Incorporation of mannose 6-phosphate receptors into liposomes

Receptor topography and binding of α -mannosidase

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A receptor that binds the lysosomal enzyme α -mannosidase via mannose 6-phosphate moieties (mannose 6-phosphate receptor) was purified from Swarm-rat chondrosarcoma and bovine liver microsomal membranes. Receptor-reconstituted liposomes were prepared by dialysis of taurodeoxycholate-dispersed lipids with purified mannose 6-phosphate receptor. Liposomes appeared by electron microscopy as 60–120 nm unilamellar vesicles. Receptor-reconstituted liposomes retained the ability to bind α -mannosidase specifically. Binding was saturable with an apparent K_d of 1 nM and was competitively inhibited by mannose 6-phosphate (K_i 2 mM). Liposomes containing entrapped ^{125}I -bovine serum albumin were used to demonstrate that treatment with 0.045% taurodeoxycholate rendered liposomes permeable to macromolecules without solubilizing the membrane. Receptor orientation in the liposome membrane was established by measuring binding of ligand to intact and detergent-treated liposomes. Unlike coated vesicles, which contain cryptic mannose 6-phosphate receptors [Campbell, Fine, Squicciarini & Rome (1983) *J. Biol. Chem.* **258**, 2526–2533], treatment of liposomes with detergent revealed no additional cryptic binding sites. In addition, treatment of liposomes with 0.75% trypsin abolished total receptor binding activity. The results suggest that the receptor is inserted with its binding site facing the outside of the liposome.

Many cell types can recognize and take up specific molecules by receptor-mediated endocytosis. Low-density lipoproteins (Anderson *et al.*, 1976), asialoglycoproteins (Wall *et al.*, 1980), α_2 -macroglobulin (Willingham *et al.*, 1979), insulin (Maxfield *et al.*, 1978), epidermal growth factor (Gorden *et al.*, 1978) and lysosomal enzymes (Rome *et al.*, 1979a) are among the many ligands that have been shown to enter cells by this process (for review, see Steinman *et al.*, 1983). The receptor, which has been shown to mediate the internalization of α -L-iduronidase (Rome *et al.*, 1979a), β -galactosidase (Willingham *et al.*, 1981) and α -mannosidase (Freeze *et al.*, 1980), recognizes and binds lysosomal enzymes that have oligosaccharide chains containing one or more mannose 6-phosphate moieties (Hasilik *et al.*, 1980; Reitman & Kornfeld, 1981). This receptor, referred to here as the mannose

6-phosphate receptor, appears to have a ubiquitous distribution (Fischer *et al.*, 1980b). The mannose 6-phosphate receptor has recently been purified from bovine liver (Sahagian *et al.*, 1981, 1982), cultured cells (Sahagian *et al.*, 1981) and Swarm-rat chondrosarcoma (Steiner & Rome, 1982). The receptor is a glycoprotein with a subunit mol.wt. of 215 000, as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Steiner & Rome, 1982). To further characterize the isolated mannose 6-phosphate receptor, the present study was designed to develop conditions for the reconstitution of the receptor into liposomes. Reconstitution of functional receptor proteins into liposomes has recently been demonstrated for the acetylcholine receptor (Anholt *et al.*, 1982), the β -adrenergic receptor (Eimerl *et al.*, 1980) and the asialoglycoprotein receptor (Baumann & Doyle, 1980). In this study, we describe a method for the reconstitution of mannose 6-phosphate receptors into chemically defined liposomes which retain the

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ability to bind the lysosomal enzyme α -mannosidase specifically.

