Linse, S., Brodin, P., Johansson, C., Thulin, E., Grundstrom, T., & Forsen, S. (1988) *Nature 335*, 651–652.
MacManus, J. P., Hutrik, C. M. L., Sykes, B. D., Szabo, A. G., Williams, T. C., & Banville, D. (1989) *J. Biol. Chem. 264*, 3470–3477.
Malik, N. A., Anantharamaiah, G. M., Gawish, A., & Cheung, H. C. (1987) *Biochim. Biophys. Acta 911*, 221–230.
Marchiori, F., Borin, G., Chessa, G., Cavaggion, G., Michelin, L., & Peggion, E. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1019–1028.
Marqusee, S., & Baldwin, R. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8898–8902.
Marsden, B. J., Hodges, R. S., & Sykes, B. D. (1988) *Biochemistry 27*, 4198–4206.
Marsden, B. J., Shaw, G. S., & Sykes, B. D. (1990) *Biochem. Cell Biol. 68*, 587–601.
Nagy, B., Potter, J. D., & Gergely, J. (1978) *J. Biol. Chem. 253*, 5971–5974.
Nicholson, H., Becktel, W. J., & Matthews, B. W. (1988) *Nature 336*, 651–656.
Palmisano, W. A., Trevino, C. L., & Henzl, M. T. (1990) *J. Biol. Chem. 265*, 14450–14456.
Pantoliano, M. W., Whitlow, M., Wood, J. F., Rollence, M. L., Finzel, B. C., Gillilard, G. L., Poulos, T. L., & Bryan, P. N. (1988) *Biochemistry 27*, 8311–8317.
Pantoliano, M. W., Whitlow, M., Wood, J. F., Dodd, S. W., Hardman, K. D., Rollence, M. L., & Bryan, P. N. (1989) *Biochemistry 28*, 7205–7213.
Potter, J. D., & Gergely, J. (1975) *J. Biol. Chem. 250*, 4628–4633.
Putkey, J. A., Sweeney, H. L., & Campbell, S. T. (1989) *J. Biol. Chem. 264*, 12370–12378.
Reid, R. E. (1987) *Int. J. Pept. Protein Res. 30*, 613–621.
Reid, R. E., & Hodges, R. S. (1980) *J. Theor. Biol. 84*, 401–444.
Reid, R. E., Gariépy, J., Saund, A. K., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2742–2751.
Reinach, F. C., & Karlsson, R. (1988) *J. Biol. Chem. 263*, 2371–2376.
Satyshur, K. A., Rao, S. T., Pyzalska, D., Drendel, W., Greaser, M., & Sundaralingam, M. (1988) *J. Biol. Chem. 263*, 1628–1647.
Serpersu, E. H., Shortle, D., & Mildvan, A. S. (1987) *Biochemistry 26*, 1289–1300.
Shaw, G. S., Hodges, R. S., & Sykes, B. D. (1990) *Science 249*, 280–283.
Shaw, G. S., Golden, L. F., Hodges, R. S., & Sykes, B. D. (1991) *J. Am. Chem. Soc.* (in press).
Shoemaker, K. R., Kim, P. S., York, E. J., Stewart, J. M., & Baldwin, R. L. (1987) *Nature 326*, 563–567.
Strynadka, N. C. J., & James, M. N. G. (1989) *Annu. Rev. Biochem. 58*, 951–998.
Tsuda, S., Hasegawa, Y., Yoshida, M., Yagi, K., & Hikichi, K. (1988) *Biochemistry 27*, 4120–4126.
Tsuji, T., & Kaiser, E. T. (1991) *Proteins 9*, 12–22.
Williams, T. C., Shelling, J. G., & Sykes, B. D. (1985) in *NMR in the Life Sciences* (Bradbury, E. M., & Nicolini, C., Eds.) pp 93–103, Plenum Press, New York.
Williams, T. C., Corson, D. C., Oikawa, K., McCubbin, W. D., Kay, C. M., & Sykes, B. D. (1986) *Biochemistry 25*, 1835–1846.

