Protein–Protein and Lipid–Protein Interactions in a Reconstituted Cytochrome P-450 Dependent Microsomal Monooxygenase

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ABSTRACT: NADPH-cytochrome P-450 reductase and cytochrome P-450, both purified from liver microsomes of phenobarbital-treated rabbits, were incorporated into dimyristoylphosphatidylcholine vesicles. The reduction of cytochrome P-450 by NADPH in the reconstituted vesicles proceeded in a biphasic fashion, and 70–80% of the absorbance change was associated with the fast phase. The Arrhenius plot of the apparent first-order rate constant of the fast-phase reduction showed a marked discontinuity around the phase transition temperature of the synthetic phospholipid; an almost 10-fold change in rate constant was associated with this discontinuity. It was, therefore, suggested that the reduction of cytochrome P-450 by reductase in this system was a diffusion-limited reaction controlled by the viscosity of the phospholipid membrane. The Arrhenius plot of overall drug monooxygenase activity catalyzed by the reconstituted vesicles showed a break but in a different way from that observed for the reduction of cytochrome P-450. This break was accompanied only by a change of the slope of the plot but not by a change in reaction rate. This difference in the two Arrhenius plots was attributed to that in the rate-limiting step of the two reactions. NADPH-cytochrome *c* reductase activity of the reconstituted vesicles, an activity catalyzed by the reductase alone, and cumene hydroperoxide dependent *N*-methylaniline demethylation activity catalyzed by cytochrome P-450 alone did not show any break in the Arrhenius plots.

The hepatic microsomal monooxygenase system, which catalyzes monooxygenation of various xenobiotics as well as endogenous lipids, consists of the terminal enzyme cytochrome P-450 and three electron-transferring proteins, i.e., NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase, and cytochrome b_5 (Sato & Omura, 1978; Taniguchi et al., 1984b). This multienzyme system is unique in that all of its component proteins are tightly bound to the endoplasmic reticulum membrane and electron transfer among them involves direct protein–protein interactions. Therefore, this system is a good model for studies of protein–protein interactions among membrane-bound proteins as well as lipid–protein interactions.

Since liver microsomes contain more than a 20 molar excess of the two cytochromes compared to the two reductases, two mechanisms have been proposed to explain how one reductase molecule can reduce more than 20 cytochrome molecules. Several workers have assumed the presence of protein clusters or complexes in which one reductase molecule is surrounded by 6–10 cytochrome molecules and electron transfer takes place directly within such clusters (Franklin & Estabrook, 1971; Ito, 1974; Peterson et al., 1976). Nonrandom distribution of these proteins in microsomal subfractions has also been reported (DePierre & Dallner, 1975). On the other hand, lateral mobility of proteins in membrane has been demonstrated for various membrane systems (Vaz et al., 1982), and random collision of membrane-bound proteins caused by their lateral motion has been proposed as a mechanism by which these proteins interact functionally (Yang, 1975; Yang et al., 1977; Duppel & Ullrich, 1976). Our previous kinetic studies on the reduction of cytochrome P-450 by NADPH-cytochrome P-450 reductase in reconstituted egg yolk phosphatidylcholine (PC)[1] liposomes have clearly shown lateral mobility of the two proteins in the liposomal membrane and provided evidence that the reduction reaction of cytochrome P-450 is effected by their random collision caused by the lateral diffusion (Taniguchi et al., 1979).

In this study, NADPH-cytochrome P-450 reductase and cytochrome P-450 were incorporated into the membrane of dimyristoylphosphatidylcholine (DMPC) liposomes to study the effects of gel to liquid-crystalline phase transition of this synthetic phospholipid on the reduction of cytochrome P-450 by the reductase and several other activities catalyzed by the reconstituted system. The results obtained indicate that the reduction of cytochrome P-450 is actually a diffusion-controlled reaction and thus support the conclusion reached in our previous study (Taniguchi et al., 1979).

Experimental Procedures

Materials. DMPC was purchased from Sigma, while egg PC was prepared by the method of Singleton et al. (1965). Cholic acid, purchased from Nissui Yakuhin Co., was recrystallized and converted to sodium salt as described (Imai & Sato, 1974a). [*N-methyl*-^{14}C]Phosphatidylcholine was obtained from Radiochemical Centre, Amersham, England. Benzphetamine hydrochloride was a generous gift from Dr. T. Kamataki, Keio University, School of Medicine, Tokyo, and NADPH was the product of Oriental Yeast Co., Tokyo. Cumene hydroperoxide obtained from Nakarai Chemical Co., Kyoto, Japan, was routinely purified by alkali extraction (Hock & Lang, 1944), whereas *N*-methylaniline from the same source was used without further purification. The other chemicals and biochemicals used are described previously (Taniguchi et al., 1979).

Purification of P-450 and Reductase. A major phenobarbital-inducible form of cytochrome P-450 was purified to gel electrophoretical homogeneity from liver microsomes of

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[1] Abbreviations: PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid.

