamide slab gel containing 0.1 % SDS. Transblots were incubated with 58.8 pmol of ^{131}I-Mal-BSA/ml (48 c.p.m./fmol) and autoradiographed as described in the Materials and methods section. (*b*) Final gel-permeation chromatogram of ^{131}I-Mal-BSA binding activity eluted by the 1 M-NaCl step (Fig. 1); 8-fold concentration chromatography on TSK G4000 column. Inset: active fraction nos. 18, 24, 28 and 36 were assayed as described for (*a*).

concentrated and repurified on a TSK G4000 column to separate traces of the 35 kDa protein. The 15 kDa binding protein could be detected in the major peak (Fig. 2).

Binding of ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL to the 35 kDa binding protein

To analyse the saturation binding of ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL to the h.p.l.c. fractions containing the separated 35 kDa protein, analysis was performed in presence and absence of excess unlabelled competitor. The maximal binding ($B_{max.}$) and the dissociation constant (K_d) were determined in the absence of the competitor by Scatchard analysis (Fig. 3). The $B_{max.}$ of the 35 kDa protein was 4500 pmol/mg of protein for ^{131}I-Mal-BSA and 2340 pmol/mg of protein for ^{131}I-acetyl-LDL. High-affinity ^{131}I-Mal-BSA binding was 2-fold higher than the binding of acetyl-LDL. The Scatchard plots show evidence of high-affinity binding sites with a K_d of 5 nM for ^{131}I-Mal-BSA binding and of 4.8 nM for ^{131}I-acetyl-LDL binding.

In the presence of excess Mal-BSA, binding of ^{131}I-acetyl-LDL was inhibited, and only unspecific binding was observed. In contrast, acetyl-LDL competed for ^{131}I-Mal-BSA binding only insignificantly. Some decrease in ^{131}I-Mal-BSA binding in the presence of acetyl-LDL does occur, but the Scatchard plots demonstrate that the dissociation constant of the ^{131}I-Mal-BSA binding is not affected by the presence of acetyl-LDL. Thus no evidence for a competitive inhibition of ^{131}I-Mal-BSA binding by acetyl-LDL could be found.

To test for ligand specificity of the 35 kDa binding protein, inhibition studies were performed at saturating ligand concentrations for the ^{131}I-labelled ligand (58.8 pmol/ml for ^{131}I-Mal-BSA and 20 pmol/ml for ^{131}I-acetyl-LDL) in the presence of increasing concentrations of unlabelled Mal-BSA or acetyl-LDL, f-BSA, PVS, polyinosinic acid, polyadenylic acid or polycytidylic acid as inhibitors (see Figs. 4*a* and 5*a*). An excess of Mal-BSA and f-BSA almost completely inhibited all of the ^{131}I-acetyl-LDL binding. PVS, polyinosinic acid and polyadenylic acid competed for 75% of the acetyl-LDL binding. However, an equal excess of polycytidylic acid did not compete for more than 25–40% of the ^{131}I-acetyl-LDL binding.

The presence of excess PVS or polyinosinic acid drastically reduced ^{131}I-Mal-BSA binding to below 5%. Polyadenylic acid inhibited 75% of ^{131}I-Mal-BSA binding. On the other hand, a 64-fold excess of acetyl-LDL and a 110-fold excess of f-BSA and polycytidylic acid up to 100 μg/ml inhibited only 25% of the ^{131}I-Mal-BSA binding.

Binding of ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL to the 15 kDa receptor protein

Saturation binding of ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL was demonstrated with the purified 15 kDa binding protein complexed with liposomes (Fig. 6). $B_{max.}$ values were 1200 pmol of ^{131}I-Mal-BSA/mg of protein and 300 pmol of ^{131}I-acetyl-LDL/mg of protein. Thus binding of ^{131}I-Mal-BSA was 4-fold higher than for ^{131}I-acetyl-LDL. The results show saturable binding of ^{131}I-Mal-BSA with an apparent K_d of about 5.6 nM, and saturable high-affinity binding of ^{131}I-acetyl-LDL with a K_d of 2.3 nM.