Experimental procedures

Materials

Sodium taurodeoxycholate, 4-methylumbelliferyl α -D-mannopyranoside, synthetic (dimyristoyl)-phosphatidylcholine (dimyristoylglycerophosphocholine), synthetic (distearoyl)phosphatidylcholine (distearoylglycerophosphocholine), cholesterol and the sodium salt of mannose 6-phosphate and glucose 6-phosphate were purchased from Sigma Chemical Corp. Chromatographically purified egg yolk L- α -phosphatidylcholine was from Calbiochem-Behring (San Diego, CA, U.S.A.). The iodinating reagent, Iodogen, was from Pierce Chemical Co. and carrier-free Na^{125}I was from Amersham Corp. Aminophenylthioether paper was from Schleicher and Schuell Inc., Keene, NH, U.S.A. Purified [^{14}C]phosphatidylcholine from rat liver was a gift from Dr. James Mead (UCLA). α -Mannosidase was purified to homogeneity from *Dictyostelium discoideum* (H. H. Freeze, R. Y. Yeh & A. L. Miller, unpublished work). All other reagents were purchased from standard commercial suppliers.

Purification of receptor

The mannose 6-receptor was purified from rat chondrosarcoma and bovine liver by the procedure of Steiner & Rome (1982) with some minor modifications. The 100000g membrane pellets were solubilized overnight at 4°C in 0.1M-sodium glycine, pH9.5, containing 0.1% Triton X-100, at 200ml/g of membrane protein, and then dialysed in 50mM-phosphate buffer, pH6.0, containing 15mM-citric acid, 0.15M-NaCl and 0.1% Triton X-100 (buffer A). All buffers contained the following proteinase inhibitors: 50mM-EDTA, 0.1M-5-amino-hexanoic acid, 0.5mM-phenylmethanesulphonyl fluoride and 5mM-benzamidine hydrochloride. Solubilized membrane proteins were recirculated over a phosphomannan-Sepharose affinity column (Steiner & Rome, 1982) for 24h, washed with 30 column vol. of buffer A containing proteinase inhibitors. The receptor was eluted as described by Steiner & Rome (1982) and concentrated to 1mg/ml in an Amicon model 202 ultrafiltration apparatus containing a PM 10 membrane.

Activity of the purified mannose 6-phosphate receptor was determined after covalent coupling of 10 μ l of receptor (1mg/ml) to activated aminophenylthioether paper discs (Renart *et al.*, 1979; Alwine *et al.*, 1979). Briefly, aminophenylthioether paper was activated to the diaminophenylthioether form and cut into 5mm-diameter discs with a paper punch. Receptor protein was applied to each diaminophenylthioether disc and allowed to react

overnight at 4°C. The reaction was quenched by the addition of 500 μ l of 100mM-glycine, pH9, containing 0.1% Triton X-100 and 1mg of bovine serum albumin/ml. The receptor-diaminophenylthioether discs were washed three times with 2ml of buffer A and then stored in buffer A containing 10mM-mannose 6-phosphate. For determination of binding activity, receptor-diaminophenylthioether discs were washed and then incubated for 2h at 25°C with 5nM- α -mannosidase in the presence and in the absence of 10mM-mannose 6-phosphate or -glucose 6-phosphate in a volume of 100 μ l. Discs were washed and assayed directly for bound α -mannosidase as described by Rome *et al.* (1979b). One unit of enzyme activity is defined as 1 μ mol of substrate hydrolysed per h at 37°C. Specific binding activity is the number of units of α -mannosidase activity bound in buffer minus units bound in the presence of mannose 6-phosphate.