Rotation and Interactions of Genetically Expressed Cytochrome P-450IA1 and NADPH–Cytochrome P-450 Reductase in Yeast Microsomes†

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ABSTRACT: Rat liver cytochrome P-450IA1 and/or yeast NADPH–cytochrome P-450 reductase was expressed genetically in yeast microsomes. The ratio of P-450IA1 to the reductase was about 17:1 and 1:2 without and with coexpression of the reductase, respectively. Rotational diffusion of P-450IA1 was examined by observing the flash-induced absorption anisotropy, $r(t)$, of the heme·CO complex. In only P-450IA1-expressed microsomes, 28% of P-450IA1 was rotating with a rotational relaxation time (ϕ) of about 1200 μs. The mobile population was increased to 43% by the presence of the coexpressed reductase, while ϕ was not changed significantly. Increased concentration of KCl from 0 to 1000 mM caused considerable mobilization of P-450IA1. The results demonstrate a proper incorporation of P-450IA1 molecules into yeast microsomal membranes. The significant mobilization of P-450IA1 by the presence of reductase suggests a possible transient association of P-450IA1 with the reductase.

Cytochrome P-450 and NADPH–cytochrome P-450 reductase are key enzymes in the hepatic microsomal monooxygenase system, catalyzing the oxidative metabolism of various drugs and xenobiotics as well as endogenous substrates (Estabrook et al., 1979; White & Coon, 1980). Among numerous isozymes of P-450, extensive evidence exists that particularly the methylcholanthrene-induced cytochrome P-450IA1 converts polycyclic aromatic hydrocarbons to highly carcinogenic compounds (Harada & Omura, 1981; Guengerich, 1987). Electron-transfer mechanisms from the reductase to cytochrome P-450IA1[1] were not established so far. Es-

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sentially two mechanisms can be envisaged: one is electron transfer within a transient complex; the other is transfer by random collisions of rotationally and laterally diffusing proteins (Ingelman-Sundberg, 1986).

Protein–protein interactions of P-450 have been extensively examined in rat liver microsomes and in reconstituted proteoliposomes by observing rotational diffusion of P-450 (Kawato et al., 1982a). Cytochrome $P\text{-}450_{PB}$ has been observed to form a transient 1:1 association with the reductase in proteoliposomes (Gut et al., 1982, 1983). Drug induction with PB, MC, and PCB caused a significant decrease in the mobility of P-450 in rat liver microsomes (Kawato et al., 1991).

Many chemically different species of P-450s present in liver microsomes prevent selective analyses of one special species of P-450. Genetic expression of liver microsomal monooxygenase systems in yeast microsomes has been successfully achieved by Ohkawa and co-workers (Sakaki et al., 1985, 1987; Murakami et al., 1990). These genetically expressed P-450 systems have a great advantage for quantitative investigation of one selected species of P-450 in eukaryotic biological membranes. Here, using the genetically expressed P-450IA1 system, we first present dynamic properties of cytochrome P-450IA1 and a possible existence of transient association of P-450IA1 with NADPH–cytochrome P-450 reductase in yeast *Saccharomyces cerevisiae* microsomal membranes.

Experimental Procedures

Genetic Expression of P-450IA1 and Reductase in Yeast Microsomes. Rat liver cytochrome P-450IA1 was genetically expressed in microsomes of the yeast *Saccharomyces cerevisiae* AH22 cell as described by Sakaki et al. (1985). Yeast NADPH–cytochrome P-450 reductase was coexpressed with P-450IA1 in yeast microsomes as described by Murakami et al. (1990). Microsomes were prepared as described by Oeda et al. (1985). The concentration of expressed P-450IA1 was about 0.5 and 0.3 nmol/mg of protein in microsomes in the absence and presence of the reductase, respectively. The molar ratio of P-450IA1 to the reductase was around 17:1 and 1:2 in the absence and presence of the expressed reductase, respectively (Murakami et al., 1990).

