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The effect of dilauroyl-L-3-phosphatidylcholine on the interaction between cytochrome *P*-450 1A1 and benzo[*a*]pyrene

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Fluorescence quenching of benzo[*a*]pyrene (BP) by cytochrome *P*-450 1A1 was used to probe the effect of the lipid, dilauroyl-L-3-phosphatidylcholine, on this substrate-enzyme interaction. In the presence of lipid, a monoclonal antibody to this *P*-450 maximally inhibited BP binding at an antibody-to-*P*-450 ratio of 1:2, corresponding to an antibody crosslinked *P*-450 complex. The antibody did not inhibit BP binding in the absence of lipid. These results indicate that when *P*-450 is subjected to the orientational constraints imposed by antibody-mediated crosslinking, the lipid alters the conformation or quanternary structure of the *P*-450 oligomer in a manner which changes its affinity for BP.

Cytochrome *P*-450; Dilauroyl-L-3-phosphatidylcholine; Benzo[*a*]pyrene

1. INTRODUCTION

The cytochromes *P*-450 are a family of hemeprotein enzymes that catalyze the oxidation of a wide array of lipophilic compounds [1–4]. These include xenobiotics, such as drugs and carcinogens, as well as endogenous compounds, such as prostaglandins, fatty acids and steroids. The various forms of *P*-450 differ in their substrate and product specificities and reactivities. For the polyaromatic hydrocarbon-metabolizing rat liver microsomal *P*-450 1A1, binding to the substrate benzo[*a*]pyrene (BP) results in excitation energy transfer from this fluorescent donor to the *P*-450 1A1 heme acceptor. This property has proven useful as a probe for BP binding to *P*-450 1A1 [5–8].

Expression of *P*-450 catalytic activities are modulated by *P*-450 interactions with essential cofactors such as NADPH-dependent cytochrome *P*-450 reductase [9] and membrane lipids [10]. We previously utilized the fluorescence quenching technique to show that the role of reductase extends beyond that of an electron transfer agent, since it enhanced binding of BP–*P*-450 to 1A1 [8]. We have now used this technique to examine the effect of the lipid, dilauroyl-L-3-phosphatidylcholine (DLPC), on the BP–*P*-450 1A1 interaction, and on the relative orientations of individual *P*-450 molecules.

2. MATERIALS AND METHODS

Rat liver microsomal *P*-450 1A1 was purified from liver microsomes of 3-methylcholanthrene-treated male rats as described [8], and its protein concentration was determined by amino acid analysis (performed by Peptide Technologies Inc., Washington, DC). Monoclonal antibody (MAb) 1-7-1 to *P*-450 1A1 [11] was purified from mouse ascites fluid [12].

Binding of BP to *P*-450 1A1 was measured by the fluorescence quenching technique, essentially as described [8]. BP and *P*-450 were incubated at 25°C for 30 min, followed by addition of DLPC and further incubation for 30 min. When present, MAb 1-7-1 was preincubated with *P*-450 1A1 for 30 min prior to addition of BP. The reaction components were initially at about fifty-fold their final concentrations, in 50 mM potassium phosphate buffer (pH 7.25), 20% glycerol. The reaction mixture was then diluted to the desired concentration with 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol, and fluorescence was immediately (after 1.5 min) measured. For kinetic dissociation experiments the fluorescence was followed for 8 h.

Fluorescence was measured at 25°C with a SLM 8000c photon counting spectrofluorometer, using previously established conditions [8]. Excitation and emission wavelengths were 388 nm and 406 nm, respectively, and both slit widths were 8 nm.

3. RESULTS

The quenching of fluorescence by the *P*-450 heme moiety was used to monitor binding of BP to *P*-450 1A1. Fluorescence quenching by *P*-450 1A1 is highly specific since neither other forms of *P*-450 [5,8] nor apo-*P*-450 1A1 [6] quench BP fluorescence. Quenching is also essentially complete, which facilities data analysis because the concentration of free BP is proportional to observed fluorescence, and the fraction of bound BP is readily calculated from the fluorescence of BP in the presence and absence of *P*-450 1A1 [8].

The effect of DLPC on binding of BP to *P*-450 1A1 is shown in Fig. 1. The fraction of bound BP did not appreciably change at DLPC concentrations up to 0.3 μg/ml, which represents a ten-fold molar excess of

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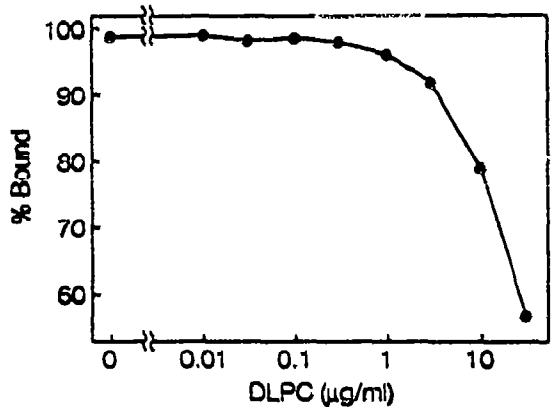

Fig. 1. Effect of DLPC on BP binding to *P*-450 1A1. The amount of bound BP was calculated from the fluorescence of BP in the absence and presence of *P*-450, as described in Materials and Methods. The solutions contained 10 nM BP, 50 nM *P*-450 1A1 and the indicted amounts of DLPC in 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol.

