Lateral heterogeneity of rat liver plasma membranes analysed by counter-current distribution

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The lateral heterogeneity of rat liver plasma membranes was examined by fragmentation and fractionation by counter-current distribution in an aqueous two-phase polymer system. The distribution pattern was analysed by plotting the relative specific activities of marker components against each other. By this analysis asialo-orosomucoid receptors were found in a domain separated from domains containing 5'-nucleotidase and leucine aminopeptidase by another domain devoid of these markers. 5'-Nucleotidase and leucine aminopeptidase resided in adjacent but separate domains. The experimental data were compared with corresponding plots of markers in model membranes. The model membranes yielded plots of different shapes depending on marker distribution and fragment size. This method of analysis should be useful for examining the lateral heterogeneity also of other membranes.

INTRODUCTION

The hepatocyte plasma membrane consists of three discrete domains, the blood-sinusoidal, the bile-canalicular and the contiguous (lateral) domain. The preparation of one or more of these domains is usually achieved by various centrifugation techniques (Neville, 1960; Aronson & Touster, 1974; Wisher & Evans, 1975; Hubbard *et al.*, 1983; Taylor *et al.*, 1983; Meier *et al.*, 1984), and they are identified by their contents of marker components, often in combination with electron microscopic examinations. One problem in the identification of the membrane fractions is the proper assignment of marker components to membranes. Thus, a low concentration of a marker in a fraction may be due to lateral heterogeneity within a membrane or, alternatively, due to contamination from other membranes.

We have recently reported on the separation and further purification of blood-sinusoidal and bile-canalicular plasma membrane fractions from rat liver by aqueous two-phase counter-current distribution (Gierow *et al.*, 1986). This method fractionates membrane material due to surface properties and, therefore, in addition to being a complement to conventional preparation methods, it might be useful for studying lateral heterogeneity of membranes.

Fragmentation of a membrane which consists of discrete domains will yield fragments with different composition depending on fragment size and the organization of the domains. The lateral heterogeneity of such a randomly fragmented membrane can be analysed after fractionation, e.g. by two-phase partitioning, by a procedure described by Albertsson (1988) and recently applied on the thylakoid membrane (Melis *et al.*, 1986; Albertsson, 1987; Albertsson & Svensson, 1987). In this analysis the concentration of one membrane marker component is plotted against that of another for each of the fractions obtained. The resulting plots will have different shapes depending on the fragment size and the lateral distribution of markers in the membrane. This approach is analogous to the use of fragmentation for sequence determination of proteins and was originally

proposed by Tiselius (1954) for structural analysis of cell components.

We have applied this procedure on rat liver plasma membranes. Differently fragmented membranes were fractionated by aqueous two-phase counter-current distribution before analysis of marker components. The lateral heterogeneity of plasma membranes is discussed on the basis of the resulting plots compared with plots of model membranes.

EXPERIMENTAL

Materials

Stock solutions of 20% (w/w) Dextran T500 (Pharmacia) and 40% (w/w) polyethylene glycol 3350 (Carbowax 3350, Union Carbide) were prepared as described by López-Pérez *et al.* (1981). [³H]AMP and [¹²⁵I]iodide were from Amersham International. Other reagents used were all of analytical grade.

Preparation of plasma membranes

Male Sprague-Dawley rats, weighing around 200 g, were starved overnight and killed the following morning by decapitation. The liver was quickly transferred to ice-cold 0.25 M-sucrose in 5 mM-Tris/HCl, pH 8.0, sliced and passed through a tissue press. After six up and down strokes in a Dounce homogenizer (loose-fitting pestle) the modified method (Wisher & Evans, 1975) of Aronson & Touster (1974) was followed. Briefly, a 105 000 g pellet was obtained, suspended in buffered 57% (w/v) sucrose by ten up and down strokes in a Dounce homogenizer (tight-fitting pestle). This suspension was used as bottom layer for discontinuous sucrose-gradient centrifugation, which finally yielded the plasma membranes (the P₂-fraction of Aronson & Touster, 1974) suspended in 15 mM-Tris/H₂SO₄, pH 7.8.

Thin-layer counter-current distribution

Plasma membranes (20 mg of protein) were added to a sample system with a total weight of 8.00 g, containing 6.0% Dextran T500, 6.0% polyethylene glycol 3350,

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15 mM-Tris/H₂SO₄, pH 7.8, and 3 mM-KCl. A standard thin-layer counter-current distribution apparatus (Albertsson, 1965, 1970) was loaded with sample system and separate top and bottom phases as described earlier (Gierow *et al.*, 1986). After completion of the separation cycle (about 13 h) the phase system was broken by the addition of 1.4 ml of 10 mM-Tris/HCl, pH 7.4, to each cavity, and the whole block was shaken for 1 min. The unloaded fractions were analysed directly for protein and enzyme activities. A representative experiment is shown in Fig. 3.