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 30 s and then reduced with a few grains of dithionite. The principle of time-resolved flash photolysis depolarization measurements was 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 (Quanta-Ray, DCR-2), and absorbance changes were measured at 450 nm selected with a Jovin-Yvon H-20 monochromator. The signal at 450 nm was divided into vertically and horizontally polarized components with a beam-splitting polarizer, which were led to Hamamatsu R567 photomultiplier tubes. The signals were analyzed by calculating the absorption anisotropy, $r(t)$, and the total absorbance change 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, absorption changes for vertical and horizontal polarization at time t after the laser flash. A slight unbalance of two photomultipliers is corrected by 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 by 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 membrane normal (Kawato et al., 1981, 1982a). A theoretical treatment of this case (Kawato & Kinosita, 1981) shows that the expected form of $r(t)$ is given by

$$r(t) = r_1 \exp(-t/\phi) + r_2 \exp(-4t/\phi) + r_3 \quad (3)$$

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. The population of mobile P-450IA1, p_m, was calculated with eq 4 on the basis of the experimentally determined minimal an-

$$\% p_m = 100\{[1 - r_3/r(0)]/(1 - 0.03)\} \quad (4)$$

isotropy of $[r_3/r(0)]_{min} = 0.03$ when all P-450IA1 was rotating in proteoliposomes (Etter et al., 1991).

CO recombination kinetics were measured in 50 mM Hepes solution without 60% sucrose. For analysis, the lifetime, τ_i, of the photodissociated state of P-450 was calculated by assuming that the total absorption change, $A(t)$, was expressed as the sum of an exponential function:

$$A(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i) \quad (5)$$

Curve fitting of the data based on eq 3 and 5 was accomplished by a PDP-11/73 minicomputer.

Other Methods. Labeling of the microsomal lipid bilayer with DPH and the steady-state fluorescence anisotropy (r^s) measurements were performed as described elsewhere (Ohta et al., 1990; Kawato et al., 1977). Samples were excited at 360 nm, and fluorescence above 420 nm was measured. Cytochrome P-450 was measured spectrophotometrically according to Omura and Sato (1964). Protein was determined according to the method of Lowry et al. (1951). Lipid phosphorus was measured according to the method of Gerlach and Deuticke (1963).

Results and Discussion

Characterization of Yeast Microsomes. The distribution of P-450IA1, proteins, lipids, and NADPH–cytochrome P-450 reductase in yeast microsomes was examined by ultracentrifugation at 155000g for 19 h in a sucrose density gradient from 30 to 50% (w/w), followed by fractionation. NADPH–cytochrome P-450 reductase was measured with its NADPH–cytochrome c reduction activity, and P-450 was measured with the absorption at 417 nm. Each of three quantities (P-450IA1, lipid phosphorus, and protein) always showed a single band appeared in the region of 37–40% in sucrose density with a peak at about 38% of sucrose. A good coincidence for these distributions implies that genetically expressed P-450IA1 molecules were incorporated in the microsomal membrane. When NADPH–cytochrome P-450 reductase was coexpressed

[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; L/P, lipid-to-protein weight ratio; MC, 3-methylcholanthrene; PB, phenobarbital; PCB, polychlorinated biphenyl; cytochrome P-450IA1, major type of rat liver P-450 in 3-methylcholanthrene-induced microsomes; cytochromes $P\text{-}450_{PB}$ and $P\text{-}450_{MC}$, major types of rat liver P-450s in PB- and MC-induced microsomes, respectively ($P\text{-}450_{PB}$ is a mixture of P-450IIB1 and P-450IIB2; $P\text{-}450_{MC}$ is a mixture of P-450IA1 and P-450IA2); $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$, cytochrome P-450s in adrenal cortex, catalyzing cholesterol side-chain cleavage and steroid 11β-hydroxylation, respectively.