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phenobarbital-pretreated rabbits by the method of Imai and Sato (1974b). The purified preparations contained 17–18 nmol of cytochrome P-450/mg of protein and were essentially free from detergents (Imai et al., 1980). NADPH-cytochrome P-450 reductase was also purified from phenobarbital-induced rabbit liver microsomes as described (Taniguichi et al., 1979, 1984b) with 2′,5′-ADP–Sepharose (Yasukochi & Masters, 1976). The final preparation had a specific NADPH-cytochrome *c* reductase activity of 55 units/mg of protein and was homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Reconstitution of Monooxygenase System. The drug monooxygenase system was reconstituted by incorporating cytochrome P-450 and NADPH-cytochrome P-450 reductase into phospholipid membranes by the cholate dialysis technique as described previously (Taniguchi et al., 1979), except that DMPC as well as egg PC was used and that Amberlite XAD-2 (10 g/L) was included in the final dialysis buffer to remove residual cholate (Taniguchi et al., 1984a).

Stopped-Flow Experiments. The reduction of cytochrome P-450 by NADPH in the reconstituted system was studied in a Union Giken RA 401 stopped-flow spectrophotometer equipped with a data processor, System 71, as described (Taniguchi et al., 1979). The reconstituted vesicles were suspended in 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM benzphetamine, and the suspension was bubbled with CO for 3 min. The suspension was mixed in the stopped-flow apparatus with an equal volume of 0.1 M potassium phosphate buffer containing 0.6 mM NADPH and 1 mM benzphetamine, which had been saturated with CO as above. An oxygen scavenging system containing glucose oxydase was used to remove residual oxygen (Taniguchi et al., 1979). The solutions were allowed to stand in the reservoir of the stopped-flow apparatus for at least 10 min at the desired temperature to attain temperature equilibrium.

Analytical Methods. Benzphetamine *N*-demethylase and *N*-methylaniline *N*-demethylase activities were assayed by measuring the formation of formaldehyde by the method of Nash (1953). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.25), 1 mM benzphetamine (or 8 mM *N*-methylaniline), 1 mM NADPH, and the reconstituted system (usually about 0.75 μM in terms of cytochrome P-450) in a final volume of 1.0 mL. The reaction was started by the addition of NADPH and stopped by the addition of 0.2 mL of 40% trichloroacetic acid. The supernatant after centrifugation was mixed with an equal amount of Nash reagent. The color development was performed at 60 °C for 20 min. For cumene hydroperoxide dependent *N*-demethylase activity, the same reaction mixture as above was used except that 2.5 mM cumene hydroperoxide was used instead of NADPH and that the color development was performed at 25 °C for 35 min to avoid decolorization by cumene hydroperoxide. Cytochrome P-450 was determined from the absorbance difference between 418 and 500 nm, with a millimolar extinction coefficient increment of 101 (Hashimoto-Yutsudo et al., 1980). NADPH-cytochrome P-450 reductase was determined by its NADPH-cytochrome *c* reductase activity measured in 0.3 M potassium phosphate buffer (pH 7.25) at 25 °C as described (Imai, 1976). Purified reductase was also determined from the intensity of flavin absorption at 455 nm, assuming a millimolar extinction coefficient of 10.7 per flavin (Iyanagi & Mason, 1973). All other methods used were described previously (Taniguchi et al., 1979).

Sucrose Density Gradient Centrifugation. The reconstituted system prepared in the presence of a trace amount of [^{14}C]-phosphatidylcholine was suspended in 0.3 mL of 50 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA and 0.1 mM dithiothreitol, and the suspension was layered over a 5-mL linear sucrose density gradient from 5 to 42% (w/w) containing 50 mM potassium phosphate buffer (pH 7.25), 0.1 mM EDTA, and 0.1 mM dithiothreitol. The sample was centrifuged in an RPS 50 II rotor of the Hitachi 55P-2 ultracentrifuge at 235000*g* for 31 h. After centrifugation, fractions were collected from the bottom of the tube and analyzed for cytochrome P-450, NADPH cytochrome *c* reductase activity, and ^{14}C radioactivity.

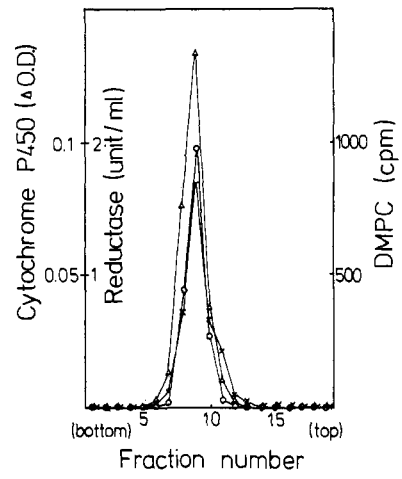

FIGURE 1: Sucrose density gradient centrifugation of reconstituted system. The reconstituted system, consisting of cytochrome P-450, NADPH-cytochrome P-450 reductase, and DMPC in ratios of 1:1:400, was layered on the top of 5–42% sucrose gradient and subjected to ultracentrifugation as described under Experimental Procedures. Each fraction collected from the bottom of the tube was assayed for the absorbance difference between 418 and 500 nm [for cytochrome P-450 (○)], NADPH-cytochrome *c* reductase activity [for the reductase (△)], and ^{14}C radioactivity [for DMPC (×)].

