Location of Functional Centers in the Microsomal Cytochrome P450 System†

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ABSTRACT: Fluorescence quenching and energy-transfer studies have been carried out to determine the position of FAD and FMN groups of NADPH–cytochrome P450 reductase and of the heme and substrate groups of cytochrome P450 with respect to the lipid/water interphase. Quenching by iodine of the fluorescence of the flavins of the reductase shows a biphasic pattern, due to the different accessibility of FAD and FMN to the solvent with Stern–Volmer constants of 7.9×10^{-4} and 2.7×10^{-3} mM^{-1}, respectively. Both prosthetic groups appear to be buried within the three-dimensional structure of the native reductase, FAD more deeply embedded than FMN and with a relative contribution to the total fluorescence of flavins of 84% (FAD) and 16% (FMN). The lack of significant energy transfer (<5%) from FAD + FMN to the rhodamine group of the N-labeled phosphatidylethanolamine incorporated in membranes reconstituted with NADPH–cytochrome P450 reductase and phosphatidylcholine points out that both groups are located at a distance greater than 5 nm from the lipid/water interphase. Steady-state fluorescence intensity and anisotropy data obtained with native and FMN-depleted NADPH–cytochrome P450 reductase show that energy transfer between both prosthetic groups occurs in the native reductase with an efficiency of ca. 31%, consistent with a separation between these groups of 2 nm as suggested earlier by Bastiaens, P. I. H., Bonants, P. J. M., Müller, F., & Visser, A. J. W. G. [(1989) *Biochemistry 28*, 8416–8425] from time-resolved fluorescence anisotropy measurements. The efficiency of energy transfer between the donor/acceptor pairs DPH/heme or 4-heptadecyl-7-hydroxycoumarin/heme allows us to estimate that the heme group is approximately 5 nm from the lipid/water interphase. The binding site of the substrate 7-ethoxycoumarin in cytochrome P450 is located at less than 5 nm from the heme group, and not at the lipid surface.

Energy transduction plays a key role in cellular metabolism and requires a high level of structural organization of the biological systems responsible for this task. An example of such energy-transducing systems is the microsomal cytochrome P450 electron-transport system [see White and Coon (1980), Coon et al. (1981), and Pompon (1987)]. The information obtained from structural and kinetic studies needs to be combined to gain a deeper understanding of these processes. Fluorescence techniques have proven to be very useful to unravel structural features of proteins [see, for example, Lakowicz (1983)], and in particular fluorescence energy transfer is a potent tool to obtain structural details in the 1–10-nm size scale (Stryer, 1978; Lakowicz, 1983; Gutiérrez-Merino et al., 1987; Gryczynski et al., 1988; Lakowicz et al., 1988). Fluorescence energy transfer can be used to precisely localize relevant functional *loci* in membrane proteins, both with respect to each other and with respect to the lipid/water interphase (Fleming et al., 1979; Gutiérrez-Merino et al., 1987; Squier et al., 1987; Jona et al., 1990). This latter point is of particular interest, for the regulatory influence of the membrane potential on the functions carried out by membrane proteins is largely dependent upon the distance of their functional centers to the membrane surface, as the electrical perturbation rapidly vanishes when separating several nanometers from it (Lee, 1977; McLaughlin, 1989; Cevc, 1990).

The two major components of the cytochrome P450 microsomal system are NADPH–cytochrome P450 reductase and cytochrome P450.

NADPH–cytochrome P450 reductase has been shown to be organized into two major structural domains: a transmembrane domain and a cytosolic domain. The transmembrane domain is composed of, at most, 45–50 amino acids of the amino-terminal region (Black & Coon, 1982), whose principal characteristics are its high hydrophobicity and the acetylation of the amino-terminal group. It is to be noted that these are properties shared with the transmembrane domain of cytochrome b_5 and of NADH–cytochrome b_5 reductase (Ozols, 1974; Mihara et al., 1978).

The cytosolic domain contains the binding sites of FAD, FMN, and NADPH, and its structural organization is only poorly resolved. On the basis of a comparative analysis of the amino acid sequences of NADPH–cytochrome P450 reductase and other FAD-dependent flavoproteins, it has been suggested that the FAD binding *loci* of NADPH–cytochrome P450 reductase are similar to those of the glutathione reductase and that the FMN binding site is similar to that of flavodoxin (Porter & Kasper, 1986; Porter et al., 1990). Bastiaens et al. (1989), using time-resolved fluorescence anisotropy measurements of native reductase and of FMN-depleted reductase, have presented experimental data supporting the existence of fluorescence energy transfer between FAD and FMN, and have estimated a separation of 2 nm between these two groups in the three-dimensional (3D)[1] structure of native reductase.

