Purification of rat liver plasma membranes by wheat-germ-agglutinin affinity partitioning

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Rat liver plasma membranes were separated from other cellular membranes by affinity partitioning in an aqueous polymer two-phase system, by using the lectin wheat-germ agglutinin covalently bound to dextran as the affinity ligand. In borate buffer the bulk of membranes partitioned in the poly(ethylene glycol)-rich top phase, whereas plasma membranes were pulled selectively into the dextran-rich bottom phase in the presence of ligand. The purity and yield of plasma membranes prepared by lectin affinity partitioning and by conventional sucrose-density-gradient centrifugation was similar, as judged from marker-enzyme activities. The affinity procedure, not dependent on lengthy centrifugations, is fast and gentle and will be advantageous when studying labile components.

INTRODUCTION

Membranes are usually fractionated on the basis of differences in physical properties. Thus, plasma membranes from rat liver are commonly purified by centrifugation techniques (Neville, 1960; Aronson & Touster, 1974; Wisher & Evans, 1975; Hubbard *et al.*, 1983), which rely on differences in size and equilibrium density for separation. These properties often partly overlap for different membranes, and may also depend on the homogenization procedure and the medium used. As a consequence, it may be difficult to obtain representative fractions of less abundant membranes, such as plasma membranes, in high purity and yield.

Two-phase partitioning in aqueous polymer systems, which separates membranes according to their surface properties (Albertsson *et al.*, 1982), is an alternative purification technique. It has been used for batch purification of plasma membranes from plant (Larsson, 1983) and mammalian cells (Brunette & Till, 1971; Lesko *et al.*, 1973; Gruber *et al.*, 1984), and for the separation of rat liver plasma-membrane domains (Gierow *et al.*, 1986). However, as the resolution of membranes by this approach is based on subtle surface differences, it is often less useful for bulk purification of membranes than for analytical purposes.

In this study we have examined the possibility of fractionating rat liver membranes by affinity partitioning in dextran/poly(ethylene glycol) two-phase systems with a lectin, wheat-germ agglutinin, covalently bound to dextran as the affinity ligand. The introduction of a biospecific ligand should allow the purification of membranes according to specific biological, rather than general physical, properties. Wheat-germ agglutinin binds reversibly to *N*-acetylglucosamine (GlcNAc), which is a common carbohydrate residue of plasma-membrane glycoproteins. This lectin, attached to Sepharose, has been used for isolating erythrocyte plasma membranes (Kaplan *et al.*, 1984). Because of unspecific interactions between membranes and Sepharose, this method is less useful for fractionating complex membrane mixtures. Affinity partitioning should therefore be a better alternative for purifying membranes.

Affinity partitioning using biospecific ligands immobilized on a phase polymer has been used successfully for the isolation of synaptic membrane domains. Thus cholinergic receptors from the electrical organ of *Torpedo californica* (Flanagan *et al.*, 1975; Johansson *et al.*, 1981) and opiate receptors from calf brain cortex (Olde & Johansson, 1985) have been purified by using bisquaternary amines and naloxone, respectively, as affinity

ligands. Here we present a rapid and efficient method for purifying rat liver plasma membranes by using the affinity-partitioning approach.

EXPERIMENTAL

Materials

Stock solutions of 20% (w/w) dextran T500 (Pharmacia Fine Chemicals AB) and 40% (w/w) poly(ethylene glycol) 3350 (Carbowax 3350; Union Carbide) were prepared as described by López-Pérez *et al.* (1981). UDP-[³H]galactose and [¹²⁵I]iodine were from New England Nuclear, and [³H]AMP was from Amersham International. Wheat-germ agglutinin, used as affinity ligand, was from Sigma Chemical Co. 2,2,2-Trifluoroethane-sulphonyl chloride (tresyl chloride) was from Synthelec, Lund, Sweden. All other reagents used were of analytical grade.

