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Rotation and Membrane Topology of Genetically Expressed Methylcholanthrene-inducible Cytochrome P-450IA1 Lacking the N-terminal Hydrophobic Segment in Yeast Microsomes*

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A modified rat liver cytochrome P-450IA1, lacking amino acids 2–30, a proposed membrane anchor for cytochrome P-450, was expressed genetically in yeast microsomal membranes. This truncated cytochrome is practically active in the deethylation of 7-ethoxycoumarin. A full-length cytochrome P-450IA1 was also expressed in yeast microsomes. Rotational diffusion of P-450IA1 was examined by observing the flash-induced absorption anisotropy $r(t)$ of the P-450·CO complex. The anisotropy decayed to a time-independent value within a 2-ms time range. Since the decay curve has the characteristics of a slow rotation of membrane-embedded cytochrome, the theoretical analysis of $r(t)$ was performed based on a "rotation-about-membrane normal" model. 41% of the shortened P-450IA1 was rotating with the rotational relaxation time ϕ of 1020 μs, whereas 27% of the full-length P-450IA1 was mobile with ϕ = 1101 μs. The high salt treatment did not remove the shortened cytochrome from the membrane and also did not drastically weaken the interactions of the cytochrome with the membrane, as judged from the slow rotation characteristics (ϕ = 830 μs). These results demonstrate that the N-terminal shortened P-450IA1 is incorporated properly into the yeast microsomal membrane and that the N-terminal hydrophobic segment is not solely responsible for attachment to the membrane, providing evidence that additional segments of P-450IA1 are involved in the membrane binding.

Cytochrome P-450 is a key enzyme in hepatic microsomal monooxygenase system, catalyzing the oxidative metabolism of various drugs, xenobiotics as well as endogenous substrates (Estabrook *et al.*, 1979; White and Coon, 1980). This enzyme comprises a superfamily of monooxygenases. Among numerous isozymes of P-450, the methylcholanthrene-inducible cytochrome P-450IA1/IA2[1] is known to convert polycyclic aromatic hydrocarbons to highly carcinogenic compounds (Harada and Omura, 1981; Guengerich, 1987). However, many chemically different species of P-450s present in liver microsomes prevent selective analyses of a special species of P-450. The rigorous characterization of a specific isoform of P-450IA1 has been established only recently by the heterologous expression in yeast microsomes (Oeda *et al.*, 1985; Sakaki *et al.*, 1985, 1987; Murakami *et al.*, 1990). These expression systems have also provided a means to examine structure-function relationships through site-directed mutagenesis and construction of chimeric proteins.

Dynamic structures of P-450 have been extensively examined in rat liver microsomes, adrenocortical microsomes, phospholipid vesicles, and yeast microsomes by observing rotational diffusion of cytochrome P-450 (Gut *et al.*, 1982, 1983; Kawato *et al.*, 1982; Iwase *et al.*, 1991; Ohta *et al.*, 1992). By observing rotational diffusion of P-450IA1 expressed in yeast microsomes, we have demonstrated a transient association of P-450IA1 with NADPH-cytochrome P-450 reductase (Iwase *et al.*, 1991). P-450IA1/IA2 has been shown to form a transient association with epoxide hydrase in phospholipid vesicles (Etter *et al.*, 1991).

Membrane topology of cytochrome P-450 has been a current topic (De Lemos-Chiarandini *et al.*, 1987; Monier *et al.*, 1988; Nelson and Strobel, 1988; Brown and Black, 1989; Vergeres *et al.*, 1990; Edwards *et al.*, 1991; Ohta *et al.*, 1992). Different from many other transmembrane proteins, microsomal cytochrome P-450 was not expected to have obvious membrane-spanning polypeptides other than the hydrophobic N-terminal segment, as judged from the computer analyses of amino acid sequences (Nelson and Strobel, 1988; Gotoh and Fujii-Kuriyama, 1989). Several biochemical investigations have suggested that liver microsomal P-450IIB1/IIB2 anchors in the membrane only via the N-terminal very hydrophobic segment and that the major part of the cytochrome exposes to the water phase (Vergeres *et al.*, 1989). However, recently genetically expressed cytochrome P-450IIE1 lacking N-terminal hydrophobic segment has been shown to tightly bind to the membrane of *Escherichia coli* (Larson *et al.*, 1991).