DLPC over *P*-450 1A1. However, BP binding decreased at higher concentrations of DLPC.

To further define the influence of lipid on the BP–*P*-450 interaction we evaluated the effect of an anti-*P*-450 1A1 monoclonal antibody (MAb 1-7-1) on BP binding in the presence of 0, 2 or 15 μg/ml of DLPC (Fig. 2A). In the presence of either concentration of DLPC, the binding curves exhibited a minimum corresponding to a 1:2 molar ratio of MAb to *P*-450. Inhibition by MAb 1-7-1 is specific since we previously showed that another MAb (1-98-1 to *P*-450 2E1) has no effect on BP binding [8], and that Mab 1-7-1 does not alter BP fluorescence in the absence of *P*-450 1A1 (data not shown). Fig. 2A also shows that DLPC was essential for MAb inhibition, since the Mab had no effect in the absence of DLPC.

The effect of lipid on the *P*-450–BP interaction was also assessed by following the time-course of BP binding after dilution of the concentrated mixture of *P*-450, BP and MAb. The effect of MAb on the binding curves obtained after the longest time (8 h) are shown in Fig. 2B. These curves are strikingly different from those at zero time (Fig. 2A), as increasing amounts of MAb uniformly increased binding. The transition between the binding profiles in Fig. 2A and B is represented by the re-equilibration kinetics of BP binding, as shown in Fig. 3. In the absence of MAb (panel A) the diluted complex slowly dissociated in both the absence and presence of DLPC, however, different BP binding profiles were observed in the presence of various Mab concentrations (panels B–F). The data firstly shows that in the absence of DLPC, addition of increasing amounts of MAb reduced the rate of BP dissociation, and hence stabilized the *P*-450–BP complex. In the presence of DLPC, however, BP binding slowly ($t_{1/2} \approx 20$ min) increased from its initially lower values. The maximal change was exhibited at the MAb:*P*-450 equivalence ratio of 0.5 (panel D), while smaller changes were observed when either *P*-450 or MAb was present in excess (panels B, C, E, F), in concordance with the data in Fig. 2A. These time-dependent increases in BP binding show that dilution strengthens the BP–*P*-450 interaction

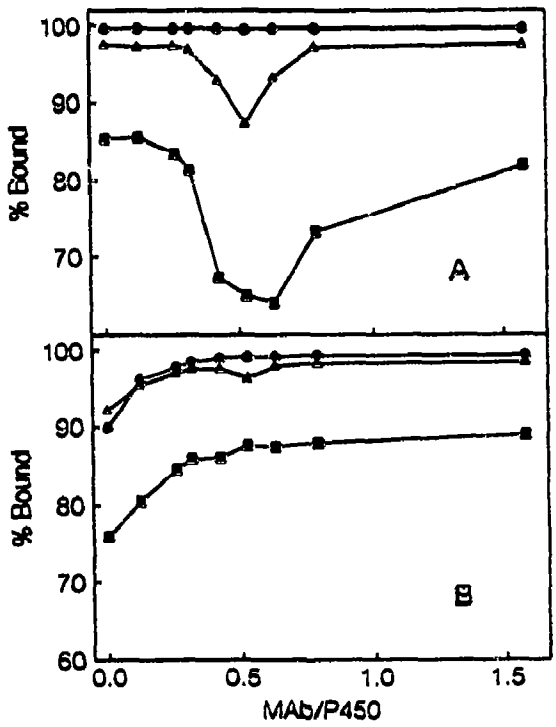

Fig. 2. Effect of MAb 1-7-1 on the BP–*P*-450 1A1 interaction. The reaction mixtures contained 100 nM *P*-450, 20 nM BP, and different amounts of MAb. Measurements of BP binding were performed without DLPC (●), and with 2 μg/ml DLPC (▲) or 15 μg/ml DLPC (■), immediately (A) and 8 h (B) after dilution of the concentrated components.