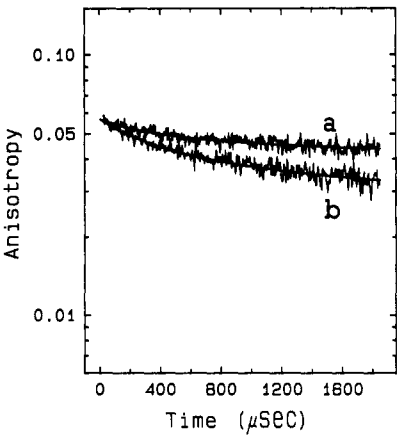

FIGURE 1: Time-dependent absorption anisotropy of cytochrome P-450IA1 in yeast microsomes with and without reductase. Samples (3–5 μM in heme) were photolyzed by a vertically polarized laser flash at 532 nm, and $r(t)$ was recorded at 450 nm as described under Experimental Procedures. Measurements were performed in 60% sucrose solution at 20 °C (~0.6 P). Curve a, microsomes with only P-450IA1 expressed; curve b, microsomes with coexpressed P-450IA1 and reductase. The zigzag lines are experimental data, and the solid curves were obtained by fitting the data to eq 3. 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 (Kawato & Kinosita, 1981).

with P-450IA1, coincorporation of these two proteins into the same microsomal membrane was also demonstrated by the coincident distribution of the reductase with P-450IA1 heme. For this coexpressed preparation, the drug oxidation activity was raised by 25-fold, implying that P-450IA1 is effectively interacting with the reductase (Murakami et al., 1990). The $L/P = 0.4$ was the same for both microsomes with and without genetically expressed reductase.

Rotation and Interactions of P-450IA1 with Reductase in Yeast Microsomes. Rotational diffusion of cytochrome P-450IA1 genetically expressed in yeast microsomes was measured at 20 °C. The $r(t)$ curves decayed within 2 ms to a time-independent value $r(\infty) = r_3$, implying the coexistence of rotating and immobile ($\phi \geq 20$ ms) populations of P-450IA1 (Figure 1). Data were analyzed according to eq 3 and 4. For only P-450IA1-expressed microsomes, the normalized time-independent anisotropy was $r_3/r(0) = 0.73 \pm 0.04$, and the rotational relaxation time was $\phi = 1180 \pm 230$ μs. Upon coexpression of the reductase, $r_3/r(0)$ decreased to 0.58 ± 0.04, while $\phi = 1276 \pm 261$ μs was not significantly different from microsomes without reductase within experimental error. The decrease in $r_3/r(0)$ indicates the mobilization of P-450IA1 from 28% to 43% for the mobile population by the presence of the reductase. No significant change in the P-450IA1 mobility was observed by the addition of substrates, such as benzo[*a*]pyrene, acetanilide, 7-ethoxycoumarin, and ethanol. A possible contribution of other P-450s originally present in yeast was negligible in the present experiments, since no significant laser flash induced absorbance change was observed for yeast microsomes where P-450IA1 was not genetically expressed.

The observed characteristics of $r(t)$ decay curves of P-450IA1 are common for proteins immersed deeply in the membrane such as $P\text{-}450_{PB}$ and $P\text{-}450_{MC}$, $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$, cytochrome oxidase, and ADP/ATP translocator in microsomes, mitochondria, or proteoliposomes. All these integral membrane proteins have both the rotating population with ϕ ranging from 100 to 1500 μs and the immobile population ($\phi \geq 20$ ms) (Greinert, 1979; Gut et al., 1982; Kawato et al., 1980, 1981, 1982a,b, 1988, 1991; Mueller et al., 1984; Ohta et al., 1990, 1991). This implies that genetically expressed P-450IA1 is likely to be properly incorporated into the yeast microsomal membrane, keeping its hydrophobic part deeply embedded in the membrane. If P-450IA1 binds simply to microsomes, P-450IA1 would move rapidly on the membrane surface, having the $r(t)$ curve with $\phi \approx 20$ μs and $r_3/r(0) = 0.00$, which is expected on analogy of such an isotropic rapid movement of glutathione peroxidase on the surface of liposomes (S. Kawato, unpublished results). The immobile population for P-450IA1 ($\phi \geq 20$ ms) is present because of forming protein microaggregates due to a high concentration of membrane proteins in yeast microsomes with $L/P = 0.4$, as shown in various types of rat liver microsomes (Kawato et al., 1982a, 1991; Gut et al., 1985).

