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SUBSTRATE BINDING SITE OF MICROSOMAL CYTOCHROME P-450 DIRECTLY FACES MEMBRANE LIPIDS

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SUMMARY: Cytochrome P-450, purified from liver microsomes of phenobarbital-treated rabbits, was incorporated into dimyristoylphosphatidylcholine liposomes. The binding of benzphetamine to the liposome-bound cytochrome P-450 was examined by measuring the benzphetamine-induced spectral change at various temperatures. The van't Hoff plot of the apparent spectral dissociation constant showed a distinct break at the temperature of phase transition of the synthetic lipid. On the other hand, no such break was observed for benzphetamine binding to microsomal bound cytochrome P-450. These results suggest that the substrate binding site of cytochrome P-450 is embedded in the apolar interior of phospholipid bilayer membranes.

Hepatic microsomal cytochrome P-450 (P-450)[2] catalyzes monooxygenation of various compounds such as drugs, carcinogens and steroids (1). Since these compounds are lipophilic and expected to accumulate in cellular membranes rather than in the cytosol, it has been suspected that the substrate binding site of P-450 is embedded in the microsomal membrane and substrates dissolved in the membrane bind to P-450 (2,3). However, little is as yet known of the topological location of the substrate binding site of microsomal P-450. One difficulty in such studies is that the substrate binding site of P-450 is thought to be itself hydrophobic and therefore it is not possible to conclude that the

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[2] Abbreviations: P-450, cytochrome P-450; DMPC, dimyristoylphosphatidylcholine.

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substrate binding site is located in the membrane from the observation that the hydrophobicity of substrates determines the tightness of their binding to P-450.

In this study, a major inducible form of P-450, purified from liver microsomes of phenobarbital-treated rabbits, was incorporated into dimyristoylphosphatidylcholine (DMPC) liposomes and the effect of gel to liquid-crystalline phase transition on the binding of benzphetamine to the liposome-bound P-450 was studied. The results obtained suggest that P-450 binds benzphetamine directly from the lipid layer and the substrate binding site is embedded in the lipid bilayer membrane.

MATERIALS AND METHODS: P-450 was purified from phenobarbital-pretreated rabbit liver microsomes as described (4). The purified preparation had a specific content of 17-19 nmol/mg protein and was practically free from detergents (5). DMPC was purchased from Sigma Chemical Co., and benzphetamine was kindly supplied by Dr. T. Kamataki of Keio University School of Medicine, Tokyo. All other chemicals were of the highest quality available commercially. The incorporation of P-450 into phospholipid liposomes was achieved by the cholate dialysis method as described (6) except that NADPH-cytochrome P-450 reductase was omitted and DMPC was used instead of egg yolk phosphatidylcholine. All spectral measurements were performed in an Aminco DW-2a spectrophotometer in split-beam and dual-wavelength modes. The spectrophotometer was interfaced on-line to a Sord M-223 mark II computer with a 12-bit A/D converter. Precise absorbance readings were obtained by averaging 100 separate measurements taken at 0.6-s intervals. Temperature was controlled by Haake F3K and Neslab RTE-8 water baths which were directly attached to thermostattable cuvettes, and the solution temperature was monitored continuously with YSI temperature probes. The spectral dissociation constant (Ks) was determined by a direct fit of the spectral data to the usual Michaelis-Menten equation using a non-linear least square method (7).

RESULTS AND DISCUSSION: The addition of benzphetamine to P-450 bound to DMPC liposomes caused a type I spectral change, which is similar to those reported for liver microsomes (8,9) and for purified P-450 preparations (10). The dependency of the induced spectral change (absorbance increment between 390 and 423 nm) on the benzphetamine concentration was analyzed as described under Materials and Methods. Figure 1 shows a typical titration curve obtained at 14.1°C. A computer simulated curve using an apparent

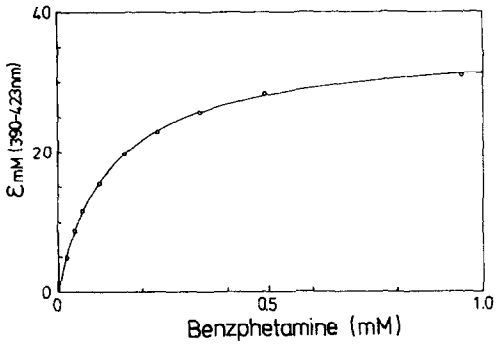

Fig. 1. Titration of DMPC-bound P-450 by benzphetamine. The reconstituted liposomes contained P-450 and DMPC at a molar ratio of 1:400. The absorbance increment between 390 and 423 nm was plotted against benzphetamine concentration added. Temperature was maintained at 14.1°C. The curve represents a computer simulated curve assuming Ks to be 0.125 mM and maximal absorbance change to be 35.3. The cencentration of P-450 was 2.5 μM.

Ks value of 0.125 mM and a maximal spectral change of 35.3 (in ε_{mM} unit) fits very well with the experimental points, indicating that a single equilibrium was involved in the substrate binding reaction. The titration curves obtained at all other temperatures examined also showed a simple single equilibrium.