4. DISCUSSION

A basic question in enzyme structure–function studies concerns the coupling between the conformation of the substrate binding site and binding of functional modulators to distal regions of the protein. In this study we examined the effect of one such modulator, DLPC, on the BP–*P*-450 1A1 interaction. Lipids such as DLPC exhibit specific interactions with *P*-450, since different lipids vary in their effect on *P*-450 activities [13,14] and conformation [15]. Lipids may also alter *P*-450 activities by directly changing *P*-450 structure, as lipid binding has been shown to alter the heme iron spin equilibrium [16,17]. A critical role of the membrane lipid bilayer is to enhance interaction of the membrane-bound *P*-450 and NADPH–cytochrome *P*-450 reductase molecules [14].

Another important role of lipids is to provide a hydrophobic milieu for lipophilic substrates near the

membrane-bound *P*-450. We observed that increasing the levels of DLPC resulted in lower BP binding to *P*-450 (Fig. 1). Decreased binding was also previously reported as an increase in the apparent K_d [5]. These results may be attributed to lowering of BP concentration in the lipid environment and increased partitioning of BP from *P*-450 to DLPC.

Previous studies using a variety of methods [10] have shown that *P*-450 forms oligomers with sizes corresponding to hexamers up to octamers, and that lipids have no effect on oligomerization [17–19]. We thus interpret our results with reference to the model in Fig. 4, which shows the complex of BP with a *P*-450 oligomer (a hexamer is shown for illustrative purposes). The three columns represent individual *P*-450 oligomers associated with 0, 0.5 and 1.0 equivalents of MAb. Since MAb-mediated crosslinking necessarily imposes conformational and/or orientational constraints on the crosslinked *P*-450 molecules, these are shown in a different and more constrained orientation than non-crosslinked *P*-450s.

The top row represents the system in the absence of DLPC. Since BP binding was the same at all MAb concentrations (Fig. 2A), the three species exhibit the same BP binding potential. In the presence of DLPC (second row), BP binding was reduced for all three species as BP was free to partition from *P*-450 to DLPC, and was lowest at a MAb:*P*-450 ratio od 0.5 (Fig. 2A). Thus minimum binding was observed under conditions favoring the bivalent MAb–*P*-450 complex (center column) while excess MAb favors the univalent MAb–*P*-450 complex (right column) whose binding behavior is similar to that of *P*-450 alone (left column).

These results thus show that MAb has no intrinsic effect on BP binding by *P*-450 either in the presence or absence of DLPC. The reduction in BP binding by the bivalent MAb–*P*-450 complex is thus a direct consequence of crosslinking, which may alter *P*-450 conformation and/or quaternary structure. The conformation may change if crosslinking enhances the interaction between the proximate *P*-450 molecules such as to perturb their BP binding sites, and crosslinking may impose orientational constraints on the individual *P*-450 monomers within the oligomer in a manner that sterically hinders transfer of BP from DLPC to the *P*-450 binding site. DLPC is responsible for the conformational or quaternary structural change of *P*-450, since no reduction in BP binding was observed in its absence.

Although the model in Fig. 4 shows MAb crosslinking of *P*-450 within a complex, intercomplex crosslinking is also possible but less likely: once a MAb molecule binds one epitope within the complex, the second MAb binding site is more proximate to an thus more likely to bind an epitope in the same complex rather than an epitope in another complex. Thus intracomplex crosslinking is favored over intercomplex crosslinking.

The role of DLPC was also examined by changing the order of mixing the reaction components. In the MAb inhibition experiments presented in Fig. 2, MAb was