Incorporation of receptor into liposomes

Receptor-reconstituted liposomes were prepared by a modification of the procedure of Baumann & Doyle (1980). Unless otherwise specified, 3mg of egg phosphatidylcholine dissolved in chloroform was dried on to the sides of a glass tube under a stream of N_2 . The lipid film was dispersed in 0.8ml of 0.01M-sodium phosphate buffer, pH6.0, containing 0.15M-NaCl (buffer B), also containing 20mM-taurodeoxycholate, and sonicated for 1min at 25°C in a high-intensity water-bath sonicator (Laboratory Supplies Company, Hicksville, NY, U.S.A.). Membrane protein or receptor protein (1mg/ml; 500 μ l) was added to the dispersed lipid, yielding a final protein/lipid ratio of 1:6. In some experiments, ^{125}I -labelled receptor protein (10⁶c.p.m.) was added to each sample to monitor protein incorporation into liposomes. All iodinations were carried out using Iodogen (Markwell & Fox, 1978). The lipid/protein mixture was dialysed for 72h against 6 litres of buffer B. The liposome vesicles that formed during dialysis were purified by isopycnic centrifugation. Solid crushed sucrose was added to the dialysed liposome suspension to yield a final sucrose concentration of 0.5g/ml. This solution was placed in an Ultra-Clear tube (11mm $\times$ 60mm; Beckman Instruments Inc., Palo Alto, CA, U.S.A.) and overlaid with 1.5ml of buffer B containing 30% sucrose followed by 1.5ml of buffer B alone. Tubes were centrifuged in a Beckman SW 56 rotor at 40000rev./min for 17h. After centrifugation, samples were collected from the bottom of the tube in 300 μ l fractions and each fraction was analysed with a gamma-counter. The position of the liposomes in the gradient could be identified either by radioactivity (when liposomes were prepared with labelled lipid or protein) or by visual observations of an opalescent band close to the top of the tube.

Fractions containing liposomes were pooled and dialysed against buffer B to remove sucrose.

Binding assay for receptor-reconstituted liposomes

Liposomes (15 μ l) were incubated with 5 nM- α -mannosidase in the presence and in the absence of 20 mM-mannose 6-phosphate or 20 mM-glucose 6-phosphate. All incubations were brought to a final volume of 50 μ l with buffer B and placed on ice for 90 min. Liposomes were aggregated by adding 25 μ l of a stearylamine solution (Eimerl *et al.*, 1980) and then 5 μ l of 1 M-MgCl₂ and placing the tubes on ice for 30 min. Liposomes containing bound α -mannosidase could be separated from unbound enzyme by centrifugation at 120 000 *g* for 10 min in a Beckman Airfuge. The supernatant was aspirated and the pellet gently washed with a 50 μ l overlay of buffer B. The pellets were dissolved in 50 μ l of buffer B containing 0.1% Triton X-100 and the bound α -mannosidase activity was assayed as described by Rome *et al.* (1979b), except that the fluorescence was measured using a Farrand MKI spectrofluorimeter (365 nm excitation, 450 nm emission).

Electron microscopy

Vesicle preparations were diluted 5-fold in buffer B and a small volume was applied to a carbon-film grid and negatively stained with 2% uranyl acetate as described previously (Valentine *et al.*, 1968; Lake & Kahan, 1975). The samples were viewed at 50 kV on a Hitachi HS-8 electron microscope.

Results

Incorporation of receptor, membrane protein and a soluble protein into lipid vesicles

Purified mannose 6-phosphate receptor was assayed for α -mannosidase binding activity, after immobilization on to diaminophenylthioether discs as described in the Experimental procedures section, to determine whether the purification procedure yielded active receptor (Table 1). Binding was slightly inhibited by glucose 6-phosphate, but was nearly 90% inhibited by mannose 6-phosphate. Receptor-diaminophenylthioether discs specifically bound 0.80 unit of α -mannosidase activity, whereas membrane protein-diaminophenylthioether discs showed no specific binding. [Membrane protein here refers to protein derived from Triton-solubilized bovine liver microsomal membranes that had been passed over the phosphomannan affinity column twice to remove all mannose 6-phosphate-receptor protein.] Such active receptor preparations were radioiodinated and used for the liposome reconstitution experiments described below. After iodination, there was no detectable decrease in binding activity (Steiner & Rome, 1982).

Fig. 1 shows the elution profiles from sucrose step

Table 1. Binding of α -mannosidase to mannose 6-phosphate receptor-diaminophenylthioether discs

Receptor-coupled discs were incubated with 5 nM- α -mannosidase and units of bound activity measured as described in the Experimental procedures section. Glucose 6-phosphate (Glc-6-P) or mannose 6-phosphate (Man-6-P) were used at concentrations of 10 mM. Control discs were prepared with receptor-free membrane protein. Values represent the averages of triplicate measurements; standard deviations were less than 10%.

