Dynamic Mobility of Genetically Expressed Fusion Protein between Cytochrome P4501A1 and NADPH−Cytochrome P450 Reductase in Yeast Microsomes

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ABSTRACT: A fusion protein of rat liver CYP1A1 with NADPH-cytochrome P450 reductase was expressed genetically in yeast microsomal membranes. This flavo-cytochrome is active in 6-hydroxylation of zoxazolamine. Rotational diffusion of the fusion protein was examined by observing the flash-induced absorption anisotropy $r(t)$ of the P450·CO complex. Theoretical analysis of $r(t)$ was performed based on a "rotation-about-membrane normal" model. The absorption anisotropy decayed within 2 ms to a time-independent value r_3. Forty percent of the fusion protein rotated with a rotational relaxation time ϕ of 1.35 ms. Treatment with high salt increased the mobile population of the fusion protein to 62% with $\phi =$ 0.96 ms. The mobile population of the fusion protein is close to that of CYP1A1 coexpressed with the P450 reductase and greater than that of CYP1A1 alone [Iwase et al. (1991) *Biochemistry 30*, 8347−8351]. The large mobile population of the fusion protein provides evidence that CYP1A1 is mobilized by forming associations with P450 reductase in microsomal membranes.

Cytochrome P450 is the terminal enzyme of the hepatic microsomal monooxygenase systems, catalyzing the oxidative metabolism of various drugs, xenobiotics as well as endogenous substrates (*1*−*3*). The monooxygenase systems consist of several membrane proteins such as NADPH-cytochrome P450 reductase, NADH-cytochrome b_5 reductase, cytochrome b_5, and cytochrome P450.

Among numerous isozymes of P450, the methylcholanthrene-inducible CYP1A1[1] is known to convert polycyclic aromatic hydrocarbons to highly carcinogenic compounds (*4*, *5*). However, many chemically different species of P450s present in liver microsomes prevent selective analysis of a special species of P450. The rigorous characterization of a specific isoform of CYP1A1 has been established by the heterologous expression in yeast microsomes (*6*−*9*). These expression systems have also provided a means to examine structure−function relationships through site-directed mutagenesis and construction of chimeric proteins (*10*−*12*).

Protein−protein interactions of cytochrome P450 have been extensively examined by Kawato and co-workers in rat liver microsomes, bovine adrenocortical microsomes, phospholipid vesicles, and yeast microsomes by observing the rotational diffusion of cytochrome P450s. It was demonstrated in lipid vesicles that NADPH-cytochrome P450 reductase forms a transient association with rat liver microsomal CYP2B1/2B2 (*13*−*16*), rabbit liver CYP1A2 (*17*), and bovine adrenocortical CYP21 (*18*). It was also demonstrated in yeast microsomes that genetically expressed rat liver CYP1A1 forms a transient association with P450 reductase (*19*). On the other hand, P450 reductase did not form such an association with bovine adrenocortical microsomal CYP17 (*18*). These results imply that the mode of interactions of cytochrome P450 with P450 reductase is significantly different depending on the chemical species of cytochrome P450.

Here, by combining the genetic expression of the fusion protein of CYP1A1 with P450 reductase and the protein rotation measurements, we demonstrated that a stable association of CYP1A1 with P450 reductase shows a high mobility of P450 in the membrane of yeast microsomes.

EXPERIMENTAL PROCEDURES

Genetic Expression of the Fusion Protein of CYP1A1 with NADPH-Cytochrome P450 Reductase in Yeast Microsomes.

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[1] Abbreviations: CYP1A1(cytochrome P4501A1) and CYP1A2-(P4501A2), the major forms of P450s in 3-methylcholanthrene-induced rat liver microsomes; CYP2B1(cytochrome P4502B1) and CYP2B2-(P4502B2), the major forms of P450s in phenobarbital-induced rat liver microsomes; CYP17(cytochrome P45017α, lyase) and CYP21(P450c21), cytochrome P450s in adrenal cortex, catalyzing steroid 17α-hydroxylation and steroid 21-hydroxylation, respectively; *L*/*P*, lipid-to-protein ratio in weight.

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The fusion protein of rat liver CYP1A1 with yeast NADPH-cytochrome P450 reductase was genetically expressed in microsomes of yeast *Saccharomyces cerevisiae* AH22 cells as described elsewhere (*11*). The cDNA fragment of yeast NADPH-cytochrome P450 reductase lacking N-terminal amino acids 1−41 was fused with rat liver CYP1A1. Microsomes were prepared as described elsewhere (*6*).