Preparation of membranes

Adult male Sprague–Dawley rats were starved overnight and killed in the morning by decapitation. The livers were immediately transferred to ice-cold 0.25 M-sucrose in 5 mM-Tris/HCl buffer, pH 8.0, and processed by the method of Aronson & Touster (1974). The microsomal P-fraction, the N₂- and P₂-fractions, highly enriched in apical and basolateral plasma membranes respectively, and the N-fraction, containing material sedimenting at low speed, were used for the partitioning experiments. Sedimented membrane fractions were resuspended to a protein concentration of about 5 mg/ml in 15 mM-boric acid adjusted to pH 7.8 with Tris base, or 15 mM-Tris adjusted to pH 7.8 with H₂SO₄.

Hepatocytes were prepared from adult male Sprague–Dawley rats (Seglen, 1973). Cells, sedimented at 75 *g* for 2 min, were resuspended in 15 mM-boric acid/Tris, pH 7.8, disrupted by N₂ cavitation (10 MPa, 10 min) and centrifuged at 1000 *g* for 5 min. The pellet was resuspended in half the volume of buffer, N₂-cavitated (5 min) and centrifuged as above. The combined supernatants were homogenized by three strokes with a tight-fitting Dounce homogenizer, then centrifuged at 4500 *g* for 5 min, and the protein content of the resulting supernatant was adjusted to about 5 mg/ml with buffer before use.

Activation of dextran

Dextran T500 was activated with tresyl chloride as described

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by Persson & Olde (1988). Dextran (5 g) was dissolved in 25 ml of dimethyl sulphoxide; 1 ml of triethylamine was added slowly, followed by 5 ml of dichloromethane. The solution was chilled on ice and 0.35 g of tresyl chloride was added dropwise with stirring. The mixture was stirred for 30 min on ice, for 60 min at 4 °C and overnight at room temperature. Then 50 ml of dichloromethane was added to terminate the reaction and to precipitate the dextran. The precipitate was washed and kneaded thoroughly with several portions of dichloromethane. The product was dried overnight at 20 °C before use.

Coupling of wheat-germ agglutinin to activated dextran

Wheat-germ agglutinin was coupled to activated dextran by the method of Nilsson & Mosbach (1981). Tresyl-dextran (3 g) was dissolved in 10 ml of coupling buffer (0.2 M-NaHCO₃, pH 7.5, adjusted with HCl) and 10 mg of wheat-germ agglutinin, dissolved in 1 ml of coupling buffer, was added. The coupling proceeded at 4 °C with gentle agitation for 24 h. The reaction was terminated by addition of 0.1 M-Tris/HCl, pH 7.5, to inactivate excess tresyl groups. Uncoupled wheat-germ agglutinin and salts were removed by ultrafiltration of the dextran-ligand mixture in a Minitan UF-unit, membrane cut-off 100 kDa (Millipore). The remaining solution, containing dextran-bound wheat-germ agglutinin, was freeze-dried and stored at -20 °C. The recovery of dextran was 40–50%.

The degree of substitution was determined by protein analysis (Bradford, 1976), with wheat-germ agglutinin as standard. Approx. 4.5 mg of wheat-germ agglutinin had bound per g freeze-dried product. Coupling of wheat-germ agglutinin at 20 °C for 22 h gave a similar degree of substitution, but the material had no affinity for plasma membranes, presumably owing to denaturation of the protein ligand. Wheat-germ agglutinin was also coupled to dextran T500 activated by CNBr (Kohn & Wilchek, 1982). Approx. 4.2 mg was bound per g of dextran. Dextran with tresyl-coupled ligand was used in all affinity-partitioning experiments presented unless otherwise indicated.

Two-phase partitioning

Two-phase polymer systems with a total weight of 2.00 g, including 0.20 g of sample, were prepared by weighing the required amount of polymer stock solutions, buffer and, when applicable, dextran-bound wheat-germ agglutinin. The two-phase systems were equilibrated overnight at 4 °C. Upon addition of sample the systems were turned upside down 20 times, vortex-mixed, and turned upside down another 20 times to ensure thorough mixing. The settling was speeded up by gentle centrifugation (Wifug Doctor, 1250 rev./min) for 5 min, and the top phase was separated from the bottom phase plus interface. The bottom phase plus interface was diluted before analysis with 0.50 ml of the buffer used in the specific system. All partitionings were performed at 4 °C.