Centrifugal counter-current distribution

The apparatus has been described in detail elsewhere (Åkerlund, 1984). Briefly the partition block consists of an outer ring for the bottom phase and an inner ring for the top phase, both making up 60 partition cavities.

Plasma membranes were either used directly, or sonicated just prior to use. A Branson Sonifier cell disruptor, model B 15, equipped with a microtip was used. The intensity output was set to 2, the pulsed duty cycle was set at 50% and the total time was set at 1.5 or

Fig. 1. Cross-section of a hypothetical membrane and plots of concentrations of domains A and B

Above: Cross-section of a hypothetical membrane consisting of three alternating membrane domains (each of size L), one containing marker A, the second devoid of markers and the third containing marker B. Below: Plots of concentrations of A against B for fragments of different sizes, obtained by random fragmentation of the membrane.

Fig. 2. Cross-section of a hypothetical membrane and plots of concentrations of domains B and C

Above: Cross-section of a hypothetical membrane consisting of domain C surrounded by domain B, both having size L . These domains are well separated from a third domain, A, by a fourth domain devoid of markers. Below: Plots of concentrations of B against C for fragments of different sizes obtained by random fragmentation of the membrane.

6 min. After sonication the membranes were added to a sample system with the same composition as above but with a total weight of 10.00 g. Bottom phase (2.00 g) was added to obtain equal volumes of the phases.

The volume of the bottom phase compartment of the partition block was 1.00 ml, but only 0.90 ml was used to keep both the interface and the bottom phase stationary. Cavities 0–4 were each loaded with 1.80 ml of well-mixed sample system, whereas the remaining cavities received 0.90 ml of bottom phase and 0.90 ml of top phase. Shaking was set to 30 s, centrifugation to 90 s, and with 53 transfers the whole cycle was completed within 2 h. 10 mM-Tris/HCl (0.90 ml, pH 7.4) was added to each cavity to break the phase system, followed by the treatment described above. Experiments representative of two to several distributions are shown in Figs. 4 to 6.

Assays

The plasma membrane markers measured were: 5'-nucleotidase (EC 3.1.3.5; Avruch & Wallach, 1971), leucine aminopeptidase (EC 3.4.11.1; Roman &

Fig. 3. Thin-layer counter-current distribution of unsonicated plasma membranes

The distribution was performed as described in the Experimental section. The specific activities in the plasma-membrane preparation were 5'-nucleotidase, 597 nmol/min per mg of protein; leucine aminopeptidase, 114 nmol/min per mg of protein; asialo-orosomucoid binding, 261 ng/mg of protein. Marker enzyme activities and protein were calculated as percentages of total amounts recovered, and expressed as fractions of the total. Recoveries were above 90 %. Protein (●), 5'-nucleotidase (□), leucine aminopeptidase (△) and asialo-orosomucoid binding (○). Panel (a) shows the distribution profile and panels (b)–(d), plots of specific activities of marker components relative to those of the plasma membrane for each fraction.

Hubbard, 1983) and ^{125}I -asialo-orosomucoid binding (Hubbard *et al.*, 1983; Gierow *et al.*, 1986). Protein was determined by the method of Lowry *et al.* (1951), modified as described previously (Gierow *et al.*, 1986).

RESULTS

Hypothetical models

The principle of using counter-current distribution as a tool in the study of domain localization of enzymes has been described by Albertsson (1988). In the present case we consider a hypothetical membrane with three domains, each having length L in cross-section (Fig. 1). Two domains, containing either component A or B, are separated by a third domain lacking these components. If this membrane is fragmented randomly to progressively smaller fragments and the concentration of component A (expressed as its specific activity) in the fragments is plotted against component B, the plots of Fig. 1 are obtained. The fragment size of $2L$ gives a straight line with a negative slope. The smallest fragment size, L or less, results in lines parallel with the x - and y -axes, or

coinciding with these axes in case components A and B are completely restricted to respective domains. Intermediate fragment sizes yield plots in which lines parallel with the axes are connected by a line with a negative slope.

A second model is shown in Fig. 2, in which the components B and C are located in adjacent domains but well separated from component A. Plotting the concentration of component B versus that of component C yields a straight line with a positive slope if the fragment size is large (more than $3L$). Reducing the fragment size results in plots of various complexities as indicated in Fig. 2.