Rotational Diffusion Measurements and Analysis. For rotational diffusion measurements, 60% (w/w) sucrose was dissolved in microsome suspensions (50 mM Hepes buffer, 1 mM EDTA, and 0.1 mM DTT, pH 7.4) in order to reduce light scattering and microsomal tumbling. The sample was bubbled with CO for 5 s and then reduced with a few grains of dithionite. The time-resolved flash photolysis depolarization measurements were performed as described elsewhere (*20*−*22*). The sample (3−5 μM in heme) was photolyzed by a vertically polarized laser flash at 532 nm from a Nd:YAG laser, and the absorbance changes were measured at 450 nm. The signals were analyzed by calculating the absorption anisotropy, $r(t)$, and the total absorbance change, $A(t)$, given by

$$r(t) = [A_V(t) - A_H(t)S]/A(t) \quad (1)$$

$$A(t) = A_V(t) + 2A_H(t)S \quad (2)$$

where $A_V(t)$ and $A_H(t)$ are, respectively, the absorption changes for the vertical and horizontal polarization at time t after the laser flash. A slight unbalance of the two photomultipliers is corrected using $S = A_{HV}/A_{HH}$, which is the ratio of the time averaged absorption changes in the vertical and horizontal components obtained with the horizontal flash excitation. In each experiment, 16 384 signals were averaged using a Toyo Technica 2805 transient memory. Analysis of $r(t)$ is based on a model of the axial rotation of cytochrome P450 about the axis perpendicular to the membrane plane (*15*, *23*). When there is a single rotating species of P450 with rotational relaxation time $\phi_{\|}$, $r(t)$ is given by

$$r(t)/r(0) = 3\sin^2\theta_N\cdot\cos^2\theta_N\cdot\exp(-t/\phi_{\|}) + 3/4\cdot\sin^4\theta_N\cdot\exp(-4t/\phi_{\|}) + 1/4(3\cos^2\theta_N - 1)^2 \quad (3)$$

where θ_N is the tilt angle of the heme plane from the membrane plane. Multiple rotating species of P450 with different $\phi_{\|}$ values are considered by analyzing the data by the following approximated equation:

$$r(t) = r_1\exp(-t/\phi) + r_2\exp(-4t/\phi) + r_3 \quad (4)$$

where ϕ is the average rotational relaxation time over multiple rotating species of P450 and r_1, r_2, and r_3 are constants. Although eq 4 is not theoretically accurate for multiple rotating species, eq 4 is used to judge to what extent the mode of rotation deviates from the rotation of a single rotating population by comparing the experimental values of ϕ, r_1/r_2, and $r_3/r(0)$ with $\phi_{\|}$, $4\cot^2\theta_N$, $1/4(3\cos^2\theta_N - 1)^2$ in eq 3. The mobile population of the fusion protein including CYP1A1, p_m (%), was calculated with eq 5 based on the experimentally determined minimal anisotropy of $[r_3/r(0)]_{min} = 0.03$ when all the CYP1A1 was rotating in proteoliposomes (*16*):

$$p_m\,(\%) = 100 \times [1 - r_3/r(0)]/(1 - 0.03) \quad (5)$$

Curve fitting of the data based on eq 4 was accomplished by a PDP-11/73 minicomputer.

Miscellaneous. Drug oxidation activity of the fusion protein was measured by observing the 6-hydroxylation of zoxazolamine (*11*). Cytochrome P450 was measured spectrophotometrically as described elsewhere (*24*). Cytochrome *c* reduction activity of P450 reductase was measured by the absorbance change at 550 nm (*25*, *26*). The steady-state fluorescence anisotropy (r^s) was measured with the fluorescent probe diphenylhexatriene using a Hitachi F-3000 fluorometer. Samples were excited at 360 nm, and fluorescence above 420 nm was measured (*22*). Lipid phosphorus was measured as described elsewhere (*27*). Western immunoblotting analysis was performed as described elsewhere (*11*). Protein was determined with the BCA protein assay using bovine serum albumin as the standard. The BCA protein assay reagent was purchased from Pierce Chemical Company (Rockford, IL). Other chemicals were of the highest grade commercially available.