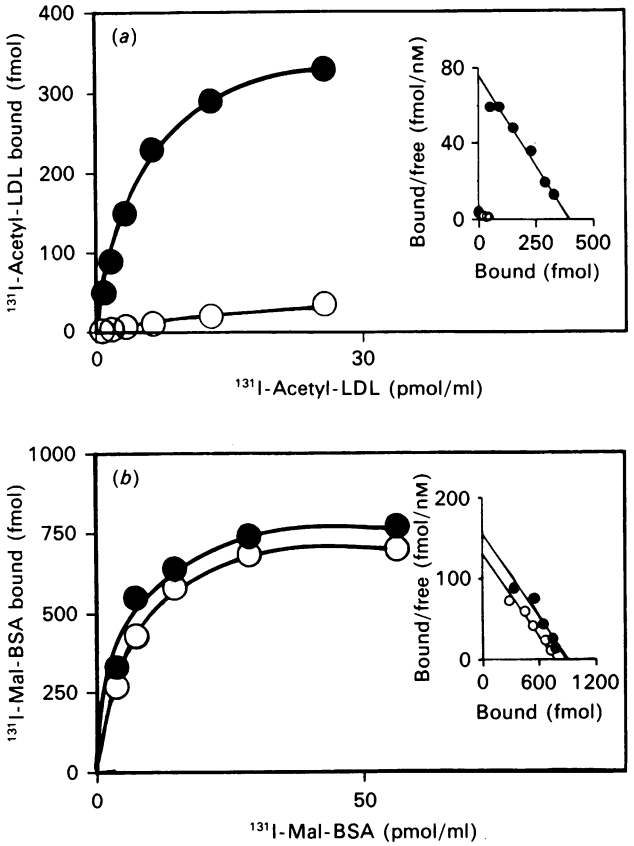

Fig. 3. Saturation analysis of the 35 kDa binding protein in presence of a second ligand

A 200 μl portion of the fraction with the 35 kDa ^{131}I-Mal-BSA binding activity obtained by affinity chromatography at 250 mM-NaCl and subsequent gel chromatography was reconstituted with egg phosphatidylcholine. Protein (0.16 μg/assay) was incubated in TNE buffer containing 2 mM-EDTA and 1 mg of BSA/ml with the indicated concentrations of ^{131}I-radioligands in the absence or presence of an excess of a second unlabelled ligand at room temperature for 30 min before filtration (to separate bound from free radioligand). (*a*) Binding of the ^{131}I-acetyl-LDL (336 c.p.m./fmol): ●, total binding in the absence of Mal-BSA; ○, total binding in the presence of 735 pmol of Mal-BSA/ml. Inset: Scatchard analysis of the total binding of ^{131}I-acetyl-LDL in the absence or presence of the second ligand. (*b*) Binding of ^{131}I-Mal-BSA (43.8 c.p.m./fmol): ●, total binding in absence of acetyl-LDL; ○, total binding in presence of 2000 pmol of acetyl-LDL/ml. Inset, Scatchard analysis of the binding data in the absence and presence of the second ligand.

Saturation binding analysis with ^{131}I-acetyl-LDL in the presence of Mal-BSA demonstrates inhibition of ^{131}I-acetyl-LDL high-affinity binding by Mal-BSA. Saturation analysis of the ^{131}I-Mal-BSA binding by acetyl-LDL shows that ^{131}I-Mal-BSA high-affinity binding is partially diminished by excess acetyl-LDL. ^{131}I-Mal-BSA binding appears to occur to at least two classes of binding sites; at one of these binding sites, Mal-BSA binding is apparently antagonized by acetyl-LDL. This binding component, detected by the decreased slope of the Scatchard curve, binds ^{131}I-Mal-BSA with the same $B_{max.}$ but with reduced affinity in the presence of excess acetyl-LDL, indicating competitive inhibition. The second class