Electron Microscopy. The reconstituted system suspended in 50 mM potassium phosphate buffer (pH 7.25) was placed on a specimen mesh coated with a poly(vinyl formal) film. The sample was then stained with 1% phosphotungstic acid (pH adjusted to 7.0 with KOH) and observed in a JEOL JEM-7 electron microscope.

RESULTS

Preparation of Reconstituted Vesicles. The reconstituted vesicles were prepared by the cholate dialysis method from NADPH-cytochrome P-450 reductase and cytochrome P-450 at a molar ratio of 1:1 together with varying amounts of DMPC. When the system reconstituted at 4 °C in the presence of a trace amount of [^{14}C]phosphatidylcholine was subjected to sucrose density gradient centrifugation, cytochrome P-450 chromophore, the reductase activity, and ^{14}C radioactivity were at the same position each forming a sharp peak, and no free proteins could be detected at the bottom of the gradient (Figure 1). It was thus clear that the two proteins had been completely incorporated into the DMPC membrane under the experimental conditions employed. Negative-staining electron microscopy indicated that the reconstituted system consisted of numerous single-walled vesicles having a diameter of 300–500 Å (Figure 2). This diameter was about one-third of that observed for the system reconstituted with egg yolk PC as lipid component (Taniguchi et al., 1979, 1984b). In view of a report that the temperature during dialysis is critical for incorporation of sarcoplasmic reticulum Ca^{2+}-ATPase into liposomes by the cholate dialysis method (Kleeman & McConnell, 1976), dialysis was also conducted

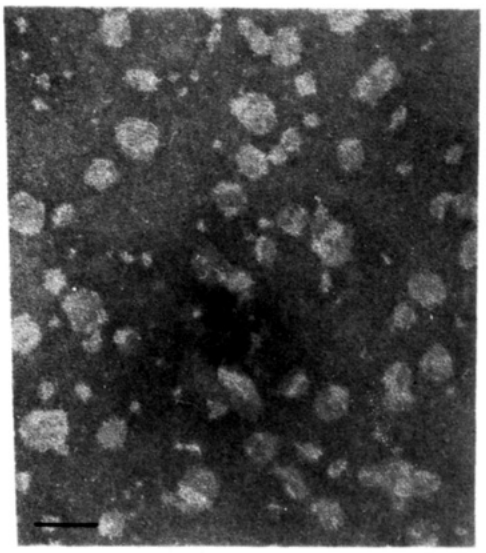

FIGURE 2: Negative-staining electron micrograph of a reconstituted system. The system consisted of cytochrome P-450, NADPH-cytochrome P-450 reductase, and DMPC at molar ratios of 1:1:400. The bar represents 1000 Å.

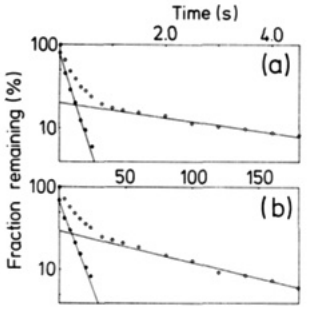

FIGURE 3: First-order plots of the reaction of cytochrome P-450 reduction in the DMPC system. Logarithm of $(A_t - A_{max})/A_{max}$ was plotted against time after the flow stop, where A_t and A_{max} are the observed absorbance changes at time t and after the completion of the reduction, respectively. Plots for the fast phase (●) were obtained by subtracting the values for the slow-phase reduction from the original data (○). (a) 33.2 °C; (b) 18.5 °C.

at 25 °C, a temperature that is higher than the phase transition temperature of DMPC (about 23 °C) (Marbrey & Sturtevant, 1976). The vesicles thus formed had larger diameters than those prepared at 4 °C, but dialysis at 25 °C led to extensive denaturation of cytochrome P-450 to the P-420 state. Therefore, we decided to reconstitute the vesicles by dialysis at 4 °C. The reconstituted systems used in the following experiments all contained the reductase, cytochrome P-450, and DMPC at a molar ratio of 1:1:400.