RESULTS AND DISCUSSION

Characterization of the Fusion Protein in Yeast Microsomes. The distribution of the fusion protein (CYP1A1 with reductase), proteins, and lipids in yeast microsomes was examined by ultracentrifugation at 155000*g* for 19 h in sucrose density gradient from 20 to 50% (w/w), followed by fractionation. CYP1A1 heme was measured at 450 nm by a CO difference spectrum, and NADPH-cytochrome P450 reductase was measured by its NADPH-cytochrome *c* reduction activity based on the cytochrome *c* reduction activity of the purified reductase (*25*). A coincidence in the distribution of the three quantities was observed by a broad band at around 29−41% in sucrose density, showing that genetically expressed fusion protein was incorporated in the microsomal membrane (see Figure 1). The presence of the fusion protein, containing both the P450 domain and the P450 reductase domain, was demonstrated by Western immunoblotting using anti-yeast reductase IgG and anti-CYP1A1 IgG (see Figure 1). Incorporation of CYP1A1 coexpressed with exogenous P450 reductase was also demonstrated by the same procedures, indicating that both CYP1A1 and P450 reductase appeared at around 37−40% in sucrose density. Since there was a negligible amount of endogenous yeast P450 and yeast P450 reductase, the heme content and NADPH-cytochrome *c* reduction activity should be dominantly contributed by genetically expressed proteins.

The concentration of the expressed fusion protein was 0.12−0.14 nmol/mg of protein in microsomes. However, we did not find any P420·CO with a CO difference spectrum. In the case of the coexpression system including CYP1A1 and exogenous yeast P450 reductase, we observed 0.15−0.25 nmol of P450/mg of protein and 0.3−0.5 nmol of P450 reductase/mg of protein in microsomes.

The 6-hydroxylation activity of zoxazolamine was 13.7 ± 0.6 nmol/nmol P450/min for the fusion protein, 10.6 ± 0.8 for CYP1A1 coexpressed with P450 reductase, and 0.50 for the CYP1A1 expressed alone, respectively, using 400 μM zoxazolamine at 10 °C. These activity values probably