Counter-current distribution

When rat liver plasma membranes were subjected to thin-layer counter-current distribution two peaks of plasma-membrane marker components were resolved (Fig. 3a). The asialo-orosomucoid receptor, marker for the blood-sinusoidal region, was highly enriched in the left-hand peak, which also contained approx. 70 % of the total protein. Both this marker and the protein diminished from left to right, the former yielding a shoulder to

Fig. 4. Centrifugal counter-current distribution of unsonicated plasma membranes

The specific activities of the plasma membrane preparation were for 5'-nucleotidase, 577 nmol/min per mg of protein; leucine aminopeptidase, 48.1 nmol/min per mg of protein; asialo-orosomucoid binding, 163 ng/mg of protein. Open circles represent values close to the detection limit. See Fig. 3 for further details.

the right (Fractions 30–35). In contrast, the bile-canalicular marker, leucine aminopeptidase, distributed into two peaks (around Fractions 6 and 44, respectively). The more general plasma-membrane marker, 5'-nucleotidase, showed three peaks of activity; two major peaks coinciding with those of leucine aminopeptidase, and a third minor peak in between (Fractions 22–24).

The specific activities of the three markers relative to those of the plasma membrane were plotted against each other as depicted above (Figs. 3b to 3d). Leucine aminopeptidase and 5'-nucleotidase activities followed each other closely, whereas asialo-orosomucoid binding decreased rather linearly with increasing 5'-nucleotidase activity. Consequently, asialo-orosomucoid binding also decreased with increasing leucine aminopeptidase activity, but in this case a more pronounced break in the negative slope was observed than with 5'-nucleotidase.

The two-phase partitioning behaviour of membranes has been shown to be a dynamic and non-equilibrium process, i.e., it can change with time both due to the kinetics of the settling of the phases (Fisher & Walter, 1984) and alterations in the membrane material (Johansson *et al.*, 1984; Heywood-Waddington *et al.*, 1986). It was therefore of interest to study the effects of the more rapid centrifugal counter-current distribution technique. The general distribution of the markers into

two peaks was similar to that obtained by the conventional thin-layer method, although with a markedly improved separation of the peaks (Fig. 4a). In addition, the right-hand peak had moved further to the right, probably due to enhanced phase separation. Some changes were noted in the marker component plots. Leucine aminopeptidase and 5'-nucleotidase activities still followed each other closely (Fig. 4b), but a pronounced break was observed in the plot of asialo-orosomucoid binding versus both leucine aminopeptidase and 5'-nucleotidase (Figs. 4c and 4d). It should be noted, however, that several measuring values from the right-hand part of the distribution profile were close to the detection limit (indicated by open circles in the plots).

Effect of sonication

As shown in the hypothetical models above, changes in fragment size may result in changes in the plots of relative specific activities. In order to study this experimentally the plasma membrane fraction was subjected to sonication for either 1.5 or 6 min. The distribution pattern soon changed markedly after the shorter treatment (Fig. 5a). Thus, 5'-nucleotidase and leucine aminopeptidase activities were shifted to the right, leaving very little activity in the left-hand peak. Protein also shifted to the right, resulting in an equal distribution of

Fig. 5. Centrifugal counter-current distribution of plasma membranes sonicated for 1.5 min

The specific activities of the plasma membrane preparation were for 5'-nucleotidase, 594 nmol/min per mg of protein; leucine aminopeptidase, 100 nmol/min per mg of protein; asialo-orosomucoid binding, 177 ng/mg of protein. See Fig. 3 for further details.

Fig. 6. Centrifugal counter-current distribution of plasma membranes sonicated for 6 min

The specific activities of the plasma membrane preparation were for 5'-nucleotidase, 609 nmol/min per mg of protein; leucine aminopeptidase, 102 nmol/min per mg of protein; asialo-orosomucoid binding, 140 ng/mg of protein. See Fig. 3 for further details.

protein between left and right. The distribution of asialo-orosomucoid receptor was affected only slightly however. A plot of the relative specific 5'-nucleotidase activity versus that of leucine aminopeptidase resulted in a broken line (Fig. 5b). Plots of asialo-orosomucoid binding against either 5'-nucleotidase or leucine aminopeptidase were more complex (Figs. 5c and 5d), each yielding one line with a positive slope, and a broken line with a negative slope.

Sonication for up to 6 min did not result in further profound changes in the distribution pattern (Fig. 6a), but increasing amounts of protein and a slightly elevated level of asialo-orosomucoid binding was recovered in the right-hand peak. The activity plots had changed into right-angled configurations (Figs. 6b to 6d).

DISCUSSION

The plot of leucine aminopeptidase activity against 5'-nucleotidase after counter-current distribution of unsonicated plasma membranes, i.e. less fragmented material, resulted in a straight line with a positive slope. Thus, the two marker enzymes co-fractionated under these conditions, suggesting that they were located together in these membrane fragments. This resembles the plot obtained for components B and C in large fragments of the model membrane of Fig. 2.