The observed mobility of cytochrome P-450IA1 in yeast microsomes is significantly lower than the mobility of $P\text{-}450_{MC}$ in rat liver microsomes, where 48% of P-450 is rotating with $\phi = 600$ μs (Kawato et al., 1991). Since the concentration of membrane proteins ($L/P = 0.4$) is the same between these two microsomes, this mobility difference is possibly in part due to the high rigidity ($r^s = 0.195$) of the yeast microsomal lipid bilayer when compared with rat liver microsomal membranes with much lower rigidity of $r^s = 0.139$. It should be noted that the fluorescence anisotropy r^s is inversely proportional to the membrane fluidity and that the theoretically maximal value is $r^s = 0.400$. Another possible explanation for this difference is that lower mobility of the present P-450IA1 is due to artificial expression resulting in oligomeric forms in yeast microsomes, whereas the higher mobility of P-450 in rat liver microsomes is due to separate distribution of cytochromes because of in situ expression.

The observed mobilization of P-450IA1 by the presence of NADPH–cytochrome P-450 reductase could be explained by formation of a transient association of these two proteins, which is an analogy of the $P\text{-}450_{PB}$·reductase association in phospholipid vesicles (Gut et al., 1982, 1983). This association has a transient character, and the lifetime of the association is not necessarily very long (for example, not longer than 1 s), but it should be longer than 20 ms in order to be realized as immobile in the present time range of 2 ms. Cytochrome $P\text{-}450_{PB}$ has been demonstrated to form a 1:1 association with the reductase in liposomes, as judged from the mobilization of $P\text{-}450_{PB}$ by the presence of an increased amount of reductase, and the immobilization of $P\text{-}450_{PB}$ by cross-linking of reductase with anti-reductase antibody (Gut et al., 1982, 1983). Association of P-450IA1 with reductase may be favored more than formation of microaggregates with other proteins or with other P-450s, leading to dissociation of P-450IA1-containing aggregates with the following scheme. The reductase has a bulky hydrophilic head and anchors to the membrane with a thin hydrophobic polypeptide (Gum & Strobel, 1979). In a hypothetical P-450IA1·reductase association, the bulky head of reductase could prevent further hydrophobic/electrostatic association between these complexes or with other proteins, supporting the observed decreased affinity of P-450IA1 with other proteins in the presence of reductase.

Effect of KCl on the Rotational Mobility of Cytochrome P-450IA1. After 30-min incubation with KCl at room temperature, the sample was transferred to a 60% sucrose solution for rotational diffusion measurements. A considerable decrease in $r_3/r(0)$ was observed by the addition of increasing amounts of KCl from 0 to 1000 mM through 100, 300, and 500 mM (final concentration), implying the progressive mobilization

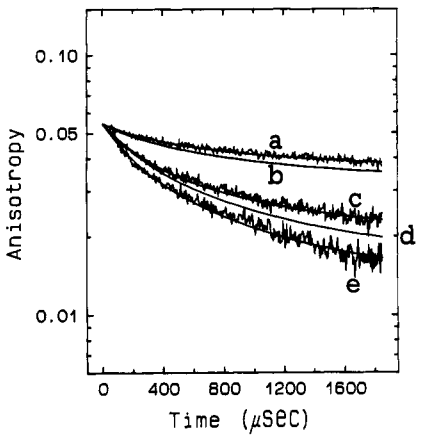

FIGURE 2: Effect of KCl on the absorption anisotropy of P-450IA1 in yeast microsomes. The final concentration of KCl is 0 (curve a), 100 (curve b), 300 (curve c), 500 (curve d), and 1000 mM (curve e). The other conditions are the same as described in Figure 1. The zigzag lines are experimental data, and the solid curves were obtained by fitting the data to eq 3. The initial anisotropies of curves a, c, d, and e are slightly normalized to the same $r(0)$ of curve b to facilitate comparison. Experimental data of curves b and d are omitted for clarity.