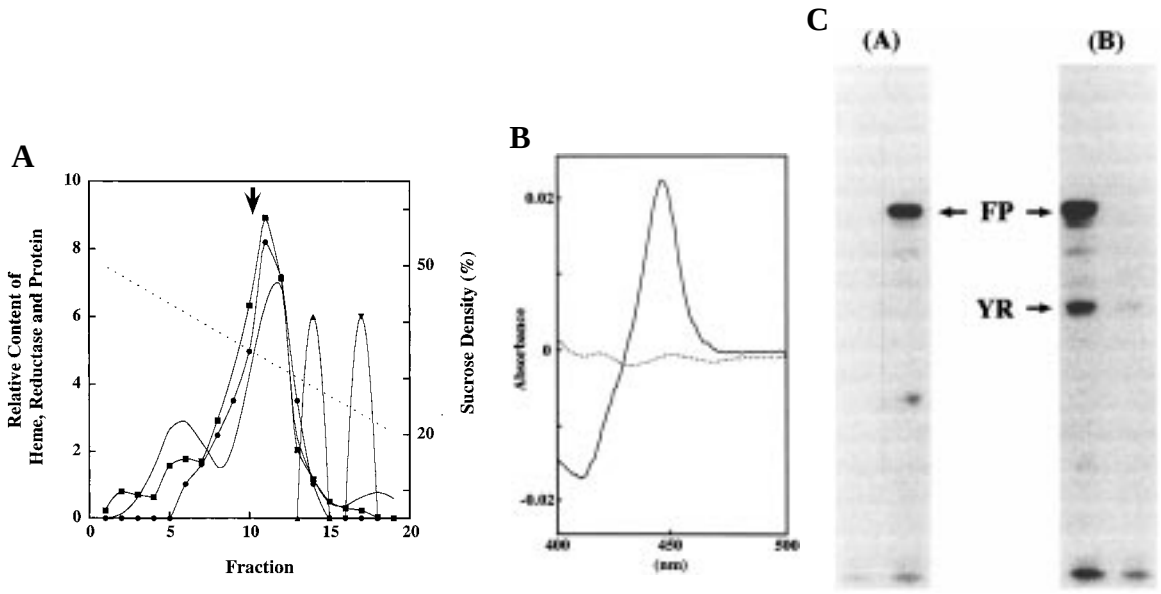

FIGURE 1: Characterization of the fusion protein in yeast microsomes. (A) Analysis of microsomes containing fusion proteins by centrifugation on a sucrose density gradient. Typical distribution profile of the heme (●), the reductase (■), and total protein (—) loaded onto a 20−50% sucrose density gradient (dotted line). Peak values of the heme content, the reductase activity and protein content were 0.2 nmol of heme, 0.6 μmol/min/mg of protein and 3 mg of protein, respectively. The arrow shows the central position of the protein distribution of control microsomes. P450 proteoliposome with $L/P = 0.8$ (▲) and P450 proteoliposome with $L/P = 2$ (▼) are shown as the sedimentation markers. The vertical axis is chosen arbitrarily to facilitate comparison. (B) CO difference spectra of the yeast microsome fractions. Solid line is CO difference spectrum of microsomes containing fusion protein; dotted line is that of control yeast microsomes. (C) Western immunoblot analysis of the yeast microsome fractions. Yeast microsomes were electrophoresed on a 10% polyacrylamide−sodium dodecyl sulfate gel and then transferred to a nitrocellulose filter. The lanes A and B were probed with anti-rat CYP1A1 IgG and anti-yeast NADPH−P450 reductase IgG, respectively. The left of panel A and the right of panel B are controls. FP and YR show the migration points of the fusion protein and yeast reductase, respectively. Molecular weight deduced from the migration of proteins consisted of that from cDNA.

reflect the relative concentration of P450 reductase to P450 and the effective accessibility of P450 reductase to CYP1A1. For the fusion protein, the ratio of the reductase to CYP1A1 was calculated to be about 1:3/4 in yeast microsomes. The ratio of the P450 reductase to CYP1A1 was about 1:3/4, 1:1/2, and 1:17 (average figures from several preparations) for the fusion protein, the coexpression system, and the only CYP1A1-expressed system, respectively. Though the relative amount of reductase to CYP1A1 was less for the fusion protein than for the coexpression system, the higher substrate hydroxylation activity of the fusion protein than that of the coexpression system would be due to the much higher effective electron-transfer probability for the permanent association of P450 reductase and P450 in the fusion protein than that for the coexpression system where P450 reductase and P450 undergo a transient association-dissociation equilibrium.

Rotation and Dynamics of the Fusion Protein. Rotational diffusion of the fusion protein genetically expressed in yeast microsomes was measured at 20 °C. The $r(t)$ curves decayed within 2 ms to a time-independent value r_3, implying the coexistence of rotating and immobile ($\phi \geq 20$ ms) populations of the fusion protein (Figure 2). Data were analyzed according to eq 4. The normalized time-independent anisotropy was $r_3/r(0) = 0.61 \pm 0.04$, and the rotational relaxation time was $\phi = 1.35 \pm 0.24$ ms (Figure 2). This corresponds to the mobile population of 40 ± 4% based on the calculation from eq 5. It should be noted that these data were obtained from several independent yeast cultures containing the fusion protein. As a comparison, we obtained $r_3/r(0) = 0.59$ with $\phi = 1.30 \pm 0.25$ ms for CYP1A1 coexpressed with P450 reductase (Figure 2). The mobile population of 40% for the fusion protein was not significantly different from that of 42% for the coexpression systems. Decay parameters were the same within experimental error for all different cultures