Reduction of Cytochrome P-450 in DMPC Vesicles. The reduction of cytochrome P-450 in the DMPC membrane was studied by mixing the vesicles with NADPH in a stopped-flow apparatus in the presence of benzphetamine, a substrate. The reduction was followed by recording the formation of the ferrous CO complex of cytochrome P-450 at 450 nm in a CO-saturated medium. As in the case of the system reconstituted with egg PC (Taniguchi et al., 1979), the reduction in the DMPC system was also a biphasic process consisting of two concurrent first-order reactions. Figure 3 shows typical first-order plots of the reduction measured at 33.2 and 18.5 °C, temperatures that are higher and lower than the phase transition temperature of DMPC, respectively. From these data the apparent first-order rate constants were determined to be 4.5 and 0.21 s^{-1} for the fast and slow phases, respectively, at 33.2 °C, while these values were determined to be 0.097

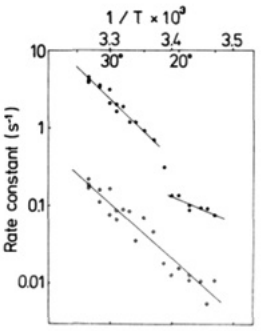

FIGURE 4: Arrhenius plot of the reduction rates of cytochrome P-450 in the DMPC system. NADPH-dependent cytochrome P-450 reduction reaction was measured with a reconstituted system, consisting of cytochrome P-450, NADPH-cytochrome P-450 reductase, and DMPC at a molar ratio of 1:1:400 at different temperatures as described under Experimental Procedures. The two apparent first-order rate constants were obtained from first-order plots as described in Figure 3. The curves were drawn by the linear regression method. The variances from the regression line were calculated to be 0.00332 and 0.00218 for the fast-phase rate constant above and below the transition temperature, respectively. When all the data were combined and fitted with a single straight line, the variance became 0.0146. On the other hand, the variance determined for the slow-phase rate constant in the whole temperature range was 0.0202, while those determined form the regression lines fitted separately above and below the temperature were 0.0164 and 0.0170, respectively. There is, therefore, no significant discontinuity in the slow-phase rate constant.

and 0.012 s^{-1} at 18.5 °C. The absorbance change associated with the fast phase was 80% at 33.2 °C and 70% at 18.5 °C. Therefore, the change of lipid component from egg PC, which has unsaturated fatty acids, to saturated DMPC does not show any significant effect on reduction kinetics. Even the change of membrane fluidity caused by the phase transition does not affect the biphasic nature of the reduction kinetics. The reduction rate, however, was affected by the fluidity change as will be described below.

Effect of Phase Transition on Cytochrome P-450 Reduction. The effect of DMPC phase transition on cytochrome P-450 reduction was then studied by measuring the reduction at various temperatures. At all the temperatures examined (14–33 °C), the reduction proceeded in a biphasic fashion and 70–80% of the absorbance change occurred in the fast phase. As shown in Figure 4, the Arrhenius plot of the fast-phase rate constant showed a marked discontinuity, the midpoint of which (22.3 °C) was very close to the DMPC phase transition temperature. This break was unique in that it was accompanied not only by a change of the slope of the plot but also by an abrupt change in the rate constant. Below this break the rate constant decreased almost 10-fold. From the slopes, the activation energies above and below the discontinuity were calculated to be 34.8 and 15.1 kcal/mol, respectively. Since it is expected that the lateral mobility of membrane-bound proteins is drastically altered at the phase transition temperature of the membrane phospholipid, the discontinuity observed suggested that the fast-phase rate of cytochrome P-450 reduction was controlled by the lateral diffusion of the reductase and cytochrome P-450 in the membrane. On the other hand, the Arrhenius plot of the slow-phase rate constant showed no significant, if any, discontinuity. This is not surprising, since the origin of the slow phase is considered to be either the spin equilibrium in the oxidized state (Backes et al., 1982) or the slow CO-independent phase observed in CO-binding kinetics (Grey, 1982), which are not affected by the diffusion rate of the proteins in membrane.

Effect of Phase Transition on Overall Monooxygenase Activity. Figure 5 shows the Arrhenius plot of NADPH-de-

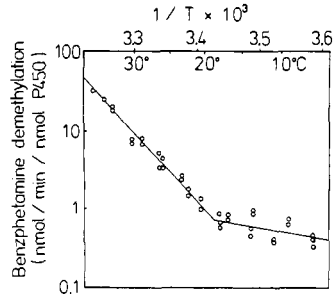

FIGURE 5: Arrhenius plot of NADPH-dependent benzphetamine *N*-demethylase in the DMPC system. The same reconstituted system used as in Figure 4 was assayed for benzphetamine *N*-demethylase activity in the presence of NADPH as described under Experimental Procedures.

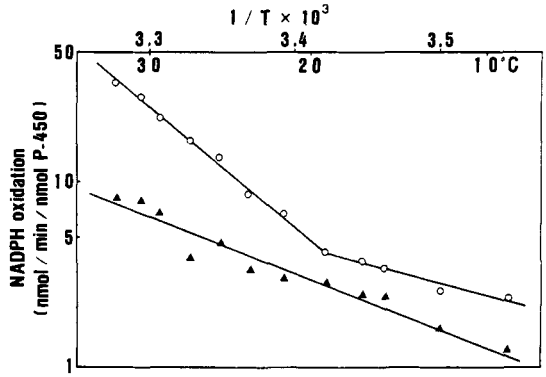

FIGURE 6: Temperature dependence of NADPH oxidation by the DMPC system. NADPH oxidation by the same reconstituted system as used in Figure 4 was assayed in the absence (▲) and presence (○) of benzphetamine by following the absorbance change at 340 nm.