Here, by combining the genetic expression of P-450IA1 lacking N-terminal segment and the protein rotation measurements, we demonstrate the tight binding of this truncated cytochrome to the membrane of yeast *Saccharomyces cerevisiae* microsomes.

EXPERIMENTAL PROCEDURES

Genetic Expression of Shortened and Full-length Cytochrome P-450IA1 in Yeast Microsomes—Rat liver cytochrome P-450IA1 lacking

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[1] The abbreviations used are: cytochromes P-450IA1 and P-450IA2, the major forms of P-450s in 3-methylcholanthrene-induced rat liver microsomes; cytochromes P-450IIB1 and P-45IIB2, the major forms of P-450s in phenobarbital-induced rat liver microsomes; cytochromes $P\text{-}450_{17\alpha,lyase}$(P-450XVIIA1) and $P\text{-}450_{C21}$(P-450XXIA1), cytochrome P-450s in adrenal cortex, catalyzing steroid 17α-hydroxylation and steroid 21-hydroxylation, respectively; cytochrome P-450cam (P-450CI), cytochrome P-450 in *Pseudomonas putida*.

amino acids 2–30 and full-length P-450IA1 were genetically expressed in microsomes of yeast *S. cerevisiae* cell as described by Yabusaki *et al.* (1988). Microsomes were prepared as described by Oeda *et al.* (1985). The concentration of expressed shortened P-450IA1 and full-length P-450IA1 was about 0.2 and 0.5 nmol/mg of protein in microsomes, respectively.

Rotational Diffusion Measurements and Analysis—For rotational diffusion measurements, 60% (w/w) sucrose was dissolved in microsome suspensions (50 mM Hepes buffer, 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 (Cherry, 1978; Kawato *et al.*, 1988; Ohta *et al.*, 1990). The sample (3–5 μM in heme) was photolyzed by a vertically polarized laser flash at 532 nm from a Nd/YAG laser, and 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 the following,

$$r(t) = [A_V(t) - A_H(t) \cdot S]/A(t) \quad \text{(Eq. 1)}$$

$$A(t) = A_V(t) + 2A_H(t) \cdot S \quad \text{(Eq. 2)}$$

where $A_V(t)$ and $A_H(t)$ are, respectively, absorption changes for vertical and horizontal polarization at time t after laser flash. A slight unbalance of two photomultipliers is corrected using $S = A_{HV}/A_{HH}$, which is the ratio of time-averaged absorption changes of vertical and horizontal components obtained with 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 P-450 about the axis perpendicular to the membrane plane (Kawato *et al.*, 1981, 1982). When there is a single rotating species of P-450 with rotational relaxation time $\phi_\parallel$, $r(t)$ is given by the following,

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

where θ_N is the tilt angle of the heme plane from membrane plane. Multiple rotating species of P-450 with different $\phi_\parallel$ values are considered by analyzing the data by the following approximated equation,

$$r(t) = r_1 \exp(-t/\phi) + r_2 \exp(-4t/\phi) + r_3 \quad \text{(Eq. 4)}$$

where ϕ is the average rotational relaxation time over multiple rotating species of P-450, and r_1, r_2, and r_3 are constants. Although Equation 4 is not theoretically accurate for multiple rotating species, Equation 4 is used to judge to what an extent the mode of rotation is deviated 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 $\phi_\parallel$, $4\cot^2\theta_N$, $1/4(3\cos^2\theta_N - 1)^2$ in Equation 3. The population of mobile P-450IA1, p_m (%), was calculated with Equation 5, based on the experimentally determined minimal anisotropy of $[r_3/r(0)]_{min} = 0.03$ when all P-450IA1 was rotating in proteoliposomes (Etter *et al.*, 1991).