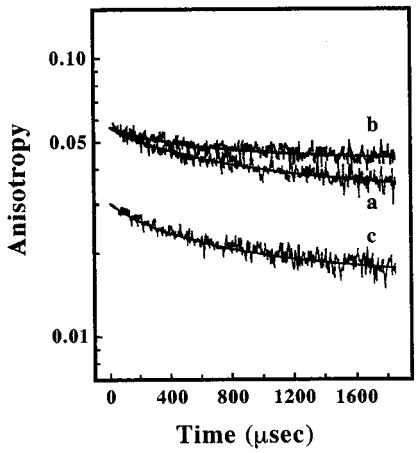

FIGURE 2: Time-dependent absorption anisotropy of the fusion protein in yeast microsomes. Samples (3−5 μM in heme) were photolyzed by a vertically polarized laser flash at 532 nm, and $r(t)$ was recorded at 450 nm as described in the Experimental Procedures. Measurements were performed in 60% sucrose solution at 20 °C (~0.6 poise). Curve a, microsomes with the fusion protein expressed; curve b, microsomes with only CYP1A1 expressed; curve c, microsomes with CYP1A1 and reductase coexpressed. The zigzag lines are experimental data and the solid curves were obtained by fitting the data to eq 4. The initial anisotropy of curve b is slightly normalized to the same $r(0)$ of curve a to facilitate comparison. This is justified by the fact that although $r(0)$ depends on the laser flash intensity, the normalized anisotropy $r(t)/r(0)$ is not affected by the different flash intensity (*23*). Curve c was displaced for illustrative purposes; otherwise, curve c is almost completely superimposed on curve a.

examined. Especially, coexpressed CYP1A1 with P450 reductase showed very similar values to those reported previously (*19*). It should be noted that, when only CYP1A1 was expressed in microsomes, the CYP1A1 showed a considerably smaller number of the mobile population of 28% (*19*). These results imply that CYP1A1 was mobilized by forming associations with P450 reductase. The ϕ value for CYP1A1 was not significantly different between these three different expression systems within experimental error. The presence of 20 μM 7-ethoxycoumarin (substrate of CYP1A1) did not significantly affect the rotational mobility of the fusion protein. The total absorption change $A(t)$ of the fusion protein·CO in microsomes showed a monoexponential decay with a lifetime of $\tau = 4.1$ ms.

There might be a possibility that a change in the heme orientation in the membrane could occur between the pure P450 and the fusion protein; thereby, the present analysis may not be correct. However, as judged from the construction of the fusion protein expression vector, the P450 moiety of the fusion protein (only C-terminal 5 amino acids are deleted) was almost the same as pure CYP1A1, and the water soluble part of the reductase (without the membrane-embedded segment) was conjugated with the P450 moiety. After trypsin treatment of the yeast microsomes, the P450 moiety of the fusion protein was observed in the microsomal fraction, and the reductase moiety was observed in the soluble fraction. Therefore, there may not be a significant change in the membrane insertion part of the fusion protein from the pure CYP1A1, having the head part of the reductase being conjugated with the water protruded part of the P450 moiety. Thus, the heme orientation of the fusion protein might not be significantly different from that of the pure CYP1A1.

It might also be the case that the rotation occurs about more than one axis due to the structure of cytochrome P450, which may not be completely buried in the membrane. However, the very high salt treatment, even with 500 mM NaCl, did not remove pure CYP1A1 and the fusion protein from the membrane, indicating that the membrane insertion is stable, being different from the cytochrome b_5 type of single anchor protein. N-Terminally truncated CYP1A1, whose N-terminal hydrophobic segment including 30 amino acids was deleted, was also highly membrane buried with almost the same rotation properties as the full-length CYP1A1 (*28*). There should be at least two membrane-embedded segments stabilizing the orientation of CYP1A1 with respect to the membrane. Therefore, the uniaxial rotation of CYP1A1 would possibly be a realistic assumption.

The lipid composition of yeast microsomes, having phosphatidylcholine (PC) (50%), phosphatidylethanolamine (PE) (20%), phosphatidylserine (PS) (10%), and other phospholipids (20%), is not very much different from that of rat liver microsomes, having PC (60%), PE (20%), PS (2%), and other phospholipids (18%) (*29*). We used 63% PC, 31% PE, and 6% PS for the proteoliposomes of CYP1A1. All of these membranes that we used would not be very different in phospholipid composition. The acyl chain composition of yeast microsomes was 14% for $C_{16:0}$, 42% for $C_{16:1}$, and 43% for $C_{18:1}$ analyzed with a HITACHI G-5000 gas chromatograph. Liver microsomes have an acyl chain composition of 29% for $C_{15:0}$, 11% for $C_{17:0}$, 15% for $C_{18:0}$, 27% for $C_{16:1}$, and 14% for $C_{20:4}$ (*30*).