Repeated affinity extraction

The crude N-fraction (Aronson & Touster, 1974) was partitioned in a two-phase system (total wt. 2.00 g), containing 5.6% dextran, including dextran-bound wheat-germ agglutinin coupled by CNBr (105 µg of ligand), 5.6% poly(ethylene glycol) and 16.5 mM-boric acid/Tris, pH 7.8. After settling, 0.9 ml of the top phase was removed and replaced by the same volume of pre-equilibrated top phase for a second extraction of the bottom phase. A third extraction was performed in the same manner, and the fractions obtained were analysed for protein and marker enzymes.

In some experiments 0.1 M-GlcNAc (total concentration in the system) was included in the last extraction with fresh top phase.

Then the systems were agitated gently for 30 min before phase separation.

Assays

Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard. The following plasma-membrane markers were measured: 5'-nucleotidase (EC 3.1.3.5; Avruch & Wallach, 1971), alkaline phosphodiesterase I (EC 3.1.4.1; Smith & Peters, 1980) and ¹²⁵I-asialo-orosomucoid binding (Hubbard *et al.*, 1983; Gierow *et al.*, 1986). Latent ¹²⁵I-asialo-orosomucoid binding, representing intracellular receptors mostly of endosomal origin (Pricer & Ashwell, 1976), was measured in the presence of 0.2% Triton X-100, with subtraction of binding in the absence of detergent. The endoplasmic-reticulum marker arylesterase (EC 3.1.1.2) was measured by the method of Shephard & Hubscher (1969), and the Golgi-membrane marker UDP-galactose:*N*-acetylglucosamine galactosyl-transferase (EC 2.4.1.38) as described by Fleischer & Smigel (1978), except that UDP-[³H]galactose was used instead of UDP-[¹⁴C]galactose (this is possible with some, but not all, batches of UDP-[³H]galactose). The mitochondrial marker succinate-cyto-

Fig. 1. Effect of polymer concentration and buffer composition on partitioning of rat liver membranes

Each point represents the amount recovered in the top phase (% of total) after partitioning of microsomes (0.7 mg of membrane protein of the P-fraction) in 2.00 g two-phase systems. After separation of the phases, the protein content (■) and the activities of alkaline phosphodiesterase (APDE, ▲) and arylesterase (AE, ●) were measured as described in the Experimental section. The specific activities of these enzymes in the microsomes were 137 and 4370 nmol/min per mg of protein respectively. (a) Polymer concentrations were varied in parallel, and the buffers used were 16.5 mM-boric acid adjusted to pH 7.8 with Tris base (—) or 16.5 mM-Tris adjusted to pH 7.8 with H₂SO₄ (---). (b) Variation of borate concentration. Boric acid was adjusted to pH 7.8 with Tris base. The concentration of the phase polymers was 6.0% (w/w).

chrome *c* reductase (EC 1.3.99.1) was assayed as described by Sottocasa *et al.* (1967). All analyses were performed in duplicate. Recoveries in the partitioning experiments were around 90%, except for galactosyltransferase, which was approx. 55%.

RESULTS

Effects of polymer concentration and buffer composition on the partitioning of membranes

The partitioning of membranes in aqueous polymer two-phase systems is influenced by both polymer concentration and added salt (Albertsson *et al.*, 1982). Thus the polymer concentration affects hydrophilic/hydrophobic interactions between polymer and particle, whereas ions, striving to distribute differently between the phases, cause an electrical potential difference across the interface, thereby affecting the partitioning of charged particles, such as membranes.

We first established conditions where the bulk of membranes partitioned in the poly(ethylene glycol)-rich top phase. This allowed us to draw plasma membranes selectively into the bottom phase by the dextran-coupled affinity ligand. In the presence of Tris/H₂SO₄ buffer, used previously for two-phase fractionation of liver membranes (Gierow *et al.*, 1986), the partitioning of membrane markers was strongly dependent on polymer concentration (Fig. 1*a*). When borate buffer was used instead, more than 90% of both the plasma-membrane marker (alkaline phosphodiesterase) and the endoplasmic-reticulum marker (arylesterase) partitioned in the top phase throughout the polymer concentration range examined (Fig. 1*a*).