pendent benzphetamine N-demethylation activity catalyzed by the reconstituted system. As can be seen, the plot of this overall monooxygenase activity exhibited a clear break at about 18–19 °C, but the shape of the plot differed from that obtained for the fast-phase reduction of cytochrome P-450 (Figure 4). In the plot for the overall activity, no discontinuity was detected, and the break consisted of only a change of the slope of the plot. The activation energies above and below the break were calculated to be 39 and 6.9 kcal/mol, respectively. A similar temperature dependence of overall monooxygenase activity was also obtained with *N*-methylaniline as substrate (data not shown), indicating that the break was not specific for particular substrates. The Arrhenius plot of NADPH-dependent benzphetamine N-demethylation activity was also determined for the system reconstituted with egg PC as the lipid component (data not shown). This plot did not show any break in the temperature range from 10 to 33 °C, and an activation energy of 30 kcal/mol was calculated. In view of the fact that egg PC is in a fluid state in this temperature range, this lack of break suggested that the break observed in the DMPC system was due to the phase transition of the synthetic phospholipid, even though the break temperature (18–19 °C) was significantly lower than the DMPC phase transition temperature (about 23 °C). As shown in Figure 6, the Arrhenius plot of the rate of benzphetamine-dependent NADPH oxidation by the DMPC-containing reconstituted system also showed a break similar to that observed for the overall monooxygenase activity. The activation energies in this case were 30 and 10 kcal/mol above and below the break, respectively. Interestingly, the Arrhenius plot of the slow NADPH oxidation in the absence of benzphetamine did not show any break, and an activation energy of 14 kcal/mol was calculated. A comparison of the data shown in Figures 5 and

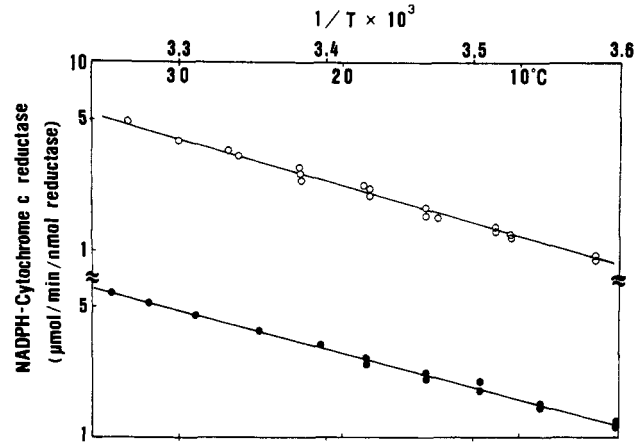

FIGURE 7: Temperature dependence of NADPH-cytochrome *c* reductase activity of the DMPC system and purified reductase. The same reconstituted system as used in Figure 4 (○) and purified membrane-free reductase (●) were assayed for NADPH-dependent cytochrome *c* reductase activity at various temperatures as described under Experimental Procedures.

6 indicated that the rate of benzphetamine-dependent oxidation of NADPH was 2- to 5-fold higher than that of the overall monooxygenase reaction (product formation). Such uncoupling of NADPH oxidation and product formation has been observed for the egg PC containing reconstituted system (Taniguichi et al., 1979), a nonvesicular reconstituted system (Imai et al., 1977), and also intact microsomes (Nordblom & Coon, 1976). The extent of uncoupling was higher at lower temperature (about 80% at 10 °C) than at higher temperature (about 60% at 33 °C).

Effects of Phase Transition on NADPH-Cytochrome c Reductase Activity and Peroxide-Dependent Drug Oxidation Activity. NADPH-cytochrome P-450 reductase can catalyze the NADPH-dependent reduction of cytochrome *c* (Sato & Omura, 1978). Figure 7 shows the Arrhenius plot of this activity catalyzed by the DMPC-containing reconstituted system. As can be seen, this plot did not show any break. A similar plot was also obtained for the same activity catalyzed by a purified, membrane-free reductase preparation. Activation energies of 11 and 10.5 kcal/mol were calculated for the membrane-bound and free reductase preparations, respectively. These results excluded the possibility that the break observed in the Arrhenius plot of NADPH-dependent monooxygenase activities catalyzed by the reconstituted DMPC system was caused by the effect of phase transition on the reductase molecule. It has been reported that cytochrome P-450 can catalyze drug oxidation in the absence of NADPH and the reductase when organic hydroperoxides or hydrogen peroxide is supplied (Nordblom & Coon, 1976; Rahimutula & O'Brien, 1974). As shown in Figure 8, the Arrhenius plot of the rate of cumene hydroperoxide dependent N-demethylation of *N*-methylaniline catalyzed by the reconstituted system was linear, indicating that this activity was not affected by the phospholipid phase transition. the activation energy of this reaction was calculated to be 21 kcal/mol. It should be noted that the rate of cumene hydroperoxide dependent reaction was more than 10 times higher than that of NADPH-supported *N*-methylaniline demethylation by the same reconstituted system, supporting the view that the peroxide-dependent reaction bypasses the rate-limiting step of the NADPH-supported reaction (Imai et al., 1977).