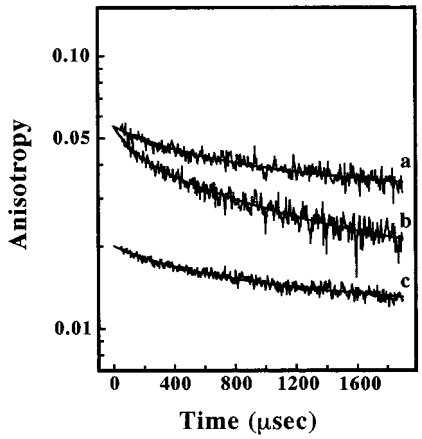

FIGURE 3: Effects of KCl and substrate on the time-dependent absorption anisotropy of the fusion protein in yeast microsomes. Curve a, microsomes with the fusion protein; curve b, microsomes incubated with 260 mM KCl (final concentration); curve c, microsomes with 20 μM 7-ethoxycoumarin (final concentration). The other conditions are the same as described in Figure 2. The zig zag lines are experimental data and the solid curves were obtained by fitting the data to eq 4. The initial anisotropy of curve b is slightly normalized to the same $r(0)$ of curve a to facilitate comparison. Curve c was vertically displaced for illustrative purposes; otherwise, curve c is almost completely superimposed on curve a.

As judged from our results, rotational mobility of membrane proteins was significantly dependent on the lipid fluidity (*19*). The mobility of CYP1A1 in yeast microsomes, with a mobile population of 28%, was considerably lower than that in rat liver microsomes with a mobile population of 48%, though the concentration of membrane proteins ($L/P = 0.4$) was almost the same between these two microsomes. However, there was a large difference in the sterol content. The larger content of ergosterol (13% of total phospholipids) in yeast microsomes than that of cholesterol in liver microsomes (4% of total phospholipids) would cause a higher rigidity of yeast microsomes (a fluorescence anisotropy of $r^s = 0.195$) than that of liver microsomes ($r^s = 0.139$) (*31*). This difference in mobility of P450 is possibly due to the high rigidity of the yeast microsomal lipid bilayer.

Effects of KCl on Rotational Mobility of the Fusion Protein. After a 30 min incubation of the fusion protein in microsomes with 500 mM KCl, the sample was transferred to 60% sucrose solution. Rotational diffusion measurements were performed at 20 °C. In the presence of KCl, the normalized time-independent anisotropy was $r_3/r(0) = 0.40 \pm 0.04$, and the rotational relaxation time was $\phi = 0.96 \pm 0.12$ ms (Figure 3). In the presence of KCl, the fusion protein was mobilized from 40 to 62%. This mobilization would be due to dissociation of the protein aggregates by weakening the electrostatic interactions (*32*). These results indicate that up to 60% of the fusion protein was immobilized in protein aggregates probably due to a high protein concentration of microsomes with a lipid-to-protein weight ratio (L/P) of 0.4. It should be noted that the high salt treatment did not significantly change the CO recombination kinetics, excluding the possible denaturation of P450 to alter the $r(t)$ curve at high KCl concentration.

Dynamic Interactions of Cytochrome P450 with NADPH-Cytochrome P450 Reductase. Kawato and co-workers extensively investigated protein−protein interactions between

microsomal P450s and NADPH-cytochrome P450 reductase in proteoliposomes and microsomes. The presence of P450 reductase mobilized rabbit liver CYP1A2 by 9% (*17*), rat liver CYP2B1/2B2 by 30% (*13*), and bovine adrenocortical CYP21 by 17% (*18*) in proteoliposomes. In the case of genetically coexpressed CYP1A1 with P450 reductase in yeast microsomes, the mobile population of CYP1A1 was increased by 15% compared with the only CYP1A1-expressed microsomes. These results imply that these cytochromes were mobilized by forming a transient association with P450 reductase. Upon formation of a small transient association, probably the immobile aggregates of the cytochrome were dissociated into small rotamers of P450 with P450 reductase, resulting in the mobilization of the cytochrome. The much higher mobility of the fusion protein than that of only CYP1A1 expressed in microsomes supports the explanation of reductase-induced mobilization of CYP1A1. The presence of a transient association between P450 and P450 reductase was practically confirmed by the observations that some of these cytochrome P450s (e.g., CYP2B1/2B2 and CYP21) were immobilized by cross-linking of P450 reductase by anti-reductase antibody (*14*, *18*).