The effect of borate concentration on the partitioning of membranes in a two-phase system containing 6.0% of each of the phase polymers was also examined (Fig. 1*b*). Approx. 90% of the plasma-membrane marker partitioned in the top phase already at 1.5 mM-borate, compared with 45% in the same system with 16.5 mM-Tris/H₂SO₄ buffer (Fig. 1*a*). However, 10 mM-borate was required to obtain 90% of the endoplasmic-reticulum marker in the top phase, in line with the comparatively

low distribution of these membranes in two-phase systems (Larsson, 1983; Gierow *et al.*, 1986). The proportion of membranes in the top phase did not increase further at higher borate concentrations. The concentration selected for further experiments (16.5 mM) was sufficient for a similar high distribution of markers for Golgi membranes and mitochondria (results not shown).

Two-phase systems buffered with boric acid/Tris had another advantage over systems buffered with Tris/H₂SO₄. When this latter buffer was used, material had a strong tendency to collect at the interface, but distributed preferentially into either of the phases in the presence of borate. This may be due to the high interfacial potential, generated because of the high affinity of borate for dextran.

Affinity partitioning of rat liver membranes

To examine the feasibility of using wheat-germ agglutinin as an affinity ligand, highly purified rat liver plasma membranes of the apical (Fig. 2*a*) and basolateral (Fig. 2*b*) domains were partitioned in two-phase systems containing increasing concentrations of wheat-germ agglutinin bound to dextran. The addition of small amounts of ligand efficiently drew the plasma-membrane markers tested of both domains from the poly(ethylene glycol)-rich top phase into the dextran-rich bottom phase. Asialo-orosomucoid receptors, marker for the basolateral domain, partitioned slightly higher than the apical markers 5'-nucleotidase and alkaline phosphodiesterase. The difference was too small, however, to exploit for the ready separation of these domains by using wheat-germ agglutinin as ligand. Approx. 7 µg of membrane protein was redistributed into the bottom phase per µg of lectin ligand added. The effect of the ligand was inhibited by 0.1 M-GlcNAc, indicating a specific interaction between ligand and membrane (Fig. 2*a*).

The membrane specificity of the ligand was tested on a microsomal fraction (the P-fraction) containing various membranes (Fig. 2*c*). The plasma-membrane markers alkaline phosphodiesterase and 5'-nucleotidase again redistributed into

Fig. 2. Affinity partitioning of rat liver membranes

Membranes were partitioned (see the Experimental section) in the presence of increasing amounts of dextran-bound wheat-germ agglutinin (WGA). The 2.00 g phase systems contained 5.6% (w/w) each of poly(ethylene glycol) and dextran in 16.5 mM-boric acid/Tris, pH 7.8. After separation of the phases, the protein content (■), alkaline phosphodiesterase (APDE, ▲), 5'-nucleotidase (5'-Nase, □), arylesterase (AE, ●) and galactosyltransferase (Gal, ○) activities, and asialo-orosomucoid binding (ASOR, △) were measured. (a) Partitioning of purified plasma membranes of the apical domain (the N₂-fraction; 1.11 mg of membrane protein added to each system). The experiment was performed in the absence or presence of 0.1 M-GlcNAc. The specific activities (nmol/min per mg of protein) of marker enzymes in the N₂-fraction were: alkaline phosphodiesterase, 4050; 5'-nucleotidase, 1140. Asialo-orosomucoid binding was 300 ng/mg of protein. Arylesterase and galactosyltransferase were not measured because of low activities. (b) Partitioning of purified plasma membranes of the basolateral domain (the P₂-fraction; 0.67 mg of membrane protein added to each system). The specific activities (nmol/min per mg of protein) of marker enzymes in the P₂-fraction were: alkaline phosphodiesterase, 680; 5'-nucleotidase, 250. Asialo-orosomucoid binding was 510 ng/mg of protein. (c) Partitioning of the microsomal fraction (the P-fraction; 0.83 mg of membrane protein added to each system). Latent asialo-orosomucoid binding was measured in a separate series (0.66 mg of membrane protein added). The partitioning behaviour of protein, alkaline phosphodiesterase and arylesterase was the same in the two experiments. The specific activities (nmol/min per mg of protein) in the preparation were: alkaline phosphodiesterase, 80; 5'-nucleotidase, 57; arylesterase, 4180; galactosyltransferase, 1.2. Latent asialo-orosomucoid binding was 150 ng/mg of protein.