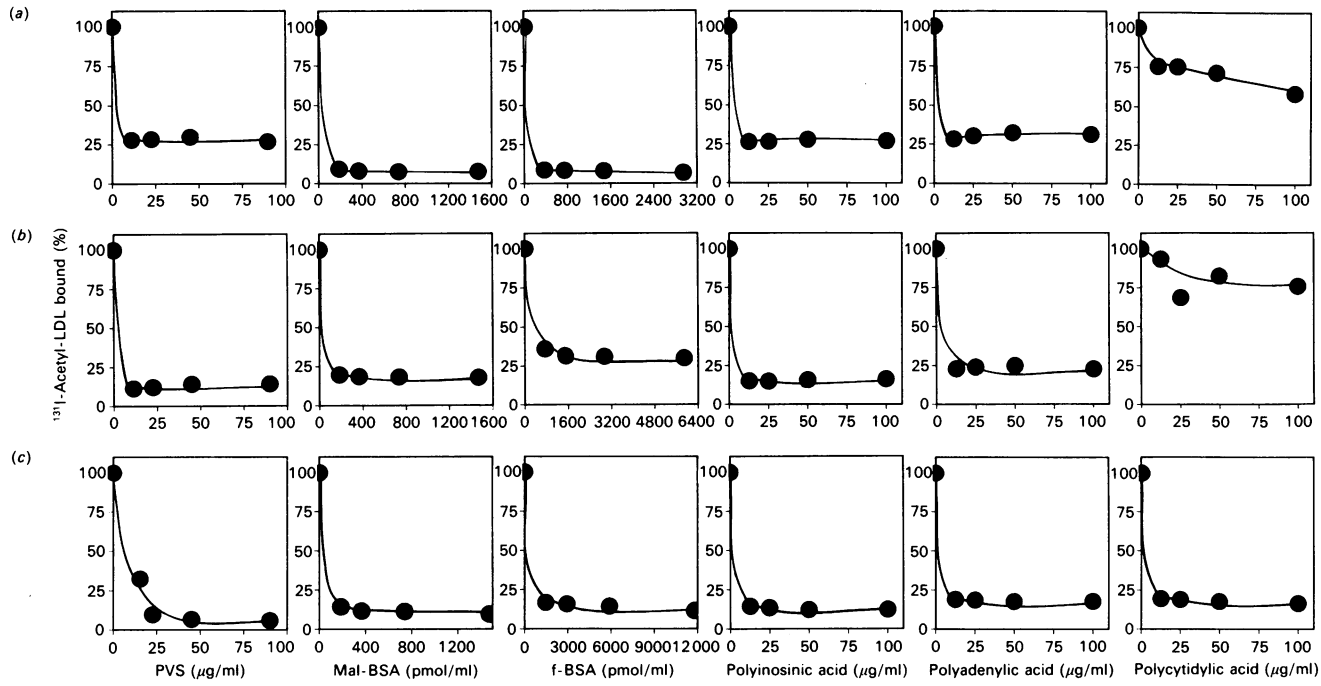

Fig. 4. Inhibition of ^{131}I-acetyl-LDL binding to reduced and separated 85 kDa, 35 kDa and 15 kDa rat hepatic receptor proteins by various concentrations of unlabelled Mal-BSA, f-BSA, PVS, polyinosinic acid, polyadenylic acid and polycytidylic acid

Liposome–protein complexes from fractions containing the 85 kDa, 35 kDa and 15 kDa receptor proteins obtained by Mal-BSA–Sepharose 4B chromatography/gel-permeation chromatography were incubated with ^{131}I-acetyl-LDL (310–448 c.p.m./fmol) in the absence or presence of various concentrations of the inhibitors as described in the legend to Fig. 3. (*a*) Inhibition of total ^{131}I-acetyl-LDL binding (5 pmol/ml) by excess inhibitor to 0.16 μg of protein containing the 35 kDa receptor protein obtained as described in the legend to Fig. 2. 100% = 0.03 pmol of ^{131}I-acetyl-LDL bound/assay. (*b*) Inhibition of total ^{131}I-acetyl-LDL binding (5 pmol/ml) by excess inhibitor to 0.25 μg of reconstituted protein containing the 15 kDa receptor protein obtained as described in the legend to Fig. 2. 100% = 0.18 pmol of ^{131}I-acetyl-LDL bound/assay. (*c*) Inhibition of total ^{131}I-acetyl-LDL binding (20 pmol/ml) by excess inhibitor to 1.7 μg of reconstituted protein containing the 85 kDa receptor protein obtained as described in the legend to Fig. 5. 100% = 0.45 pmol of ^{131}I-acetyl-LDL bound/assay.

of binding sites, however, appears to bind ^{131}I-Mal-BSA with unchanged affinity in the presence of acetyl-LDL, as the part of the curve representing this binding class has the same slope as the binding curve in absence of an inhibitor.

To investigate inhibition of ^{131}I-Mal-BSA binding to the 15 kDa protein, Mal-BSA, acetyl-LDL and polyanions were tested as competitors for ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA binding. Inhibition studies were carried out at ^{131}I-acetyl-LDL concentrations of 5 pmol/ml (Fig. 4*b*). There is a complete inhibition of ^{131}I-acetyl-LDL binding to the level of non-specific binding in the presence of excess PVS, polyinosinic acid, Mal-BSA, f-BSA or polyadenylic acid. However, polycytidylic acid inhibited just 25% of the ^{131}I-acetyl-LDL binding.

^{131}I-Mal-BSA binding could be completely inhibited by PVS and polyinosinic acid, and by 75% by polyadenylic acid (Fig. 5*b*). Acetyl-LDL, however, inhibited just 25–40% of ^{131}I-Mal-BSA binding. A 110-fold excess of f-BSA and high concentrations of polycytidylic acid (up to 100 μg/ml) failed to compete for more than 12% of the radioligand binding.

Partial purification of the 85 kDa protein with high-affinity binding for ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL

To purify the 85 kDa protein, liver membranes were solubilized with Triton X-100 in conjunction with GSH/GSSG to stabilize this protein. Mal-BSA–Sepharose was again used for affinity chromatography.

The liver membrane proteins were solubilized (4 °C, 30 min) with 1% Triton X-100 in TNE buffer containing 5 mM-GSH/5 mM-GSSG, the proteinase inhibitors PMSF and Trasylol, and 0.1 mM EDTA. The extract was centrifuged at 100000 ***g*** for 30 min and the supernatant was fractionated on a Mal-BSA–Sepharose column. After washing with buffer B containing 0.1% Triton X-100, 5 mM-GSH/5 mM-GSSG/0.1 mM-EDTA, 80% of the applied binding capacity was retained by the column. By increasing the ionic strength in the column buffer to 250 mM-NaCl and 5 mM-GSH without GSSG, a ^{131}I-Mal-BSA binding activity with a molecular mass of 85 kDa was eluted (Fig. 7). In this fraction, 1.6 mg of protein was recovered, with a specific binding activity of 200–250 pmol of ^{131}I-Mal-BSA/mg of protein, from 40 g of tissue.

A second peak with ^{131}I-Mal-BSA binding activity containing the 15 kDa protein was again eluted by a further increase of ionic strength in the column buffer to 1 M-NaCl.