	α -Mannosidase bound (units/disc)			Specific binding (units)
	Buffer	Glc-6-P	Man-6-P	
Receptor disc	0.91	0.81	0.11	0.80
Control disc	0.10	0.10	0.12	0

gradients of a number of different liposome preparations. Reconstitution of liposomes with ¹²⁵I-labelled mannose 6-phosphate receptor resulted in the incorporation of nearly all of the ¹²⁵I-labelled receptor into the liposomes (fractions 11–14, Fig. 1a). When the liposomes were prepared with [¹⁴C]phosphatidylcholine and unlabelled membrane protein, the distribution of radioactivity was the same (Fig. 1b). In the absence of phospholipid, free ¹²⁵I-labelled membrane protein remained at the bottom of the gradient (Fig. 1c). To determine the degree of protein-entrapment within liposomes, membrane protein-reconstituted vesicles were prepared in the presence of 0.01 mg of ¹²⁵I-labelled bovine serum albumin/ml (400 $\times$ 10⁶ c.p.m.). After centrifugation, most of the ¹²⁵I-bovine serum albumin was found at the bottom of the gradient (Fig. 1d), indicating that it was not associated with liposomes. However, 10% of the added ¹²⁵I-bovine serum albumin was associated with the liposomes (fractions 11–14, panel D). These fractions were pooled, treated with 0.05% taurodeoxycholate and re-isolated by isopycnic centrifugation. The ¹²⁵I-bovine serum albumin, previously liposome-associated, appeared at the bottom of the gradient (Fig. 1e). ¹²⁵I-labelled-membrane-protein-reconstituted liposomes were also treated with taurodeoxycholate and re-isolated by centrifugation. Unlike the experiments with ¹²⁵I-bovine serum albumin, labelled membrane protein did not appear at the bottom of the gradient. Instead the elution patterns of the taurodeoxycholate-treated (Fig. 1f, continuous line) and untreated liposomes (Fig. 1f, broken line) were identical. The peak liposome fractions in Fig. 1(f) were slightly displaced from those seen in Figs. 1(a)–1(d) because a smaller sample volume (1 ml) was applied to the bottom of each gradient.

Structure of liposomes

Plate 1 is an electron micrograph of negatively stained liposomes prepared from egg phosphatidyl-

Fig. 1. Elution profiles of ^{125}I -protein-reconstituted liposomes

Liposomes were prepared as described in the Experimental procedures section. Fractions were collected from gradients and counted for radioactivity. The percentage of the total recovered radioactivity was calculated for each fraction. (a), Liposomes reconstituted with ^{125}I -labelled receptor; (b), liposomes prepared with ^{14}C -labelled phospholipid and unlabelled protein; (c), ^{125}I -labelled membrane protein only; (d), liposomes prepared in the presence of ^{125}I -bovine serum albumin and unlabelled membrane protein; (e), fractions 10–14 from (d) after treatment with 0.045% taurodeoxycholate; (f), ^{125}I -labelled membrane protein-reconstituted liposomes (—) and ^{125}I -labelled-membrane-protein-reconstituted liposomes after treatment with 0.045% taurodeoxycholate (....).

choline and cholesterol (4:1, w/w) in the presence and in the absence of mannose 6-phosphate receptors purified from bovine liver. Receptor-reconstituted liposomes appeared to be fairly homogeneous, with diameters of 60–120 nm. Protein-free liposomes appeared different, having a more hetero-

Table 2. Binding of α -mannosidase to liposomes reconstituted with mannose 6-phosphate receptors from rat chondrosarcoma or bovine liver

Liposomes were incubated with 5 nM- α -mannosidase as described in the Experimental procedures section. Glucose 6-phosphate (Glc-6-P) or mannose 6-phosphate (Man-6-P) were used at concentrations of 20 mM. Specific binding refers to the α -mannosidase bound per μg of liposome-associated protein in buffer, minus that bound per μg in the presence of 20 mM-mannose 6-phosphate. Control liposomes refers to liposomes reconstituted with membrane protein. The specific activity of the pure α -mannosidase was 0.5 unit of enzyme activity per ng of enzyme.