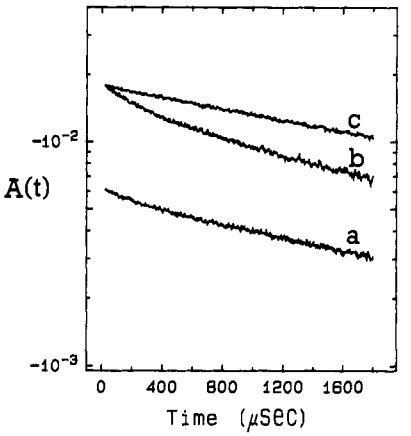

FIGURE 3: Total absorption decay of cytochrome P-450·CO in yeast microsomes. Measurements were performed in 50 mM Hepes buffer solution at 20 °C. Curve a, P-450IA1 without substrate; curve b, P-450IA1 with 1% (v/v) ethanol; curve c, P-450IA1 with 20 μM 7-ethoxycoumarin. Theoretical curves, obtained by fitting the data to eq 5, were omitted for clarity. The initial absorption $A(0)$ of curve b was normalized to that of curve c, and $A(0)$ of curve a was vertically displaced to facilitate comparison.

of P-450IA1 by KCl (Figure 2). By going from 0 to 1000 mM, $r_3/r(0)$ was decreased from 0.73 to 0.25, corresponding to an increase in the mobile population of P-450IA1 from 28% to 77%. This might be due to dissociation of nonspecific protein aggregates including P-450IA1 or oligomers of P-450IA1 by weakening electrostatic interactions between proteins (Ohta et al., 1991). Since complete recovery of $r(t)$ was observed after removal of KCl, the present mobilization of P-450 was not caused by a release of peripheral proteins from microsomes. If genetically expressed P-450IA1 is not deeply incorporated into the lipid bilayer, just electrostatically binding to the membrane and moving on the lipid bilayer, by the presence of such a high concentration of 1 M KCl, P-450IA1 should be removed from the membrane, resulting in very rapid rotation with ϕ smaller than 10 μs observed when the membrane was completely solubilized by cholate (Kawato, unpublished results). However, this possibility can be ruled out, because KCl did not greatly speed up the rotation (i.e., not largely decreased ϕ) of the mobile P-450. The observed slow rotation with ϕ = 800 μs at 1000 mM KCl is too slow to be caused by solubilization of simply bound P-450IA1 from the microsomal membrane.

When the KCl concentration was increased from 0 to 1000 mM, no significant change was observed in both CO recombination kinetics and P-450·CO difference spectra, excluding the possible denaturation of P-450 to alter $r(t)$ curves at high KCl concentrations. No fluidization was observed in the lipid phase by the presence of KCl, excluding the possibility that the KCl fluidized the lipid bilayer and thereby mobilized P-450.

Recombination Kinetics of CO to Reduced Cytochrome P-450IA1. The total absorption change $A(t)$ of cytochrome P-450IA1·CO in microsomes showed a curve close to the monoexponential decay with a lifetime of τ = 2714 μs, both in the presence and in the absence of genetically expressed NADPH–cytochrome P-450 reductase (Figure 3). Addition of ethanol showed clear double-exponential recombination of CO with τ_1 = 280 μs (24%) and τ_2 = 2514 μs (76%). A complete monoexponential recombination of CO with τ = 3322 μs was observed upon binding of 7-ethoxycoumarin to P-450IA1. The slight deviation of $A(t)$ from a complete monoexponential decay for P-450IA1 without exogenous substrates might be due to the presence of a little amount of P-450IA1 bearing endogenous substrates besides substrate-free P-450IA1.