Binding of ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL to the 85 kDa receptor protein

Again, saturation binding of ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL was analysed by using liposome–protein

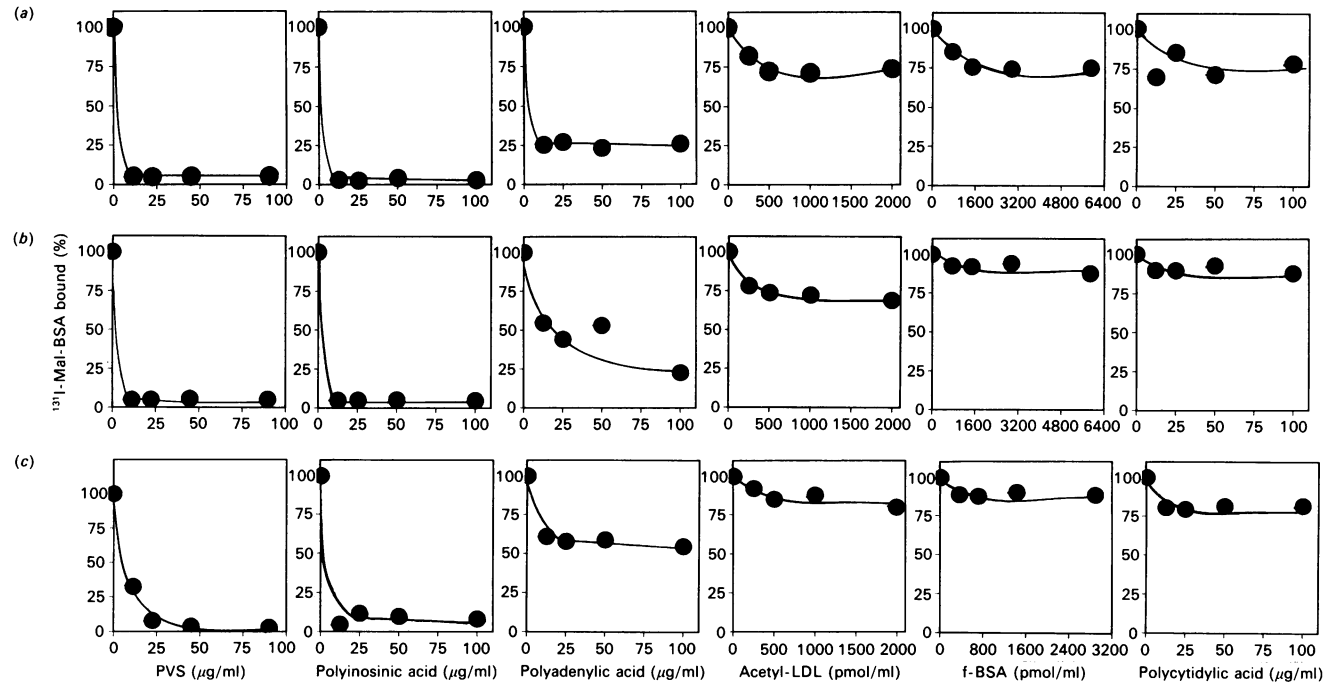

Fig. 5. Inhibition of ^{131}I-Mal-BSA binding to reduced and separated 85 kDa, 35 kDa and 15 kDa rat hepatic receptor proteins by various concentrations of unlabelled acetyl-LDL, f-BSA, PVS, polyinosinic acid, polyadenylic acid and polycytidylic acid

Liposome–protein complexes with the 85 kDa, 35 kDA and 15 kDA ^{131}I-Mal-BSA binding proteins were obtained and incubated with ^{131}I-Mal-BSA (42.2–87.6 c.p.m./fmol) and excess polyanionic competitors under the conditions described in the legends to Figs. 3 and 4. (*a*) Inhibition of total ^{131}I-Mal-BSA binding (29.4 pmol/ml) by excess inhibitor to 0.16 μg of protein containing the 35 kDa receptor protein obtained as described in the legend to Fig. 2. 100% = 0.13 pmol of ^{131}I-Mal-BSA bound/assay. (*b*) Inhibition of total ^{131}I-Mal-BSA binding (29.4 pmol/ml) by excess inhibitor to 0.25 μg of protein, containing the 15 kDa binding protein obtained as described in the legend to Fig. 2. 100% = 0.6 pmol of ^{131}I-Mal-BSA bound/assay. (*c*) Inhibition of total ^{131}I-Mal-BSA binding (58.8 pmol/ml) by excess inhibitor to 1.7 μg of reconstituted protein containing the 85 kDa binding protein obtained as described in the legend to Fig. 7. 100% = 2.05 pmol of ^{131}I-Mal-BSA bound/assay.