Source	α -Mannosidase bound (ng/ μg)			Specific binding (ng/ μg)
	Buffer	Glc-6-P	Man-6-P	
Chondrosarcoma-receptor liposomes	1.36	0.91	0.17	1.19
Liver receptor liposomes	0.61	0.65	0.23	0.38
Control liposomes	0.10	0.15	0.15	0

geneous appearance and a larger average diameter (70–200 nm). Liposomes prepared without cholesterol had the same appearance in electron micrographs (results not shown).

There were many small regularly shaped structures of approx. 6 nm diameter barely visible on the surface of receptor-reconstituted liposomes (Plate 1A, inset, arrows). Since these structures do not appear in control liposomes (Plate 1B, inset), they may represent mannose 6-phosphate receptors.

Measurement of receptor binding activity

The receptor-reconstituted liposomes were routinely purified on sucrose gradients and the two peak fractions were pooled and dialysed (see the Experimental procedures section). Approx. 80% of the added receptor or membrane protein was incorporated into liposomes, yielding a protein concentration of approx. 0.5 mg/ml. When receptor-reconstituted liposomes were assayed for binding of α -mannosidase there was only a slight variation observed between different liposome preparations. Liposomes reconstituted with rat chondrosarcoma receptor bound 1.36 ng of α -mannosidase per μg of receptor (Table 2). Binding was nearly 90% inhibited by the addition of mannose 6-phosphate, whereas glucose 6-phosphate inhibited only slightly. Specific binding was 1.19 ng/ μg for chondrosarcoma receptor-reconstituted liposomes. Mannose 6-phosphate receptors were isolated from bovine liver to compare binding properties with the chondrosarcoma receptor. Liver receptor-reconstituted liposome binding was mannose 6-phosphate-specific but lower than that seen for chondrosarcoma receptor-lipo-

EXPLANATION OF PLATE 1

Electron micrograph of negatively stained liposome preparations

(A), Receptor-reconstituted liposomes prepared with phosphatidylcholine and cholesterol (4:1, w/w); (B), liposomes prepared with phosphatidylcholine and cholesterol (4:1, w/w) in the absence of protein. The insets are enlargements of the boxed areas in the main micrographs. Arrows indicate 5 nm particles in the receptor-reconstituted liposomes. Magnification of main micrographs $\times 101\,750$; magnification of insets, $\times 296\,000$. The bar represents 200 nm.

Fig. 2. Taurodeoxycholate treatment of ^{125}I -bovine serum albumin-entrapped or ^{125}I -labelled-receptor-reconstituted liposomes

Liposomes were treated with increasing concentrations of taurodeoxycholate for 30 min on ice, collected by centrifugation and counted for radioactivity. The radioactivity in the untreated control liposomes was set at 100% and all samples were normalized to this value. Symbols: $\circ$, ^{125}I -labelled-receptor-reconstituted liposomes; $\bullet$, ^{125}I -bovine serum albumin-entrapped liposomes.

somes (Table 2). Liposomes prepared with receptor-free membrane protein showed no specific binding (Table 2). Receptor-reconstituted liposomes prepared with (dimyristoyl)phosphatidylcholine, (distearoyl)phosphatidylcholine, cholesterol or combinations of these did not show altered receptor binding activity (results not shown). However, the incorporation of receptor protein into liposomes prepared using these lipids was only 30–50% as efficient as the incorporation seen when liposomes were prepared from egg phosphatidylcholine.