Discussion

Previous studies on the kinetics of cytochrome P-450 reduction by NADPH-cytochrome reductase in reconstituted

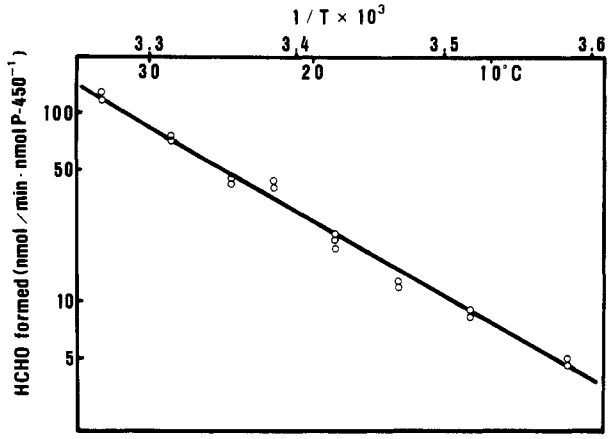

FIGURE 8: Arrhenius plot of cumene hydroperoxide dependent *N*-methylaniline *N*-demethylase of the DMPC-reconstituted system. The same reconstituted system as used in Figure 4 was assayed for *N*-demethylase activity in the presence of cumene hydroperoxide as described under Experimental Procedures.

systems clearly showed that the electron transfer between the reductase and cytochrome P-450 occurs in a one to one complex of cytochrome P-450 and the reductase (Taniguchi et al., 1979; Oprian et al., 1979). When these two proteins are mixed with detergents or dilauroylphosphatidylcholine (the latter acts like a detergent due to its high critical micellar concentration), no bilayer structures are formed, and the reductase and cytochrome P-450 exist as a stable complex (French et al., 1980), in which the electron transfer from the reductase to cytochrome P-450 takes place directly (Oprian et al., 1979). On the contrary, when both proteins were incorporated into liposomal membrane, no such stable complex formation was observed (Taniguchi et al., 1979), which suggested rapid lateral mobility of the two proteins in the reconstituted membrane.

In the present work we have further studied the interaction of the reductase with cytochrome P-450 by examining the effect of phospholipid phase transition on cytochrome P-450 reduction and overall drug monooxygenase activities catalyzed by the system reconstituted with DMPC as the lipid component. As shown in Figure 4, the rate constant of fast-phase reduction of cytochrome P-450 in the DMPC system undergoes an abrupt and almost 10-fold change at the phase transition temperature of DMPC. Since the viscosity of phospholipid bilayer membranes is drastically altered at their phase transition temperatures and the lateral mobility of membrane-bound proteins depends mainly on the membrane viscosity (Vaz et al., 1982), the above finding can be best explained by assuming that the reduction reaction is controlled by the diffusion rates of the reductase and cytochrome P-450 in the DMPC membrane. In other words, it can be concluded that the rate-limiting step of the reduction process is the formation of a reductase–cytochrome P-450 complex within which the electron transfer from the reduced reductase to oxidized cytochrome P-450 takes place. This conclusion is further supported by the finding that the reduction of cytochrome *c* catalyzed by the DMPC-reconstituted system exhibits a linear Arrhenius plot (Figure 7). In this reaction, the membrane-bound reductase interacts with water-soluble cytochrome *c* instead of membrane-bound cytochrome P-450. Therefore, this reaction should not be affected by the changes in the physical state of the membrane. It should be mentioned in this connection that the Arrhenius plot of NADH-cytochrome *c* reductase activity catalyzed by DMPC liposomes containing NADH-cytochrome b_5 reductase and cytochrome b_5 is similar in shape to that obtained in this study and an abrupt change in reaction rate is observable at the phase transition temperature (Strittmatter & Rogers, 1975).

By using the fluorescence photobleaching recovery technique Wu and Yang (1984) have recently determined the diffusion constant of cytochrome P-450 in DMPC and reported that the phase transition is accompanied by a 10–20-fold change in the diffusion constant, a change that is roughly compatible with that observed in this study. The reaction rate estimated from the diffusion constant determined by Wu and Yang (1984) is about 3 orders of magnitude higher than the value experimentally determined in this study. This discrepancy may be explained by factors such as molecular orientation factors, but it should be also noted that different reconstitution methods were employed in the present kinetic experiments and in the photobleaching experiments. While we incorporated the reductase and cytochrome P-450 into single-walled DMPC liposomes by the cholate dialysis method, Wu and Yang (1984) prepared their system by incubating cytochrome P-450 with preformed multilayered DMPC. This difference in preparation method appears to be critical in view of the observation that the conformation of cytochrome b_5 that has been bound to preformed liposomes is quite different from that incorporated into liposomes by the cholate dialysis technique (Enoch et al., 1979). To resolve the problem, it is necessary to determine the diffusion constant of cytochrome P-450 incorporated into DMPC liposomes by the cholate dialysis method.