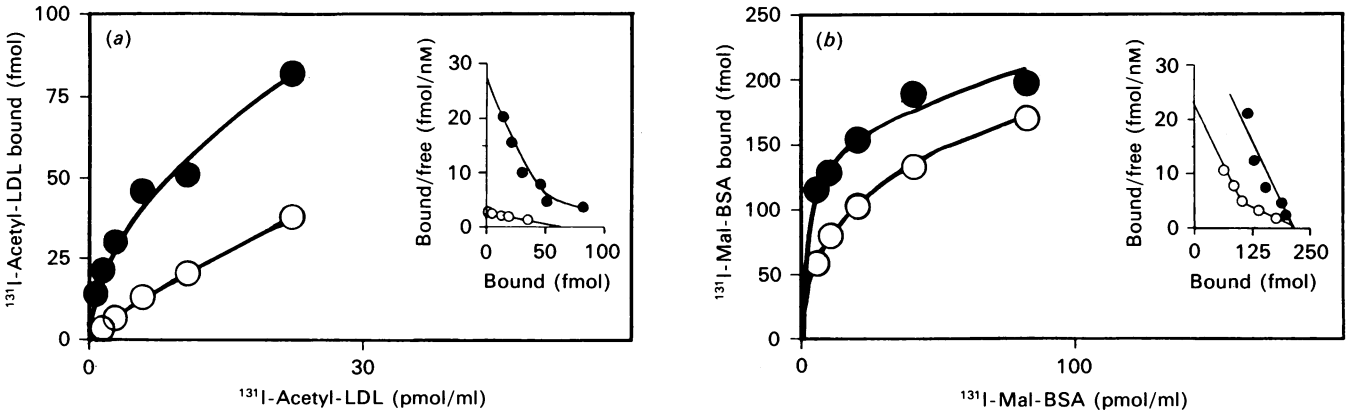

Fig. 6. Saturation analysis of the 15 kDa receptor protein in the presence of a second ligand

A 200 μl portion of the 1 M-NaCl step fraction was concentrated and subjected to gel chromatography on the TSK G4000 h.p.l.c. column as described in the legend to Fig. 2. The resulting fractions containing ^{131}I-Mal-BSA binding activity at the 15 kDa receptor protein were pooled and reconstituted with egg phosphatidylcholine. Protein (0.16 μg/assay) was incubated with the indicated concentrations of ^{131}I-radioligands in the absence or presence of an excess of a second unlabelled ligand as described in the legend to Fig. 3. (*a*) Binding of ^{131}I-acetyl-LDL (336 c.p.m./fmol): ●, total binding in the absence of Mal-BSA; ○, total binding in the presence of 1470 pmol of Mal-BSA/ml. Inset: Scatchard analysis of the binding of ^{131}I-acetyl-LDL in the absence and presence of the second ligand. (*b*) Binding of ^{131}I-Mal-BSA (43.8 c.p.m./fmol): ●, total binding in the absence of acetyl-LDL; ○, total binding in the presence of 2000 pmol of acetyl-LDL/ml. Inset: Scatchard analysis of the binding data in the absence and presence of the second ligand.

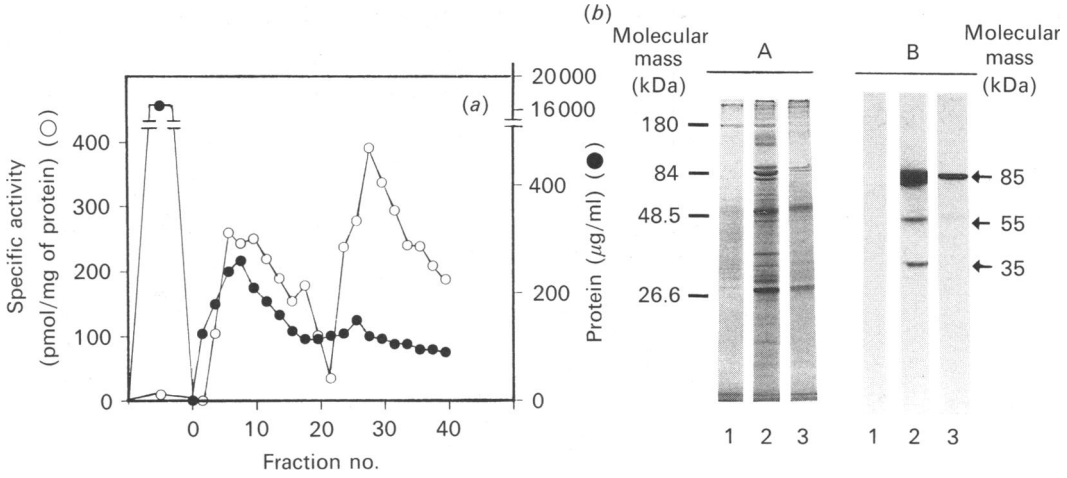

Fig. 7. Purification of a reduced 85 kDa Mal-BSA binding protein by Mal-BSA affinity chromatography and revelation of ^{131}I-Mal-BSA binding