$$p_m(\%) = 100 \times [1 - r_3/r(0)]/[1 - 0.03] \quad \text{(Eq. 5)}$$

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

Other Methods—Drug oxidation activity of cytochrome P-450IA1 was measured by observing *O*-deethylation of 7-ethoxycoumarin (Sakaki *et al.*, 1985). Cytochrome P-450 was measured spectrophotometrically according to Omura and Sato (1964). Protein was determined with the BCA protein assay using bovine serum albumin as standard. BCA protein assay reagent was purchased from Pierce Chemical Co.

RESULTS AND DISCUSSIONS

Characterization of Cytochrome P-450IA1 in Yeast Microsomes—The distribution of shortened P-450IA1, proteins, and lipids in yeast microsomes was examined by ultracentrifugation at 155,000 × *g* for 19 h in sucrose density gradient from 25 to 50% (w/w), followed by fractionation. A good coincidence in the distribution of three quantities was observed with a dominant band at around 32% in sucrose density, showing that genetically expressed P-450 lacking the N-terminal hydrophobic segment was incorporated in the microsomal membrane. Incorporation of full-length P-450IA1 was also demonstrated

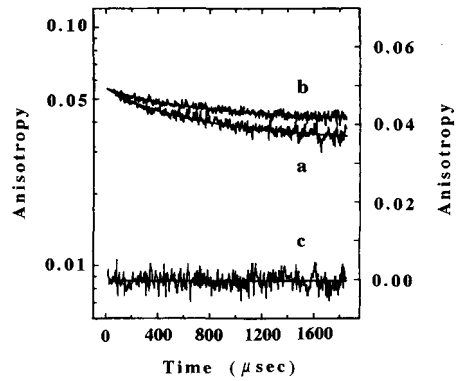

FIG. 1. **Time-dependent absorption anisotropy of shortened and full-length cytochrome P-450IA1 in yeast microsomes.** Samples (3–5 μM in heme) were photolyzed by a vertically polarized laser flash at 532 nm, and $r(t)$ was recorded at 450 nm as described under "Experimental Procedures." Measurements were performed in 60% sucrose solution at 20 °C (~0.6 poise). *Curve a*, microsomes with shortened P-450IA1 expressed; *curve b*, microsomes with full-length P-450IA1 expressed; *curve c*, P-450cam with yeast microsomes. The logarithmic scale (*left*) is chosen for *curves a* and *b* for theoretical comparison. The linear scale (*right*) is chosen for *curve c*, because the $r(t)$ of P-450cam is extremely noisy on a logarithmic scale due to the zero anisotropy. The *zigzag lines* are experimental data, and the *solid curves* were obtained by fitting the data to Equation 4. The initial anisotropy of curve a is slightly normalized to the same $r(0)$ of *curve b* 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 (Kawato and Kinosita, 1981).

with the same procedures. With the CO difference spectra of the shortened P-450IA1, we observed about 8.9 nmol of the cytochrome in the precipitated microsomal fraction and about 0.6 nmol in the supernatant upon ultracentrifugation at 170,000 × *g* for 60 min after incubating microsomes with 800 mM NaCl for 30 min at room temperature. These results imply that more than 90% of P-450IA1 lacking the N-terminal segment is hydrophobically incorporated in the microsomal membrane. Note that more than 90% of the full-length P-450IA1 was also shown to be incorporated in microsomal membranes. On the other hand, water-soluble P-450cam was clearly separated from microsomes by ultracentrifugation after incubation of P-450cam with yeast microsomes, even without any salt treatment. The *O*-deethylation activity for 7-ethoxycoumarin of shortened P-450IA1 was decreased to 1.1 nmol/min/nmol of P-450, which was around one-third that of full-length P-450IA1.