The apparent activation energy of the reduction reaction is lower at temperatures below the break than at the temperatures above (Figure 4). The diffusion of lipid analogues in phospholipid bilayer membranes in the gel phase (below the phase transition temperature) shows, on the contrary, a higher apparent activation energy compared to that in the liquid-crystalline phase (above the phase transition temperature) (Vaz et al., 1982). The lateral diffusion of cytochrome P-450 determined by the fluorescence photobleaching recovery technique shows similar characteristics, although the temperature dependence is rather weak in both phases and the exact activation energy cannot be determined due to rather high scattering of the data (Wu & Yang, 1984). One possible explanation of the low activation energy observed in the reduction reaction below the phase transition temperature is the presence of "defects" or inhomogeneities in bilayer membranes (Vaz et al., 1982). Due to structural and steric reasons, proteins, which do not fit into the crystalline packing of the acyl chains of the phospholipids in the gel phase, may be expected to be squeezed out of the ordered gel phase domains. Protein-rich domains would then be formed and may be expected to have a local structure of the low degree of order similar to that in the liquid-crystalline phase. The formation of such protein-rich domains is actually observed with other membrane proteins such as Ca^{2+}-dependent ATPase (Kleeman & McConnell, 1976). The partial fluorescence recovery observed in the gel phase (Wu & Yang, 1984) suggests that cytochrome P-450, too, is forced out of the gel phase bilayer (Vaz et al., 1982). In such domains the temperature dependence of the diffusion rate may be different from that in the bulk gel phase domains. The photobleaching technique, on the other hand, measures the diffusion of the fluorescence label in a relatively large area, and the observed diffusion rate reflects the diffusion of such clusters or domains in the bilayer membranes rather than the diffusion of proteins within the clusters.

In contrast to the case of cytochrome P-450 reduction, the Arrhenius plot of overall monooxygenase activity catalyzed by the reconstituted DMPC system does not show an abrupt change in rate constant, and only a change in the slope of the

plot is observable at about 19 °C, a temperature that is significantly lower than the phase transition temperature of DMPC. It is likely that this difference is related to the difference in the rate-limiting step in the two types of reaction. The rate-limiting step of cytochrome P-450 reduction (introduction of the first electron required for overall monooxygenase activity) seems to be the diffusion of the two proteins, whereas that of the overall monooxygenation reaction is thought to be the introduction of the second electron to a ternary complex of ferrous cytochrome P-450, substrate, and molecular oxygen as discussed previously (Imai et al., 1977). The finding that the phase transition of DMPC is not accompanied by a change in the rate of overall monooxygenase activity can be explained by assuming that the formation of the reductase–cytochrome P-450 complex is faster than the introduction of the second electron. It is not surprising that benzphetamine-dependent NADPH oxidation shows an Arrhenius plot that is similar to that of overall monooxygenase activity (break point at about 19 °C) because of two activities are two different measures of the same reaction except that not all of the NADPH oxidized is coupled to product formation.

Since the overall monooxygenase activity catalyzed by cytochrome P-450 incorporated into egg PC membrane showed a linear Arrhenius plot, the change in the activation energies observed in the DMPC system must be due to the phase transition of the lipid, although the temperature where the change was observed (18–19 °C) is significantly lower than that of the phase transition temperature (23 °C). As will be reported elsewhere,[2] conformational changes of cytochrome P-450 embedded in DMPC membrane were detected at around 19 °C with the spin-label technique. It is, therefore, reasonable to assume that this conformational change is responsible for the alteration of activation energy near the phase transition temperature. Nonlinear Arrhenius plots were also observed in various monooxygenase activities catalyzed by microsome-bound cytochrome P-450 (Duppel & Ullrich, 1976; Yang et al., 1977). The activation energy below the break temperature, however, was in all cases higher than that at higher temperatures. These authors attributed the observed break to the phase transition of microsomal membranes, although there is no clear-cut phase transition in the microsomal membranes in the temperature range studied. The presence of various isozymes of cytochrome P-450, which have distinct but somewhat overlapping substrate specificity, may explain the observed nonlinear Arrhenius plot. The monooxygenase activities measured in these studies are usually catalyzed by several isozymes (Imai, 1979). The Arrhenius plot is, therefore, not necessarily linear. Another possible explanation is the presence of cytochrome b_5. Since the second electron is mainly supplied from cytochrome b_5 to cytochrome P-450 and the introduction of the second electron is considered to be the rate-limiting step (Imai et al., 1977; Taniguchi et al., 1984b), the observed change in the activation energy may reflect the change in the interaction between cytochrome b_5 and cytochrome P-450. In any case, the results obtained in the present reconstituted system cannot be directly compared to those obtained in intact microsomes.