The present study demonstrates that genetically expressed P-450 systems can be very useful to analyze protein–protein interactions of selected species of the cytochrome in biological membranes. Although P-450 proteoliposomes are also useful for this purpose, they have a disadvantage of the absence of many membrane proteins other than monooxygenases. Rotational diffusion analysis for other genetically expressed monooxygenase systems in membranes such as P-450IA1 without N-terminal polypeptide (Yabusaki et al., 1988), P-450IIE1 without N-terminal polypeptide (Coon, 1990), and P-450IA1–reductase fused enzyme (Sakaki et al., 1985, 1987; Murakami et al., 1987, 1990) may provide us useful new information.

Registry No. Cytochrome P450, 9035-51-2; NADPH–cytochrome P450 reductase, 9039-06-9.

REFERENCES

Cherry, R. J. (1978) *Methods Enzymol. 54*, 47–61.
Coon, M. J. (1990) in *Symposium on Molecular Biology of Cytochrome P-450* (Omura, T., Ed.) pp 7–9, Tokyo.
Estabrook, R. W., Werringloer, J., & Peterson, J. A. (1979) *ACS Symp. Ser. 97*, 149–179.
Etter, H. U., Richter, C., Ohta, Y., Winterhalter, K. H., Sasabe, H., & Kawato, S. (1991) *J. Biol. Chem.* (in press).
Gerlach, E., & Deuticke, B. (1963) *Biochem. Z. 337*, 477–479.
Greinert, R., Staerk, H., Stier, A., & Weller, A. (1979) *J. Biochem. Biophys. Methods 1*, 77–83.
Guengerich, F. P., Ed. (1987) in *Mammalian Cytochromes P-450*, Vol. 2, CRC Press, Boca Raton, FL.
Gum, J. R., & Strobel, H. W. (1979) *J. Biol. Chem. 254*, 4177–4185.
Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., & Kawato, S. (1982) *J. Biol. Chem. 257*, 7030–7036.
Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., & Kawato, S. (1983) *J. Biol. Chem. 258*, 8588–8594.
Gut, J., Kawato, S., Cherry, R. J., Winterhalter, K. H., & Richter, C. (1985) *Biochim. Biophys. Acta 817*, 217–228.
Harada, N., & Omura, T. (1981) *J. Biochem. 89*, 237–248.
Ingelman-Sundberg, M. (1986) in *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Ortiz de Mon-

tellano, P. R., Ed.) pp 119–160, Plenum Press, New York.
Kawato, S., & Kinosita, K., Jr. (1981) *Biophys. J. 36*, 277–296.
Kawato, S., Kinosita, K., Jr., & Ikegami, A. (1977) *Biochemistry 16*, 2319–2324.
Kawato, S., Sigel, E., Carafoli, E., & Cherry, R. J. (1980) *J. Biol. Chem. 255*, 5508–5510.
Kawato, S., Sigel, E., Carafoli, E., & Cherry, R. J. (1981) *J. Biol. Chem. 256*, 7518–7527.
Kawato, S., Gut, J., Cherry, R. J., Winterhalter, K. H., & Richter, C. (1982a) *J. Biol. Chem. 257*, 7023–7029.
Kawato, S., Lehner, C., Mueller, M., & Cherry, R. J. (1982b) *J. Biol. Chem. 257*, 6470–6476.
Kawato, S., Mitani, F., Iizuka, T., & Ishimura, Y. (1988) *J. Biochem. 104*, 188–191.
Kawato, S., Ashikawa, I., Iwase, T., & Hara, E. (1991) *J. Biochem. 109*, 587–593.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951), *J. Biol. Chem. 193*, 265–275.
Mueller, M., Krebs, J. J. R., Cherry, R. J., & Kawato, S. (1984) *J. Biol. Chem. 259*, 3037–3043.
Murakami, H., Yabusaki, Y., Sakaki, T., Shibata, M., & Ohkawa, H. (1987) *DNA 6*, 189–197.
Murakami, H., Yabusaki, Y., Sakaki, T., Shibata, M., & Ohkawa, H. (1990) *J. Biochem. 108*, 859–865.
Oeda, K., Sakaki, T., & Ohkawa, H. (1985) *DNA 4*, 204–210.
Ohta, Y., Mitani, F., Ishimura, Y., Yanagibashi, K., Kawamura, M., & Kawato, S. (1990) *J. Biochem. 107*, 97–104.
Ohta, Y., Yanagibashi, K., Hara, T., Kawamura, M., & Kawato, S. (1991) *J. Biochem. 109*, 594–599.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.
Sakaki, T., Oeda, K., Miyoshi, M., & Ohkawa, H. (1985) *J. Biochem. 98*, 167–175.
Sakaki, T., Shibata, M., Yabusaki, Y., & Ohkawa, H. (1987) *DNA 6*, 31–39.
White, R. J., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–356.
Yabusaki, Y., Murakami, H., Sakaki, T., Shibata, M., & Ohkawa, H. (1988) *DNA 7*, 701–711.