Fig. 3. Affinity partitioning of hepatocyte membranes

Post-mitochondrial supernatant from isolated hepatocytes (1.27 mg of protein) was partitioned in the presence of increasing amounts of dextran-bound wheat-germ agglutinin (WGA) under the conditions described in the legend to Fig. 2. Symbols are as in Fig. 2. The specific activities (nmol/min per mg of protein) in the preparation were: alkaline phosphodiesterase (APDE), 63; 5'-nucleotidase (5'-Nase), 13; arylesterase (AE), 1620; galactosyltransferase (Gal), 0.35.

the bottom phase in the presence of increasing amounts of wheat-germ-agglutinin-dextran. In contrast, the endoplasmic-reticulum marker arylesterase remained in the top phase even at a ligand concentration (112.5 µg of wheat-germ agglutinin per 2.00 g system) high enough to direct more than 90% of the plasma-membrane markers to the bottom phase. The distribution of latent asialo-orosomucoid receptors, presumably located in endosomes (Pricer & Ashwell, 1976), and of the Golgi-membrane marker galactosyltransferase was relatively unaffected by the ligand.

Affinity partitioning of membranes from isolated hepatocytes

The effect of wheat-germ-agglutinin-dextran on the partitioning of membranes present in a postmitochondrial supernatant from isolated hepatocytes was also examined (Fig. 3). The partitioning behaviour of various marker enzymes was similar to that of the microsomal fraction (cf. Fig. 2c). Thus most of the alkaline phosphodiesterase and 5'-nucleotidase were drawn into the bottom phase even by a low concentration of affinity ligand.

Arylesterase remained in the top phase throughout the concentration interval examined, whereas galactosyltransferase partly redistributed into the bottom phase in a ligand-concentration-dependent manner.

Repeated affinity extraction

The degree of purification of plasma membranes that could be obtained by affinity partitioning was compared with that obtained by conventional sucrose-gradient centrifugation. For this purpose the crude N-fraction (Aronson & Touster, 1974) containing plasma membranes, endoplasmic reticulum, mitochondria and Golgi membranes (cf. Lundberg *et al.*, 1985) was used. This fraction was subjected either to centrifugation in a discontinuous sucrose gradient (Aronson & Touster, 1974), or to two-phase affinity partitioning followed by two re-extractions of the lectin-containing bottom phase with fresh top phase (Table 1).

Table 1 shows that both the enrichment and the yield of the plasma-membrane markers alkaline phosphodiesterase and 5'-nucleotidase by affinity partitioning was similar to that obtained in the sucrose gradient. The contamination by markers for other membranes was similar in the two cases.

The reversibility of the plasma-membrane-ligand interaction was studied by including 0.1 M-GlcNAc in the last extraction step. Then approx. 90% of the alkaline phosphodiesterase and 5'-nucleotidase remaining in the wheat-germ-agglutinin-containing bottom phase was released into the top phase. It is thus possible to separate purified plasma membranes from the affinity ligand by partitioning under these conditions.

DISCUSSION

The results presented confirm that affinity partitioning in aqueous polymer two-phase systems is a useful method for the purification of membranes. By using the lectin wheat-germ agglutinin as affinity ligand, plasma membranes from rat liver as well as from isolated hepatocytes were separated efficiently from other cellular membranes. The degree of purification and yield of liver plasma membranes by affinity partitioning were similar to those with conventional sucrose-gradient-centrifugation method.