(*a*) Chromatogram of a Mal-BSA–Sepharose CL 4B affinity column. A 125 ml portion of membrane protein (20 mg/ml) solubilized with 1% Triton X-100 in 50 mM-Tris/HCl pH 8/150 mM-NaCl/0.1 mM-EDTA/5 mM-GSSG/5 mM-GSH was applied to a Mal-BSA–Sepharose 4B column containing 1.6 g of Mal-BSA–Sepharose. The column was equilibrated in 50 mM-Tris/HCl (pH 8)/50 mM-NaCl/0.1 mM-EDTA/5 mM-GSH/0.1% Triton X-100. The fractions were eluted by 250 mM-NaCl (fraction nos. 1–20) and subsequently by 1 M-NaCl (fraction nos. 22–40) as described in the Materials and methods section (procedure B). Single fractions of 1.4 ml were collected. ^{131}I-Mal-BSA binding was assayed by the filtration assay. (*b*) ^{131}I-Mal-BSA binding proteins from fractions containing binding activity. Lanes 1–3 contain aliquots of the fractions nos. 1–2, 5–6 and 11–12 from the 250 mM-NaCl step, which were separated in a 11% polyacrylamide slab gel in Laemmli (1970) buffer containing 0.1% SDS and 1% β-mercaptoethanol. Group A, proteins stained by silver stain; group B, autoradiographs of transblots incubated with 58.8 pmol of ^{131}I-Mal-BSA/ml (48 c.p.m./fmol) as detailed in the Materials and methods section. Left hand side, molecular mass markers; right hand side, binding proteins.

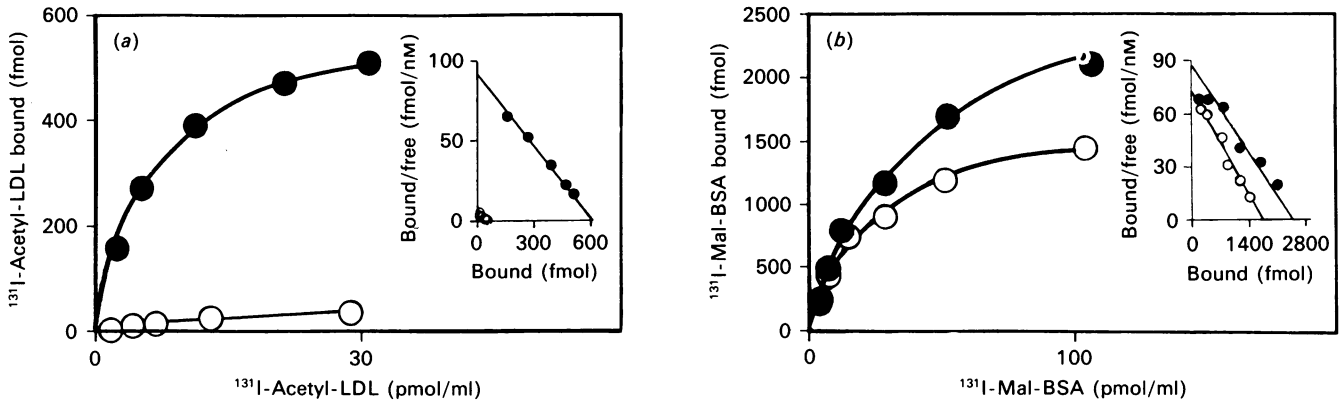

Fig. 8. Saturation analysis of the 85 kDA receptor in presence of a second ligand

The proteins of the fractions containing the ^{131}I-Mal-BSA binding activity obtained by Mal-BSA affinity chromatography with the 250 mM-NaCl step were complexed with liposomes. Protein (0.7 μg/assay) was incubated with the indicated concentrations of ^{131}I-radioligands as described in the legend to Fig. 3 in the absence and presence of an excess of a second unlabelled ligand. (*a*) Binding of ^{131}I-acetyl-LDL (420 c.p.m./fmol): ●, total binding in the absence of Mal-BSA; ○, total binding in the presence of 1470 pmol of Mal-BSA/ml. Inset: Scatchard analysis of the total binding of ^{131}I-acetyl-LDL in the absence or presence of the second ligand. (*b*) Binding of ^{131}I-Mal-BSA (87.6 c.p.m./fmol): ●, total binding in the absence of acetyl-LDL; ○, total binding in the presence of 2000 pmol of acetyl-LDL/ml. Inset: Scatchard analysis of the binding data in the absence or presence of a second ligand.

complexes containing the partially purified 85 kDa protein (250 mM-NaCl fraction of the Mal-BSA–Sepharose 4B column) in the presence or absence of excess competitor (Fig. 8). The maximal binding capacities were 3700 pmol of ^{131}I-Mal-BSA/mg of protein and 850 pmol of ^{131}I-acetyl-LDL/mg of protein respectively for the two ligands. The binding capacity for ^{131}I-Mal-BSA is apparently 4–5-fold higher than for ^{131}I-acetyl-LDL. The results show saturable high-affinity binding of ^{131}I-Mal-BSA with an apparent K_d of about 30 nM, and saturable high-affinity binding of ^{131}I-acetyl-LDL with an apparent K_d of 6.5 nM.

^{131}I-Acetyl-LDL saturation binding was totally inhibited by excess Mal-BSA. No high-affinity binding could be demonstrated in the presence of Mal-BSA. In contrast, no competition for ^{131}I-Mal-BSA binding by acetyl-LDL could be demonstrated. The Scatchard plot of the binding data indicate that the dissociation constant of ^{131}I-Mal-BSA binding sites is not changed by excess acetyl-LDL, since there is no significant change in the slope of the curve. It appears that a decrease in ^{131}I-Mal-BSA binding caused by excess acetyl-LDL at high radioligand concentrations might result from competition between acetyl-LDL and ^{131}I-Mal-BSA for unspecific binding sites.