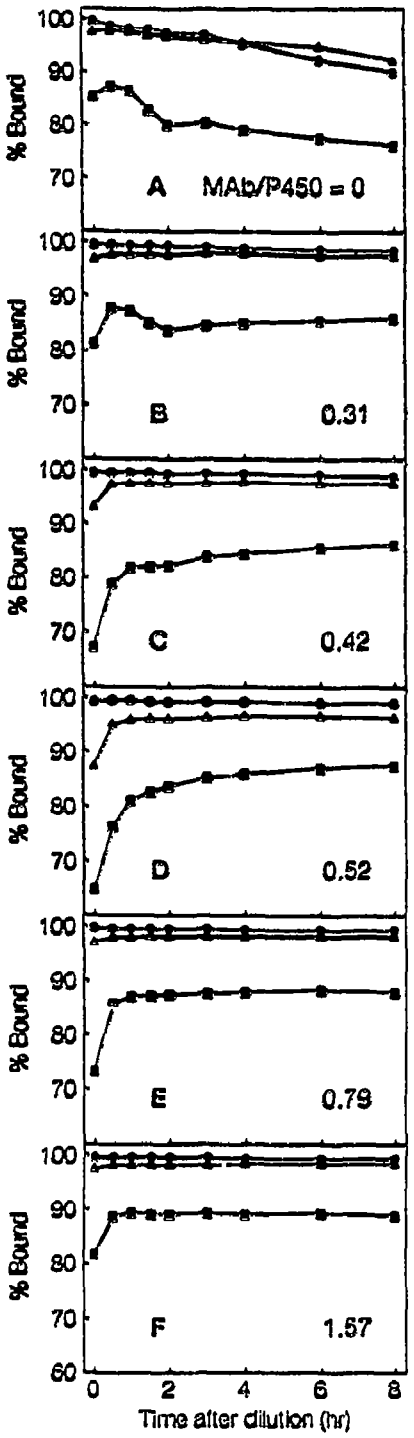

Fig. 3. Effect of DLPC and MAb 1-7-1 on progress-time curves for the interaction between BP and *P*-450 1A1. The pre-equilibrated BP–*P*-450 complex was diluted fifty-fold and BP binding was measured. Reaction conditions were the same as in Fig. 2 and were performed without DLPC (●), and with 2 μg/ml DLPC (▲) or 15 μg/ml DLPC (■). Measurements were performed the MAb/*P*-450 ratios shown in each panel.

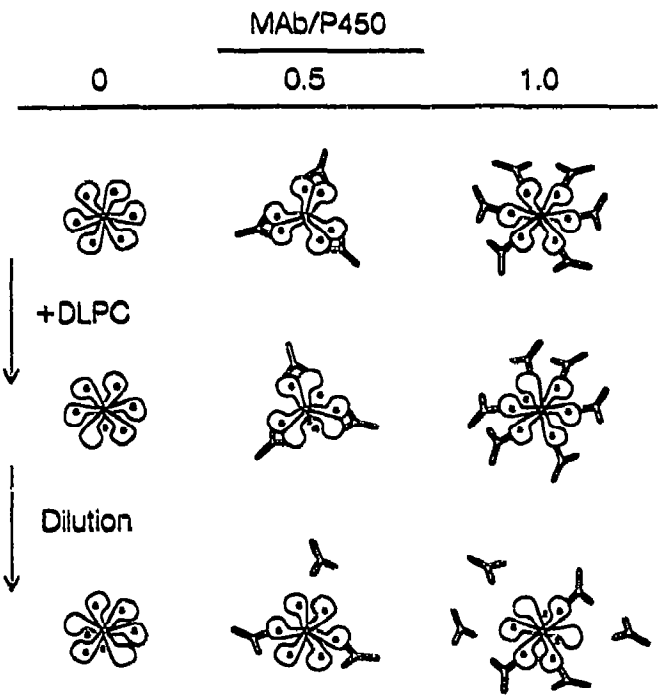

Fig. 4. Schematic model of MAb-*P*-450-BP interactions. The filled circles represent BP and the shaded region represents DLPC molecules. The species in the three columns represent individual *P*-450 oligomeric species associated with 0, 0.5 and 1.0 molecules of MAb. Top row, complexes without DLPC; second row, complexes in the presence of DLPC; bottom row, complexes after dilution, showing dissociation of MAb from *P*-450.

equilibrated with *P*-450 prior to addition of DLPC. However, if *P*-450 was mixed with 15 μg/ml DLPC prior to addition of MAb, the inhibition profile at zero time did not exhibit a minimum and resembled that in Fig. 2B (data not shown). Thus bivalent MAb–*P*-450 complexes are only produced in the absence of DLPC. When DLPC is present, the orientation of *P*-450 monomers within the oligomer is presumably infavorable for bivalent MAb binding.

After the concentrated mixture of MAb, *P*-450 and BP (second row) is diluted, re-equilibration occurs (bottom row). In the absence of MAb (left column), BP as expected dissociated from *P*-450 (Fig. 3, top panel), however, dilution under conditions favoring bivalent MAb complexes (center column) increased BP binding. We postulate that dilution shifts the equilibria toward dissociation of MAb from *P*-450, which allows a time-dependent relaxation to *P*-450 conformation or quaternary structure that more effectively binds BP. The BP binding kinetics (Fig. 3) indicate that this process has a $t_{1/2}$ of about 20 min. A similar time scale was reported for changes in catalytic activity following incubation of *P*-450 with lipid [20]. This result was attributed to slow changes in *P*-450–lipid interactions, and may arise from a lipid-induced conformational change or reorientation of *P*-450 monomers.

We have thus utilized MAb-mediated crosslinking as a sensitive probe for the effect of lipid on *P*-450 structure. Our data demonstrate that lipid modifies the conformation and/or orientation of *P*-450s within the oligomer, since MAb could inhibit BP binding to oligomeric *P*-450 in the presence of lipid, but had no effect when added to *P*-450 in the absence of lipid. Such effects on the quaternary structure of *P*-450s are important because *P*-450 substrates are often sequentially metabolized by multiple *P*-450s and the relative orientation of *P*-450s within an oligomer may alter metabolic flow and the ultimate disposition of a substrate [21].

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