The rate of NADPH-cytochrome *c* reductase activity of the reconstituted DMPC system exhibits a linear Arrhenius plot. In this reaction, the membrane-bound reductase interacts with water-soluble cytochrome *c*. Therefore, this activity should not be affected by changes in the physical state of the membrane, unless such changes induce conformational alterations in the reductase molecule. The lack of such conformational changes can be concluded from the linearity of the Arrhenius plot and also from the finding that the cytochrome *c* reductase activities of the membrane-bound and free reductase show practically the same activation energy. A linear Arrhenius plot and similar activation energy have also been reported for NADPH-cytochrome *c* reductase activity of liver microsomes (Schenkman, 1972). It should also be noted that the hydrophilic catalytic domain of the reductase has an independent conformation from the hydrophobic membrane-bound domain (Black & Coon, 1982). The lack of any break in the Arrhenius plot of slow NADPH oxidation by the reconstituted system in the absence of substrate may indicate that this activity is, at least, partly catalyzed by the reductase, which has been shown to be autoxidizable (Yasukochi et al., 1979). An interesting finding is that cumene hydroperoxide dependent drug oxidation activity of the reconstituted system also shows a linear Arrhenius plot. It seems that the aforementioned conformational change of cytochrome P-450 induced by the phase transition does not affect the activation energy of this reaction. This may be due to the fact that the peroxide-dependent reaction bypasses rate-limiting step(s) of the NADPH-supported reaction. Whether the two reactions share an identical iron–oxo intermediate is still uncertain (Black & Coon, 1981).

Finally, the liposomal reconstituted system used in this and our previous studies (Taniguchi et al., 1979, 1984b) is quite different from those prepared by mixing the reductase and cytochrome P-450 with detergents or dilauroylphosphatidylcholine. In these systems no bilayer structures are formed, and the reductase and cytochrome P-450 exist as a stable complex. In the liposomal reconstituted systems, on the other hand, these two proteins can move rapidly on the plane of membrane, and the lateral mobility of these proteins enables the interaction with other electron-transferring proteins such as cytochrome b_5 (Taniguchi et al., 1984b). In this respect, the liposomal reconstituted system is a better model for studies of the microsomal monooxygenase system, although one should be careful in applying the conclusions obtained in the liposomal reconstituted system to liver microsomes, which are much more complex in structure than the reconstituted system.

Registry No. DMPC, 18194-24-6; NADPH-cytochrome P-450 reductase, 9039-06-9; cytochrome P-450, 9035-51-2; monooxygenase, 9038-14-6; benzphetamine *N*-demethylase, 37237-40-4.

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The 43-Kilodalton Protein of *Torpedo* Nicotinic Postsynaptic Membranes: Purification and Determination of Primary Structure†

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ABSTRACT: The primary structure of the 43-kilodalton peripheral membrane protein (43-kDa protein) of *Torpedo* nicotinic postsynaptic membrane has been determined. The 43-kDa protein, which was isolated by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis, has an amino terminus resistant to Edman degradation, while the sequence at the carboxyl terminus is Tyr-Val. An amino acid sequence of 405 residues was obtained by NH_2-terminal sequence analysis of complementary peptides generated by digestion with trypsin, chymotrypsin, *Staphylococcus aureus* V8 protease, and endoproteinase Lys-C, as well as by chemical cleavage at methionine. This sequence of molecular mass 45 618 daltons lacks the amino terminus but extends to the carboxyl terminus of the 43-kDa protein. Unusual structural features of the 43-kDa protein include two regions of ~80 residues, each containing 10% cysteine, as well as stretches predicted to exist as amphipathic α-helices. Other than the group blocking the amino terminus, no evidence was found for posttranslational modification of amino acids. The 43-kDa protein may represent a novel protein family because a computer search of this sequence with the National Biomedical Research Foundation data base (Release 12.0) did not reveal any significant homology to known protein sequences.

In highly purified postsynaptic membranes isolated from *Torpedo* electric tissue, the nicotinic acetylcholine receptor (AChR)[1] constitutes as much as 40% of the membrane protein. The predominant nonreceptor protein is a protein of molecular mass 43 000 (43-kDa protein or ν_1) that is a peripheral protein on the cytoplasmic surface (Neubig et al., 1979; Wennogle & Changeux, 1980; Elliott et al., 1980; St. John et al., 1982). By biochemical and immunological criteria, this protein is distinct from actin and creatine phosphokinase, major constituents of the electrocyte cytoplasm that are also characterized by M_r ~43 000 and that are present in variable amounts in preparations of postsynaptic membranes (Gysin

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[1] Abbreviations: AChR, nicotinic acetylcholine receptor; 43-kDa protein, the basic, membrane-bound 43-kilodalton protein of *Torpedo* postsynaptic membranes; LIS, lithium diiodosalicylate; IAA, iodoacetamide; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; [^{14}C]CAM-43K, [^{14}C](carboxamidomethyl)-43-kDa protein; DFP, diisopropyl phosphorofluoridate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane; TPCK, N^{α}-(*p*-tosyl)-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid.

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