It should be noted, on the other hand, that the mobility of rabbit liver CYP2B4 and bovine adrenocortical CYP17 was not affected by the presence of P450 reductase (*17*, *18*). This implies that CYP2B4 and CYP17 probably do not form a transient association with P450 reductase. Lateral collision-controlled electron transfer would occur between P450 reductase and cytochrome P450(2B4, 17α). On the other hand, electron transfer would occur within the associations between P450 reductase and cytochrome P450(1A1, 1A2, 2B, 21). The observed association between P450 reductase and P450(1A1, 1A2, 2B, 21) should have a transient nature and might not be stable over a time range of, for example, seconds or minutes. This is because these cytochrome P450s functionally interact with several other proteins including cytochrome b_5. In fact, it was shown by mobility and chemical cross-linking experiments that CYP1A2 forms a transient association with cytochrome b_5 (*17*, *33*). If the association of P450 reductase with P450 is very stable, cytochrome b_5 would have difficulty in donating electrons to P450.

Significance of Fusion Protein Experiments. We selectively examined the dynamic and rotational behavior of the permanent 1:1 complex of CYP1A1 with P450 reductase using the fusion protein. Consistent with our explanation of the reductase-induced mobilization of P450 by dissociating protein aggregates and forming a transient association, the mobile population is much greater for the fusion protein than that for only CYP1A1-expressed microsomes (*19*). The fusion protein could be very mobile to have a mobile population greater than that for coexpression systems, because the number of the reductase−P450 complexes should be greater for the fusion protein-expressed microsomes than the CYP1A1- and reductase-coexpressed microsomes. Practically, the mobile population of 40% for the fusion protein is, however, almost the same as that of CYP1A1 coexpressed with the P450 reductase. The reason would be due to the high density of the proteins in microsomal membrane ($L/P = 0.4$), preventing full mobility even for the fusion protein. Another possibility is that the incorporation of fusion protein was not very homogeneous but was incorporated in part as aggregates as judged from the rather broad and inhomogeneous distribution of the microsome band in the sucrose density gradient analysis and from the considerable mobilization induced by KCl. Anyway, because the fusion protein was much more mobile than only the CYP1A1-expressed system in microsomes, it is proved that the stable complex of CYP1A1 with P450 reductase has much less tendency toward aggregation than CYP1A1 without P450 reductase.

The rate of electron transfer from NADPH to the heme domain through the reductase domain of the fusion protein was greatly increased by about 20 times the rate from independent P450 reductase to CYP1A1 as judged from stopped flow kinetic measurements (*11*). The rate constant for the reduction of the heme of the fusion protein with NADPH in the presence of zoxazolamine was larger than 50 s^{-1}. The V_{max} value of the zoxazolamine 6-hydroxylation activity was, however, increased only 1.4 times compared with the coexpressed CYP1A1 and P450 reductase system. Only a slight increase in the hydroxylation activity of the fusion protein even with a very rapid electron transfer was interpreted as being due to a slow release of hydroxylated products from the P450 domain, which step is rate-limiting regarding the hydroxylation reaction of the fusion protein.

We cannot conclude for the fusion protein whether the P450 reductase domain and the P450 domain undergo rapid collisions due to independent flexible motion of domains or whether they form a tight contact without flexible motion. There are several lines of indirect results which support the importance of intramolecular flexible motion in the fusion protein for efficient electron transfer. A certain length of the amino acid chain, longer than 2 amino acids, was necessary to retain a high electron-transfer activity for the artificial fusion protein of cytochrome *c* and cytochrome peroxidase. A natural fusion protein P450BM3 was not crystallized as a form of a holo P450BM3 molecule (J. Peterson, unpublished results), but good crystals were obtained for the separated P450 domain (*34*). This might be due to an internal flexibility of two domains interfering with the crystallization of the holo enzyme. Intramolecular flexibility may allow correct facing of the donor and acceptor amino acids between two redox domains.

If there is a considerable angular flexibility for the P450 domain, then we should have a significantly lower $r(0)$ value than that of pure CYP1A1 with $r(0) = 0.053$, because such a rapid intramolecular rotation should contribute to a decrease in $r(t)$ in the time range of submicroseconds. However, the present $r(0)$ value of around 0.051 was almost the same as those of the coexpression microsomes and the only CYP1A1-expressed microsomes within experimental error, implying that the angular freedom of flexible motion of the P450 domain would not be large.

Other than P450BM3, NO synthase is also a natural fusion protein between P450 and P450 reductase (*35*), playing an important role in signal transduction in the central nervous system by producing NO gas from L-arginine. The biological significance of the fusion structure in NO synthase could be examined by the present rotation experiments with respect to intramolecular electron-transfer mechanisms and possible interactions with the cell membrane.

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