Rotation and Interactions of Shortened and Full-length P-450IA1—Rotational diffusion of full-length and shortened cytochrome P-450IA1 genetically expressed in yeast microsomes was measured at 20 °C. The $r(t)$ curves for both P-450IA1s decayed within 2 ms to a time-independent value r_3, implying the co-existence of rotating and immobile ($\phi \geq 20$ ms) populations of P-450IA1 (Fig. 1). Data were analyzed according to Equation 4. For shortened P-450IA1, the normalized time-independent anisotropy was $r_3/r(0) = 0.60 \pm 0.04$, and the rotational relaxation time was $\phi = 1020 \pm 196$ μs (Fig. 1). As a comparison, full-length P-450IA1 has $r_3/r(0) = 0.74 \pm 0.04$ and $\phi = 1101 \pm 138$ μs. It should be noted that these data were obtained from several independent yeast cultures for both shortened and full-length P-450IA1. Decay parameters were the same within experimental error for all different cultures. Especially full-length P-450IA1 showed very similar values to those reported previously (Iwase *et al.*, 1991). Based on the calculation from Equation 5, assuming the same heme orientation for both the shortened and the full-length P-450IA1s, the

shortening of the N-terminal hydrophobic segment increased the mobile population of P-450IA1 from 27 ± 4% to 41 ± 4%, whereas ϕ was not significantly changed within the experimental error. The mobility of the shortened P-450IA1 is rather similar to that of the full-length P-450IA1 simultaneously expressed with NADPH-cytochrome P-450 reductase in which 43% of P-450IA1 was mobile with ϕ = 1276 μs (Iwase *et al.*, 1991). It should be noted that even a high salt treatment with 800 mM NaCl did not change slow rotation characteristics of the shortened P-450IA1 (ϕ = 830 μs and $r_3/r(0)$ = 0.44), although some mobilization was observed. In contrast, the water-soluble P-450cam showed the rapid isotropic rotation as judged from the $r(t)$ = 0.00 (ϕ < 1 μs) in the presence of microsomal membranes (Fig. 1).

The observed $r(t)$ decay curve of shortened P-450IA1 has common characteristics for P-450 immersed deeply in the membrane, such as full-length P-450IA1, P-450IA2, $P\text{-}450_{17\alpha,\text{lyase}}$, and $P\text{-}450_{C21}$, in microsomes or proteoliposomes. All these integral membrane proteins have both the rotating population with ϕ ranging from 100 to 1200 μs and the immobile population ($\phi \geq$ 20 ms) (Etter *et al.*, 1991; Iwase *et al.*, 1991; Kawato *et al.*, 1991; Ohta *et al.*, 1992). This implies that the P-450IA1 without the distinct N-terminal hydrophobic segment is likely to be incorporated into the yeast microsomal membrane, keeping its hydrophobic part embedded in the membrane. If shortened P-450IA1 binds simply to microsomes, the shortened P-450IA1 would move very rapidly on the membrane surface having the $r(t)$ curve with $\phi \leq$ 10 μs and $r_3/r(0)$ = 0.00, which is expected on an analogy of such an isotropic rapid movement of glutathione peroxidase on the surface of liposomes.[2] The immobile population for shortened and full-length P-450IA1 ($\phi \geq$ 20 ms) is present because of forming protein microassociations due to a high concentration of membrane proteins in yeast microsomes with the lipid to protein ratio of 0.4 in weight, as shown in various types of rat liver microsomes (Kawato *et al.*, 1982, 1991; Gut *et al.*, 1985).