Eucaryotic cytochromes P450 are organized into 2 major structural domains: the transmembrane domain, formed at most by the 45–50 amino acids of the amino-terminal region, and the cytosolic domain, where substrate and heme bind (Brown & Black, 1989). Regarding this latter domain, the

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[1] Abbreviations: cmc, critical micelle concentration; DPH, 1,6-diphenyl-1,3,5-hexatriene; 4H7HC, 4-heptadecyl-7-hydroxycoumarin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RPE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; 3D, three dimensional.

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particular orientation of the heme group with respect to the lipid/water interphase is a matter of debate (Gut et al., 1982; Kawato et al., 1982; Nelson & Strobel, 1988; Edwards et al., 1991). Considering the overall dimensions of the cytochrome P450, an orientation of the heme group parallel to the membrane surface necessarily implies that the heme group is relatively close to the lipid/water interphase. This would favor the hypothesis that the hydrophobic substrates will have access to the catalytic site via diffusion through the lipid bilayer (Parry et al., 1976; Ebel et al., 1978; Kühn-Velten et al., 1989). However, several groups (Kawato et al., 1982; Gut et al., 1982; Edwards et al., 1991) have independently reported experimental evidence favoring the hypothesis of a heme group located almost perpendicular to the membrane surface, and far from the lipid/water interface. Kunz et al. (1985) have provided experimental evidence suggesting that the heme group is located about 6 nm away from the membrane surface. This latter conclusion has been achieved indirectly, and therefore the location of the heme group with respect to the lipid/water interface deserves to be unambiguously established, for the putative modulation of the cytochrome P450 by the surface density of charge of the microsomal membrane will be largely dependent upon this parameter.

Regarding the position of the binding site(s) of substrates with respect to the heme group, there are again somewhat contradictory reports (Kunze et al., 1983; Ortiz de Montellano et al., 1983; Ortiz de Montellano & Komives, 1985). On the one hand, using alkene and alkyne substrate able to produce alkyl derivatives of the heme group, it was concluded that heme and substrate *loci* must be quite close each other (Kunze et al., 1983; Ortiz de Montellano et al., 1983; Ortiz de Montellano & Komives, 1985). On the other hand, Omata and Ueno (1985) and Omata et al. (1986), using fluorescent substrates, like aflatoxin B_1, 7-ethoxycoumarin, and benzo[*a*]pyrene, from energy-transfer experiments have estimated that their binding sites are about 4–6 nm separated from that of the heme group.

In this paper, we report fluorescence quenching and energy-transfer studies directed to determine the position of FAD, FMN, and heme groups with respect to the lipid/water interphase, and the separation between the binding sites of the pairs FAD/FMN and heme/substrate in the 3D structure of cytochrome P450 reductase and in cytochrome P450, respectively.

MATERIALS AND METHODS

Rat hepatic microsomes were prepared according to Lu and Levin (1972), except that 1 mM β-mercaptoethanol and 1 mM penylmethanesulfonyl fluoride were added to all the preparation buffers, to prevent against oxidation and proteolysis, respectively. To enhance the content of cytochrome P450, the rats were treated with phenobarbital by intraperitoneal injection of 60 mg/kg body weight per day during 4 days, and 24 h after the last dose, the rats were sacrified by cervical dislocation. Microsomal preparations were characterized as indicated by Lake (1987). Briefly, protein concentration was estimated using the method of Lowry (Lowry et al., 1951), cytochrome P450 content was measured from the difference of absorbance at 450 nm between the cytochrome P450 reduced and saturated with CO and the reduced form of cytochrome P450 (Omura & Sato, 1964a,b), the content of cytochrome P420 was evaluated as indicated by Estabrook et al. (1972) and only preparations of microsomes having less than 5% of total cytochrome in the 420 form have been used in this study, and the content of cytochrome b_5 was measured as in Tredger et al. (1984).

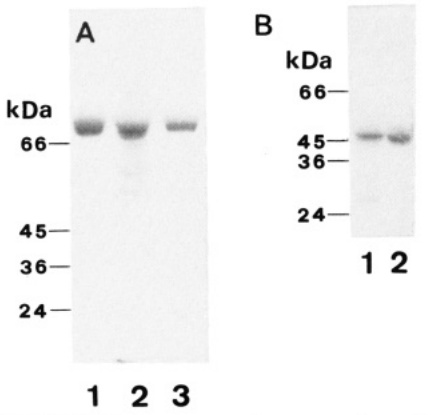

FIGURE 1: SDS–PAGE of representative preparations of the proteins used in this study. The standard molecular mass markers used were bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), and trypsinogen (24 kDa). (A) Electrophoresis in 7.5% (w/v) polyacrylamide of purified NADPH–cytochrome P450 reductase. The different lanes correspond to (1) 21 μg of reductase, (2) fraction eluted from the 2′,5′-ADP–Sepharose column, and (3) 10 μg of reductase. (B) Electrophoresis in 10% (w/v) polyacrylamide of purified cytochrome P450. Lanes 1 and 2 correspond to 5 and 15 μg of purified cytochrome P450, respectively.