Determination of receptor topography

^{125}I -Bovine serum albumin-entrapped or ^{125}I -labelled-receptor-inserted liposomes were incubated for 90 min on ice with increasing concentrations of taurodeoxycholate to determine if there was a concentration of taurodeoxycholate that could render the vesicles permeable without solubilizing the membrane proteins. ^{125}I -Bovine serum albumin entrapped in liposomes was released rapidly at taurodeoxycholate concentrations above 0.02% (Fig. 2). At 0.045% taurodeoxycholate, greater than 90% of the ^{125}I -bovine serum albumin was released, whereas approx. 80% of the ^{125}I -labelled receptor remained liposome-associated. We assumed that since 0.045% taurodeoxycholate allowed the release of ^{125}I -bovine serum albumin from the liposomes, then it could also allow added α -mannosidase to enter the liposomes and bind to any receptors that might be facing the inside of the vesicles. Indeed, when similar

Table 3. Binding of α -mannosidase to receptor-reconstituted liposomes in the presence and in the absence of taurodeoxycholate

Chondrosarcoma receptor-reconstituted liposomes and coated vesicles isolated from rat liver (Campbell *et al.*, 1983) were assayed for α -mannosidase binding activity in the presence and in the absence of 0.045% taurodeoxycholate (see the Experimental procedures section). Specific binding represents α -mannosidase bound in buffer only per μg of protein (either liposome-associated or coated vesicle) minus that bound in the presence of 20 mM-mannose 6-phosphate. The percentage of receptors exposed is calculated as $100 \times$ the amount of bound α -mannosidase in the absence of taurodeoxycholate (tDOC) divided by that bound in the presence of 0.045% taurodeoxycholate. Values represent the averages of duplicate samples from two separate experiments.

	Specific binding (ng/ μg)		Receptor exposed (%)
	No tDOC	tDOC	
Receptor-reconstituted liposomes	0.37	0.37	100
Coated vesicles	0.027	0.37	7

measurements were performed with coated vesicles from rat liver and calf brain, receptor binding sites were found to be cryptic and were only detected when vesicles were disrupted with 0.045% taurodeoxycholate (Baumann & Doyle, 1980; Table 3). When receptor-reconstituted liposomes were assayed in the presence and absence of taurodeoxycholate no cryptic binding sites were discovered. In addition, binding activity was destroyed by treating intact liposomes with 0.75% trypsin for 30 min (results not shown), whereas cryptic receptors in coated vesicles were protected from the effects of trypsin (Campbell *et al.*, 1983). Trypsin did not enter the liposomes since treatment of ^{125}I -bovine serum albumin-entrapped liposomes did not result in the release or degradation of labelled bovine serum albumin (results not shown).

Quantification of receptor binding

Fig. 3 shows a concentration curve for the binding of α -mannosidase to chondrosarcoma receptor-reconstituted liposomes made from egg phosphatidylcholine in the presence and in the absence of 20 mM-mannose 6-phosphate. The apparent K_d value for α -mannosidase binding was estimated from Scatchard analysis (Scatchard, 1949) to be 1 nM. This value is similar to the K_d calculated for the binding of α -L-iduronidase to soluble chondrosarcoma receptor (Steiner & Rome, 1982), chondrosarcoma microsomes (Rome & Miller, 1980) and intact fibroblasts (Rome *et al.*, 1979a). A similar K_d was measured with bovine receptor liposomes prepared from (dimyristoyl)phosphatidylcholine

Fig. 3. Binding of α -mannosidase to receptor-reconstituted liposomes as a function of enzyme concentration. Portions (15 μ l) of chondrosarcoma receptor-reconstituted liposomes were assayed for α -mannosidase binding (see the Experimental procedures section) in the presence (○) and in the absence (●) of 20 mM-mannose 6-phosphate.

Fig. 4. Inhibition of α -mannosidase binding to receptor-reconstituted liposomes by mannose 6-phosphate. Chondrosarcoma receptor-reconstituted liposomes (15 μ l) were incubated with increasing concentrations of mannose 6-phosphate in the presence of 0.5 nM- α -mannosidase (▲), 2 nM- α -mannosidase (●) or 5 nM- α -mannosidase (○). Values are plotted as the reciprocal of bound α -mannosidase (ng^{-1}) at each mannose 6-phosphate concentration versus the concentration of mannose 6-phosphate.