A prerequisite for using dextran-linked ligand was to find conditions under which the bulk of membranes would partition in the poly(ethylene glycol)-rich top phase. This was attained by adding borate. As borate has affinity for saccharides (Zittle,

Table 1. Comparison of plasma membranes purified by sucrose-gradient centrifugation and repeated affinity extraction

Plasma membranes were purified as described previously (the N₀-fraction; Aronson & Touster, 1974) or by repeated affinity extraction (see the Experimental section) of the N-fraction. Yields and relative specific activities (RSA) were calculated relative to the homogenate (sum of the 3000 g supernatant and pellet obtained as described by Aronson & Touster, 1974). The results are based on two parallel preparations each using two livers. Elution with GlcNAc, based on one preparation, was done after only two extractions with top phase. Additional purifications by affinity extraction, not included in the Table, gave similar results. The values given are means $\pm$ deviations from the mean. The average specific activities (nmol/min per mg of protein) in the homogenates were: 5'-nucleotidase, 16; alkaline phosphodiesterase, 50; arylesterase, 850; galactosyltransferase, 0.39; succinate-cytochrome *c*-reductase, 54. The homogenates contained 0.15 g of protein/g of liver. Abbreviation: N.D., not determined.

Component	Sucrose-gradient centrifugation		Repeated affinity extraction		Affinity extraction and elution with GlcNAc	
	RSA	Yield (%)	RSA	Yield (%)	RSA	Yield (%)
Protein		0.44 $\pm$ 0.02		0.47 $\pm$ 0.03		0.68
5'-Nucleotidase	75 $\pm$ 1	33 $\pm$ 1	61 $\pm$ 9	29 $\pm$ 6	42	29
Alkaline phosphodiesterase	63 $\pm$ 1	28 $\pm$ 1	43 $\pm$ 4	21 $\pm$ 3	31	21
Arylesterase	0.66 $\pm$ 0.05	0.29 $\pm$ 0.02	0.46 $\pm$ 0.04	0.22 $\pm$ 0.03	1.08	0.74
Galactosyltransferase	16 $\pm$ 1	7 $\pm$ 1	6 $\pm$ 1	3 $\pm$ 1	8	3
Succinate-cytochrome <i>c</i> reductase	< 0.5	< 0.2	< 0.5	< 0.2	N.D.	N.D.

1951), it will distribute preferentially in the dextran-rich bottom phase. The result is a phase, containing a negatively charged polyelectrolyte, forcing the negatively charged membranes to the top phase by electrostatic repulsion.

The partitioning experiments with post-mitochondrial supernatant of isolated hepatocytes and the crude N-fraction from liver indicate the possibility of obtaining highly purified plasma membranes directly from a crude homogenate by affinity partitioning. As phase separation is rapid, the method should allow the fast purification of plasma membranes; it should be possible to purify these membranes in 20 min or less, depending on the number of extractions required. A further advantage of the method is that hydrostatic forces due to high-speed centrifugation are avoided. Thus membrane preparation by affinity partitioning should be of interest when labile components are to be examined.

In this work we have used wheat-germ agglutinin as a ligand for the purification of plasma membranes from liver cells by affinity partitioning. In principle, it should be possible to use this lectin for purifying plasma membranes from other sources, or, in general, to purify membranes having lectin-binding sites by using other lectins of suitable specificity. As the association constant of wheat-germ agglutinin for saccharides is in the range 10^4 – 10^5 M⁻¹ (Lotan & Sharon, 1973; Nagata & Burger, 1974), high-affinity binding appears not to be a prerequisite for affinity partitioning of membranes. In this respect partitioning may be less demanding than affinity chromatography, which seems to require 10-fold higher association constants or more for binding (Baenziger & Fiete, 1979). It is not clear at present, however, how the partitioning behaviour of membranes is affected by the number and density of binding sites, or by the strength of their interactions with the ligand. Multiple binding sites of high density, with similar affinities for the ligands to those in the present study, also applied when membranes enriched in cholinergic and opiate receptors were purified by affinity partitioning (Flanagan *et al.*, 1975; Johansson *et al.*, 1981; Olde & Johansson, 1985). It will be of interest to examine whether relatively few high-affinity binding sites, as in antigen–antibody or hormone–receptor interactions, will be sufficient for the purification of membranes by affinity partitioning.

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