To examine further the ligand specificity of the 85 kDa binding activity, inhibition studies were performed at saturating ligand concentrations for binding of 58.8 pmol of ^{131}I-Mal-BSA/ml and of 20 pmol of ^{131}I-acetyl-LDL/ml in the presence of increasing concentrations of Mal-BSA or acetyl-LDL, f-BSA, PVS, polyinosinic acid, polyadenylic acid or polycytidylic acid as inhibitors (Figs. 4*c* and 5*c*). An excess of Mal-BSA, f-BSA, PVS, polyinosinic acid or polyadenylic acid inhibited essentially all of the ^{131}I-acetyl-LDL binding. Excess PVS and polyinosinic acid reduced ^{131}I-Mal-BSA binding to below 5% (Fig. 5*c*). Polyadenylic acid inhibited 50% of the ^{131}I-Mal-BSA binding. On the other hand, a 34-fold excess of acetyl-LDL, a 55-fold excess of f-BSA or high concentrations of polycytidylic acid (up to 100 μg/ml) inhibited only 25% of the ^{131}I-Mal-BSA binding.

DISCUSSION

Mal-BSA–Sepharose affinity chromatography has been used to separate the reduced 85 kDa, 35 kDa and 12–15 kDa binding proteins. Ligand blotting with ^{131}I-Mal-BSA was used to reveal the fractionation of these binding proteins. After partial purification of microgram quantities of these binding proteins, they were used for biochemical binding analyses. The filter membrane assay with reconstituted proteins has allowed us to monitor binding activity and to analyse the specificity of binding to the ligands in greater detail.

The reduced binding proteins were purified by at least 440-fold by Mal-BSA affinity chromatography. Most of the binding activity was adsorbed, and 70% could be recovered by stepwise increases of NaCl concentration in the elution buffer. Most of the 35 kDa protein and very small amounts of the 85 kDa protein were partially purified in the presence of β-mercaptoethanol as reducing agent and of OG as non-ionic detergent for solubilization of the membrane. The fact that both species eluted at the same ionic concentration suggested that they have similar affinity for Mal-BSA. In contrast, in the presence of Triton X-100 and GSH/GSSG, mainly the 85 kDa protein with traces of the 35 kDa protein were obtained by affinity chromatography. The use of β-mercaptoethanol/OG appears to be a necessary condition for demonstration of the 35 kDa protein and of a decrease in the binding capacity of the 85 kDa binding protein species. Further studies, e.g. immunoblotting with specific antibodies, might demonstrate a structural relationship between these proteins. Potentially, the 85 kDa activity could contain the 35 kDa protein in a disulphide-bridged dimer or heteromer. The 15 kDa protein, and in most of our experiments a 12 kDa protein, were observed in the presence or absence of proteinase inhibitors. The 15 kDa protein exhibits binding with higher affinity than the fractions containing the 35 kDa protein and/or the 85 kDa protein. It seems unlikely that the 15 kDa protein simply represents proteolysis products of binding proteins with a higher molecular mass.

Despite the efficiency of Mal-BSA chromatography in the purification of scavenger receptor activity, this procedure alone yields preparations which are far from being homogeneous. The 35 kDa protein which was eluted from the Mal-BSA–Sepharose column was contaminated with the 85 kDa protein and with some proteins without detectable binding in the ligand blots. Given that most of the contaminating proteins had different molecular masses, we attempted to purify further the binding proteins by gel chromatography. The sequential use of Mal-BSA–Sepharose chromatography and gel chromatography separated the 85 kDa, 35 kDa and 15 kDa binding proteins, as judged by the ligand blotting. A total of 85% of the applied proteins was recovered. Gel chromatography gives a further 6-fold increase in specific activity. With the assumption of a molecular mass of 35 kDa for the binding protein, the 35 kDa protein eluted from the TSK G4000 column is approx. 18000-fold purified as calculated from the binding data of the Scatchard plots.

The binding proteins differ in their molecular mass, their dissociation constants of acetyl-LDL and Mal-BSA binding, and their behaviour in competition experiments with acetyl-LDL and Mal-BSA. Using the filter assay, we were able to measure a K_d of 30 nM for ^{131}I-Mal-BSA high-affinity binding, and 6.5 nM for ^{131}I-acetyl-LDL high-affinity binding to the 85 kDa protein fraction. For the fraction with the 35 kDa protein, binding data indicated a K_d of 4.8 nM for ^{131}I-Mal-BSA and 5.0 nM for ^{131}I-acetyl-LDL. In a previous study, a K_d of 17.3 nM for the Mal-BSA binding at this protein was found (Ottnad *et al.*, 1988). It appears that in the reconstituted system, ^{131}I-Mal-BSA binding to the 35 kDa protein occurs with higher affinity than in the ligand blot and the immobilized protein. The 15 kDa protein displayed saturable high-affinity binding with a K_d of 2.3 nM for ^{131}I-acetyl-LDL and 5.6 nM for ^{131}I-Mal-BSA. When the reduced proteins in the fractions containing the binding proteins were saturated with ^{131}I-acetyl-LDL and ^{131}I-Mal-BSA, there was a 4:1 binding ratio for Mal-BSA:acetyl-LDL at the 85 kDa and the 15 kDa binding proteins, and a 2:1 binding ratio at the 35 kDa binding protein. It is interesting to note that the 35 kDa protein on the one hand and the 85 kDa and the 15 kDa binding proteins on the other hand display ligand binding in distinct stoichiometries with respect to Mal-BSA and acetyl-LDL. This finding shows that the binding proteins can be functionally distinguished.