Upon salt treatment of shortened P-450IA1 in microsomes by incubating with 800 mM NaCl followed by removal of the salt with ultracentrifugation, the mobile population increased from 41 to 58% with a slightly increased speed of ϕ = 830 ± 180 μs. It should be noted that, however, almost no release of shortened P-450IA1 was observed by this high salt treatment as judged by ultracentrifugation. This mobilization might be due to dissociation of nonspecific protein associations, including the cytochrome or oligomers of the cytochrome by weakening electrostatic interactions between proteins (Ohta *et al.*, 1991). If the shortened P-450IA1 is not hydrophobically incorporated into the lipid bilayer, just electrostatically binding to the membrane and moving on the lipid bilayer, by such a high salt treatment, this P-450IA1 should be removed from the membrane, resulting in a considerable decrease in the total absorption signal. However, this possibility was ruled out, because little decrease in the total absorption signal was observed by the NaCl treatment. It should be noted that the high salt treatment did not significantly change the CO recombination kinetics and P-450·CO difference spectra, excluding the possible denaturation of P-450 to alter $r(t)$ curves at high NaCl concentrations. The total absorption change $A(t)$ of shortened cytochrome P-450IA1·CO in microsomes showed a monoexponential decay with lifetime of τ = 3.3 ± 0.5 ms, which is almost the same as that of the full-length P-450IA1. No fluidization was observed in lipid phase by the high salt treatment, excluding the possibility that the NaCl treatment fluidized the lipid bilayer and thereby mobilized P-450 (Ohta *et al.*, 1991).

Possible Membrane Topology of Microsomal Cytochrome P-450—The present study demonstrates that cytochrome P-450IA1 is embedded in the membrane not only with the N-terminal segment but also with the other anchor peptide. We also showed that genetically expressed P-450 systems are useful to analyze membrane topology of a selected species of the cytochrome in biological membranes. The alcohol-inducible rat liver cytochrome P-450IIE1 lacking N-terminal segment was also expressed and efficiently incorporated in the membrane of *E. coli* (Larson *et al.*, 1991). This shortened P-450IIE1 showed almost the same drug oxidation activity as that of the full-length P-450IIE1 expressed in the *E. coli* membrane. These results are consistent to our present observation, implying that the membrane embedded segment other than the N-terminal hydrophobic anchor should be present for some of microsomal type of cytochrome P-450s (*e.g.* P-450IA1 and P-450IIE1). As judged from computer analyses of amino acid sequences (Nelson and Strobel, 1988; Gotoh and Fujii-Kuriyama, 1989), microsomal cytochrome P-450s could have only one membrane-spanning segment of the N-terminal very hydrophobic peptides. However, P-450s might have some membrane-embedded segments, although not membrane-spanning, keeping tight interactions with the membrane (Monier *et al.*, 1988). It should be noted that the N-terminal anchor model has been proposed and examined mainly for phenobarbital-inducible cytochrome P-450IIB1/IIB2 (Edwards *et al.*, 1991; Vergeres *et al.*, 1991) using peptide-specific IgG mapping, trypsinolysis, and computer calculations on the basis of the amino acid sequences. Until we draw a general conclusion for the membrane topology of microsomal cytochrome P-450, we need to obtain experimental topological information for, at least, several other types of P-450. We can add a related new demonstration that adrenocortical microsomal cytochromes $P\text{-}450_{C21}$ and $P\text{-}450_{17\alpha,\text{lyase}}$ are largely embedded in the membrane with not only N-terminal segment but also other segments in phospholipid vesicles (Ohta *et al.*, 1992). Exhaustive trypsinolysis of these cytochromes at 37 °C for 2 h did not remove any peptides of P-450 from the membrane. Rotational diffusion of these cytochromes showed a typical membrane-embedded type axial rotation about the axis perpendicular to the membrane plane.

To further elucidate the membrane topology of cytochrome P-450, rotational diffusion analysis for other genetically expressed monooxygenase systems in membranes such as P-450IIE1 without N-terminal polypeptide (Larson *et al.*, 1991), P-450IA1-reductase-fused enzyme (Murakami *et al.*, 1987; Yabusaki *et al.*, 1988), or site-directionally mutated P-450IA2 (Shimizu *et al.*, 1991) may provide us useful new information.

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[2] S. Kawato, unpublished results.

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