(results not shown). As mentioned previously, binding was inhibited by mannose 6-phosphate. The inhibition was demonstrated to be competitive (Fig. 4), with a K_i value for mannose 6-phosphate of 2 mM. This value is approximately an order of magnitude higher than the K_i values measured for mannose 6-phosphate inhibition of binding to mannose 6-phosphate receptors in fibroblast and Swarm-rat chondrosarcoma membranes [0.1 mM (Rome *et al.*, 1979a) and 0.5 mM (Rome & Miller, 1980) respectively].

Discussion

Studies of the binding properties of the pure mannose 6-phosphate receptor with lysosomal enzymes have been difficult to perform due to physical and chemical similarities between receptor-bound and free ligand. Reconstitution of the receptor into liposomes has allowed us to carry out these studies since we can now quantitatively separate the free ligand from ligand that is receptor-bound. Receptors inserted into liposomes with very high efficiency (Fig. 1) and retained the ability to bind α -mannosidase (Table 2). Most liposome-associated receptors were oriented with binding sites facing towards the outside of the vesicle, as evidenced by the absence of measurable cryptic binding sites when liposomes were assayed under conditions that rendered them permeable to α -mannosidase (Table 3). It is unclear why the mannose 6-phosphate receptor would insert into liposomes in a non-random orientation. The asialoglycoprotein receptor, however, was also reported to insert preferentially into liposomes with 70% of the binding sites facing the outside of the vesicle (Baumann & Doyle, 1980). In addition, liposomes containing acetylcholine receptors prepared in the absence of cholesterol have nearly all ^{125}I - α -bungarotoxin-binding sites externally located (Anholt *et al.*, 1982). If rotation of receptors within the liposome membrane were possible, then receptors initially inserted at random might be induced to re-arrange when ligand is added. Although this explanation is possible, we consider it unlikely in the light of the destruction of all binding sites by trypsin (in the absence of ligand) and the apparent lack of rotation seen in coated vesicle membranes (Campbell *et al.*, 1983).

The binding activity of reconstituted receptors appeared to be unaffected by the type of phospholipid used to prepare the vesicles and the reconstitution procedure itself did not reduce the affinity of the receptor toward α -mannosidase (Fig. 3). The apparent K_d was similar to that measured for the mannose 6-phosphate receptor in whole cells (Rome *et al.*, 1979a; Rome & Miller, 1980; Steiner & Rome, 1982) and purified membrane preparations (Rome & Miller, 1980; Fischer *et al.*, 1980a). The calculated K_i for mannose 6-phosphate, however, was four times higher in liposomes than chondrosarcoma membranes (Rome & Miller, 1980). The calculated K_i for receptor-diaminophenylthioether discs was even higher (1 mM; C. H. Campbell, unpublished work). This apparent requirement for increasing concentrations of mannose 6-phosphate to inhibit binding may reflect a subtle change in the receptor after removal from its physiological environment.

Negatively stained preparations of mannose 6-phosphate-receptor-reconstituted liposomes viewed by electron microscopy appeared similar in size to

vesicles prepared with the acetylcholine receptor (Anholt *et al.*, 1982). Unlike vesicles reconstituted with acetylcholine receptors, which contain typical 8 nm doughnut-shaped receptor particles, mannose 6-phosphate receptor particles in our liposomes were difficult to identify definitively. Structures were seen, however, in reconstituted vesicles that were not present in the control vesicles.

The reconstitution of purified mannose 6-phosphate receptors into defined lipid vesicles will allow us to probe numerous physical and chemical properties of the receptor that could not be studied in a soluble system. In addition to the quantitative binding studies described here, we are attempting to carry out structural studies of the reconstituted receptor with the goal of defining the binding and membrane anchoring domains. Receptor-reconstituted liposomes should also serve as an ideal system for examining the effects of structural alteration on functional properties of the receptor.

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