Polyanionic competitors interfered with ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL binding at the 85 kDa, 35 kDa and 15 kDa binding proteins. The inhibition pattern for ^{131}I-Mal-BSA binding of polyanionic compounds was different from that with ^{131}I-acetyl-LDL. The competition for ^{131}I-Mal-BSA and ^{131}I-acetyl-LDL binding by the polyanions demonstrated a similar sensitivity of the ligand binding for three of the five polyanions tested for the 85 kDa, 35 kDa and 15 kDa binding proteins. This might indicate that the binding proteins are closely related entities. However, there were also notable differences between the three binding activities. Polycytidylic acid competes for >75% of the ^{131}I-acetyl-

LDL binding at the 85 kDa protein but only for 25–30 % binding at the 35 kDa and 15 kDa binding proteins. Mal-BSA and f-BSA compete for ⩾90 % of the ^{131}I-acetyl-LDL binding to the 35 kDa protein and only for 75 % of the binding to the 15 kDa binding proteins. PVS and polyinosinic acid completely inhibited ^{131}I-Mal-BSA binding to the 85 kDa, 35 kDa and 15 kDa binding proteins. However, with respect to ^{131}I-Mal-BSA binding, polyadenylic acid inhibits 50 % of binding to the 85 kDa protein and 75 % binding to the 35 kDa and the 15 kDa proteins; f-BSA and polycytidylic acid appear to compete more effectively for the binding at the 35 kDa protein (up to 25 %) than at the 15 kDa protein.

Although in the experiments with the non-reduced protein we found evidence for competitive inhibition between Mal-BSA and acetyl-LDL at a common binding site (Dresel *et al.*, 1987), the results obtained with the reduced proteins in this study demonstrate a more complex inhibition. Mal-BSA has a very strong inhibitory potency for ^{131}I-acetyl-LDL binding as observed in the saturation binding experiments with the 85 kDa, 35 kDa and 15 kDa binding proteins, despite the fact that ^{131}I-acetyl-LDL binds with higher affinity to its binding sites than does Mal-BSA. On the other hand, ^{131}I-Mal-BSA high-affinity binding is only insignificantly inhibited by excess acetyl-LDL at the 85 kDa and 35 kDa binding proteins. Thus it seems unlikely that Mal-BSA and acetyl-LDL compete for a common binding site at the 85 kDa and 35 kDa proteins. We think that the best explanation for the inhibition of acetyl-LDL binding by Mal-BSA is that Mal-BSA binding induces an obliteration of the major high-affinity acetyl-LDL binding sites by a non-competitive inhibition. A mixed type of competition for a site of Mal-BSA binding by acetyl-LDL might be seen at the 15 kDa binding protein; however, not all of the high-affinity Mal-BSA binding can be blocked by excess acetyl-LDL, indicating the presence of a second class of binding sites. It is necessary to validate the use of the known inhibitors of the acetyl-LDL receptors for each binding site. The simple demonstration of a blockade of the formation of a ligand–receptor complex by addition of a second ligand or polyanion does not establish a competitive inhibition of binding to the reduced proteins.

It is intriguing to consider that the acetyl-LDL and Mal-BSA binding proteins may have a role *in vivo* in the scavenger function of the rat liver. The 250 kDa binding protein is probably a functional scavenger receptor *in vivo*. The binding stoichiometry of acetyl-LDL and Mal-BSA *in vitro* resembles hepatic uptake *in vivo* after intravenous administration of saturating amounts of the ligands (Dresel *et al.*, 1987). A comparable protein has been localized on the surface of sinusoidal endothelial cells of the liver (Kodama *et al.*, 1988).

The reduced binding proteins analysed in this study exhibit high-affinity binding for acetyl-LDL and Mal-BSA. It remains to be determined whether the 85 kDa, 35 kDa and 15 kDa proteins observed are reduced subunits of high-molecular-mass receptors. Kodama *et al.* (1988) demonstrated a reduced 77 kDa protein derived from a purified 220 kDa acetyl-LDL receptor of bovine liver. However, the reduced 77 kDa protein did not bind detectable acetyl-LDL in a ligand blot. These authors suggested a trimeric 77 kDa subunit structure for the non-reduced acetyl-LDL receptor. If their suggestion is correct, and if the 250 kDa protein of rat liver is closely related to the bovine 77 kDa trimer, it appears unlikely that the active binding proteins characterized in this study are subunits of the 250 kDa protein, since, in contrast with the 77 kDa protein, they exhibit high-affinity binding in the reduced state. Further studies will be required to analyse the structure of the hepatic scavenger receptors.

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