When the apparent Ks value was plotted against the temperature as shown in Figure 2, a clear break was observed at 22-23°C which corresponds closely to the phase transition temperature of DMPC. Both above and below the break the experimental points

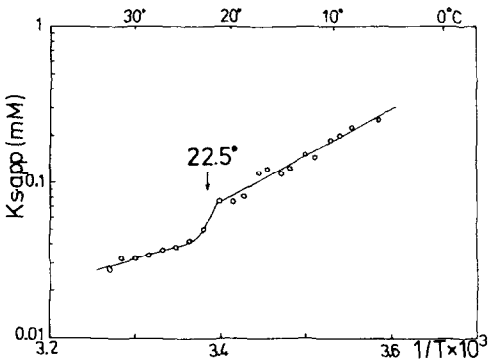

Fig. 2. Van't Hoff plot of the apparent dissociation constant of benzphetamine to DMPC-bound P-450. The dissociation constants were determined as described in Figure 1. The arrow shows the midpoint of the break observed.

fell on a straight line, and the dissociation constant decreased as the temperature was increased. These findings are compatible with the assumption that the substrate binding site of P-450 is located in the membrane and only the substrates dissolved in the lipid phase can bind to P-450. The reasoning for this statement is as follows. Benzphetamine, a hydrophobic compound, can be dissolved in both the water and lipid phases and the partition between the two phases is determined by its partition coefficient. It has been shown that partition coefficient of small lipid-soluble compounds are dependent on the fluidity of membranes and these small molecules are expelled from lipid membranes below the phase transition temperature (11,12). It is, therefore, expected that a significant portion of benzphetamine dissolved in DMPC membrane is excluded from the membrane and that the concentration of benzphetamine in membrane phase decreases when the temperature becomes lower than the phase transition temperature of DMPC. Under this circumstance, if the substrate binding site of P-450 is located in the lipid phase, the apparent dissociation constant caluculated from the concentration of benzphetamine added to the reaction mixture should increase below the phase transition temperature in agreement with the results shown in Figure 2. On the other hand, if the binding site faces the water phase, the apparent dissociation constant should decrease below the phase transition temperature because the actual concentration of benzphetamine in the water phase increases under this condition.

Another possible explanation of the results is to assume that a conformational change is induced in P-450 by the phospholipid phase transition, leading to a change in the dissociation constant. However, this possibility seems unlikely for the following reasons. Firstly, lipid-induced conformational changes

of membrane-bound proteins so far reported are accompanied by a change in slope of Arrhenius plots without any discontinuity (13,14). Secondly, our spin-label studies have shown that the conformational change caused by DMPC phase transition does not take place at the temperature where the break in Figure 2 was observed (H. Taniguchi, Y. Imai, R. Sato and S. Ohnishi, manuscpipt in preparation).

To see if the break observed was actually caused by the phase transition of DMPC, intact liver microsomes prepared from phenobarbital-treated rabbits were titrated with benzphetamine at various temperatures and the van't Hoff plot of the apparent dissociation constant was drawn. As shown in Figure 3, in this case no break was observed and the dissociation constant decreased continuously as the temperature was increased. Since microsomal membranes do not show any phase transition in the temperature range studied (15), it is clear that the break observed in Figure 2 was not due to an intrinsic property of P-450 but due to the phase transition of the DMPC membrane.

It has been reported that the pKa value of benzphetamine is around neutral (16) and that uncharged benzphetamine rather than the charged one binds to P-450 (17). The benzphetamine

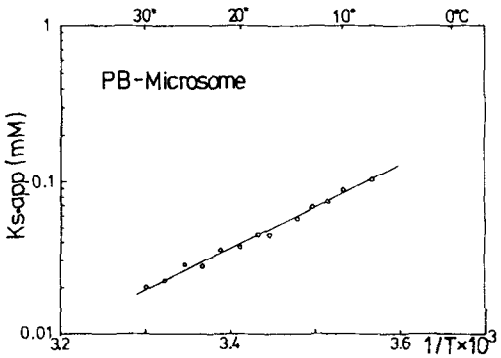

Fig. 3. Van't Hoff plot of the apparent dissociation constant of benzphetamine to microsome-bound P-450. The dissociation constant were determined as described in Figure 1, and in Materials and Methods.

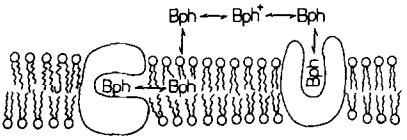

Fig. 4. Two possible schemes describing the substrate binding reaction of microsomal P-450.

binding reaction of membrane-bound P-450 may, therefore, be illustrated as shown on the left-hand side of Figure 4. The scheme shown on the right-hand side assumes that the substrate binding site of P-450 is exposed to the water phase. In these schemes the partition of charged benzphetamine between the water and lipid phases and the proton dissociation equilibrium in the lipid phase are neglected, because the concentration of charged species in the lipid phase should be very low.

A relationship between the hydrophobicity of a substrate and the apparent dissociation constant has been pointed out by several workers (17,18). However, this does not necessarily imply that the membrane lipids act as a pool of membrane-soluble substrates, because the substrate binding site of P-450 is also thought to be hydrophobic (17). In this work, we utilized reconstituted liposomes, in which the lipid composition can be easily manupilated, to study the effect of membrane fluidity on substrate binding to P-450. By changing the temperature it is thus possible to alter the partition coefficients of substrates between the water and lipid phases. The results obtained by this technique are consistent with the idea that the substrate binding site of P-450 in the DMPC liposomes, and most likely also in intact microsomes, directly phases the membrane lipid phase and P-450 binds substrates dissolved in the membrane as shown in Figure 4. These findings are physiologically important, because most substrates of P-450 are lipophilic and accumulate

in cellular membranes such as the endoplasmic reticulum rather than in the cytosol.

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