^{31}P NMR Saturation-Transfer Study of the in Situ Kinetics of the Mitochondrial Adenine Nucleotide Translocase†

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ABSTRACT: The exchange of intramitochondrial ATP (ATP_{in}) for extramitochondrial ATP (ATP_{out}) was measured by using ^{31}P NMR spectroscopy over a range of temperatures in isolated rat liver mitochondria oxidizing glutamate and succinate in the presence of external ATP but no added ADP (state 4). The rate of this exchange is more than an order of magnitude faster than rates reported previously that were determined by using isotopic techniques in the presence of oligomycin, the potent ATPase inhibitor. Differences are ascribed in part to the low levels of matrix ATP present in oligomycin-treated mitochondria. The addition of oligomycin to mitochondrial suspensions decreases intramitochondrial ATP levels from 17 ± 3 (SEM) nmol/mg of protein in state 4 to 1.51 ± 0.1 nmol/mg of protein in the presence of inhibitor at 8 °C. Simultaneously, transporter flux falls from 960 ± 55 nmol/min·mg to undetectable levels (less than 300 nmol/min·mg). Although transport rates are much faster when measured by saturation-transfer than by conventional isotopic methods, the enthalpy values obtained by determining the effect of temperature on flux are very similar to those reported in the past that were determined by using isotopic techniques. Intramitochondrial ATP content regulates the rate of the ATP_{in}/ATP_{out} exchange. At 18 °C, the concentration of internal ATP that produces half-maximal transport rate is 6.6 ± 0.12 nmol/mg of mitochondrial protein. The relationship between substrate concentration and flux is sigmoidal and is 90% saturated at 11.3 ± 0.18 nmol/mg of mitochondrial protein. Since the measured rates of exchange of ATP_{in} for ATP_{out} are almost 10 times faster than the ATP synthase (ATP/P_i) exchange rates, the translocase cannot limit net ATP/P_i exchange in state 4. It may, nonetheless, limit net synthesis of ATP under other conditions when matrix ATP concentration is lower than in state 4 and when external ADP is present at higher concentrations than in these experiments.

Chance and Williams (1956) proposed that mitochondrial respiration is tightly controlled by ADP availability. The site of control by ADP remains uncertain. The adenine nucleotide translocase, the electrogenic mitochondrial membrane transporter that catalyzes the exchange of nucleotides, has been suggested as a rate-controlling step for net ATP synthesis (Heldt, 1966, 1967; Heldt & Klingenberg, 1968; Kemp et al., 1969). Previous work aimed at identifying the important determinants of mitochondrial respiration has generated two main hypotheses. The first is the "near-equilibrium hypothesis" of Ericinska and Wilson (1982), which suggests that the electron transport chain and the cytosolic phosphorylation

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