The plots of asialo-orosomucoid binding against either leucine aminopeptidase or 5'-nucleotidase activity instead yielded negative slopes with breaks. A negative slope is consistent with the presence of the receptors and the marker enzymes in different fragments, i.e. asialo-orosomucoid binding is located in a domain that is separate from the domain containing leucine aminopeptidase and 5'-nucleotidase (cf. components A and B in Fig. 1). The break in the slope suggests the possibility of a more complete separation of the markers upon further fragmentation of the membrane.

Fragmentation of the plasma membrane by sonication for 1.5 min before counter-current distribution resulted in a change in the appearance of the plot of leucine aminopeptidase against 5'-nucleotidase activity from a straight line with a positive slope to a broken line. A further shift to a right-angled plot was obtained by prolonged sonication. This configuration is consistent with the presence of 5'-nucleotidase and leucine aminopeptidase in close but separate domains (compare Fig. 6b with the plot of fragment size 2L of Fig. 2, reversing the axes).

The plots of asialo-orosomucoid binding against the marker enzymes obtained after 1.5 min of sonication were complex but similar to fragment size 1.5 L of Fig. 1. After the longer sonication, however, they shifted to a right-angled configuration similar to that of small fragments of the model membrane of Fig. 1. This is consistent with the location of asialo-orosomucoid receptors in a domain separated from domains containing 5'-nucleotidase and leucine aminopeptidase by an additional domain lacking these markers.

Leucine aminopeptidase has been localized exclusively to the bile-canalicular membrane region (Roman & Hubbard, 1983), whereas the subcellular distribution of 5'-nucleotidase seems less clear. The latter enzyme has been reported to be roughly equally distributed between bile-canalicular and blood-sinusoidal regions (Matsuura *et al.*, 1984), and also, in low concentrations, in the

contiguous area (Wisher & Evans, 1975). On the other hand, Bartles *et al.* (1985) suggested a localization predominantly in the bile-canalicular region. Our results support the latter suggestion, since only a small amount of either enzyme distributed with the asialo-orosomucoid receptors. These receptors reside mainly in the blood-sinusoidal region (Matsuura *et al.*, 1982; Hubbard *et al.*, 1983), although some sites of unknown significance have been detected in bile-canalicular membranes (Matsuura *et al.*, 1982). However, the experiment with counter-current distribution of unsonicated membranes indicated that no binding components were present in the bile-canalicular region.

The specific activities of the marker components after counter-current distribution are depicted in the Figures. The highest specific activities of 5'-nucleotidase and leucine aminopeptidase (approx. 2000 and 500 nmol/min per mg of protein, respectively) were observed in the right-hand peak after thin-layer or centrifugal counter-current distribution without prior sonication. This represents a purification over the homogenate of around 70- and 50-fold, respectively, and 3- to 5-fold over the plasma membrane fraction used for the distribution. The contamination of membranes containing asialo-orosomucoid receptors in the right-hand peak was low, particularly after centrifugal counter-current distribution. The specific activities of the right-hand peak diminished, although the total activities increased, when sonication preceded the distribution step. This treatment did not affect the enzyme activities. Sonication rather caused the shift of material, both containing and devoid of the marker enzymes, from left to right, thereby increasing the amount of protein to the right.

Contrary to the marker enzymes, the specific binding of asialo-orosomucoid was highest in counter-current fractions obtained after sonication. The treatment itself increased the specific binding 1.6-fold, in line with the report by Baenziger & Fiete (1980) that sonication unmasks hidden non-plasma membrane asialo-orosomucoid binding sites. However, the subsequent counter-current distribution increased the specific binding another 4-fold in the left-hand peak fractions, yielding an over 100-fold purification compared with the homogenate.

The separation time of centrifugal counter-current distribution is shorter than that of thin-layer counter-current distribution. This is an advantage when labile material is used. However, thin-layer counter-current distribution might give additional separation because of the less complete separation of the phases and the presence of lower phase droplets in the upper phase as has been demonstrated by Fisher & Walter (1984). This may explain why the asialo-orosomucoid binding marker gave a bimodal distribution in the thin-layer counter-current distribution (Fig. 3), but only one peak in the centrifugal counter-current distribution (Fig. 4).

Counter-current distribution is a useful method for separation of cell organelles and membrane vesicles, particularly since it separates according to their surface properties. It is not only useful as a preparative method for purification but also, in conjunction with the plotting method, as an analytical tool to gain information of the lateral heterogeneity of membranes. The plotting method gives additional information compared to the counter-current distribution diagram and can help in building a model of the membrane. This approach, here exemplified with a rat-liver plasma-membrane fraction, should be

applicable for studies on the domain organization of biological membranes in general.

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