Purification of NADPH–Cytochrome P450 Reductase. NADPH–cytochrome P450 reductase has been purified from hepatic microsomes pepared from phenobarbital-treated rats following Yasukochi and Masters (1976), with modifications of the detergents used in different steps of the purification, to avoid problems in the fluorescence measurements of FAD and FMN produced by some nonionic detergents (e.g., commercial Triton X-100 shows fluorescence emission at 600 nm when exciting at 450 nm, and this masks the signal of FAD and of FMN). Cholate and Tween 20 at concentrations of 0.625 and 1.25 mg of detergent/mg of protein, respectively, were used to solubilize the microsomes. In the buffer used to elute the ion-exchange DEAE-Sepharose CL6B column (first step), we have used 0.8% v/v Tween 20 and 0.1% v/v cholate, and from then on 0.1% v/v Tween 20 in all the subsequent steps (affinity column of 2′,5′-ADP–Sepharose 4B, dialysis, and Sephadex G50 column). Purified reductase migrated as a single band (>95%) in SDS–PAGE (see Figure 1). The activity of NADPH–cytochrome P450 reductase has been measured using cytochrome *c* and potassium ferricyanide as exogenous electron acceptors, as in Masters et al. (1967), Vermilion and Coon (1979), and Lake (1987). Currently, we have obtained activity values of the purified reductase of 27–35 μmol of substrate oxidized min^{-1} (mg of protein)$^{-1}$ under optimal conditions (pH 7.7, 37 °C, in 0.3 M potassium phosphate), quite similar to the activity reported in Lindstrom and Aust (1984), Ryan et al. (1985), and Taniguchi et al. (1987), and the content of flavins averaged 1.8–1.9 mol of flavins/mol of reductase (averaged from six different preparations).

The preparation of FMN-depleted NADPH–cytochrome P450 reductase was carried out as in Calhoun et al. (1987). The FMN-depleted enzyme was extensively dialyzed against KBr-free buffer to ensure a good depletion of Br^- from the solution, for this anion will interfere in fluorescence quenching measurements. Routinely we have obtained about 8–10% reductase activity remaining after this treatment, in agreement with earlier findings (Calhoun et al., 1987).

Purification of Cytochrome P450. Cytochrome P450 has been purified as in Kuwahara et al. (1984) and Ryan et al. (1985), from hepatic microsomes of phenobarbital-treated

rats (see above), except that the detergents have been changed to optimize the functionality of cytochrome P450 under conditions allowing for measurements of the fluorescence of flavins in reconstituted systems of the reductase and of cytochrome P450. Briefly, sodium cholate (1.5 mg of detergent/mg of protein) was used to solubilize hepatic microsomes, and in all subsequent steps, Tween 20 was used as the detergent. Tween 20 (0.1% v/v) was used to equilibrate the phenyl-Sepharose CL4B column (first step) and 0.5% v/v Tween 20 to elute cytochrome P450 from this column; 0.2% v/v Tween 20 was present in the buffers used to run the hydroxyapatite column; 0.6% v/v Tween 20 was used in the DEAE-Sepharose CL-6B step and 0.2% v/v Tween 20 in the CM-Sepharose CL-6B step. The purified cytochrome P450 was stored at −70 °C until use in 50 mM potassium phosphate (pH 7.2)/20% glycerol/1 mM EDTA/1 mM β-mercaptoethanol and 0.1% v/v Tween 20.

Purified cytochrome P450 ran basically as a single electrophoretic band (>95%) in SDS–PAGE (see Figure 1), and was found to contain 11–12 nmol of cytochrome P450/mg of protein, averaged from six different preparations. On the other hand, the absorption spectrum of the oxidized form of cytochrome P450 is identical to that reported for isoenzyme *a* (Kuwahara et al., 1984), showing an absorption maximum centered at 416 nm indicative of an iron atom in the low-spin form. The activity of the purified cytochrome P450 was assayed with 7-ethoxycoumarin as the substrate in reconstituted systems containing cytochrome P450 and the reductase, and following the conversion of 7-ethoxycoumarin to 7-hydroxycoumarin by fluorescence, for 7-hydroxycoumarin is a highly fluorescent product at 460 nm (Ullrich & Weber, 1972; Prough et al., 1978).

Reconstitution. Cytochrome P450 and NADPH–cytochrome P450 reductase have been reconstituted in lipid bilayers of defined chemical composition as in East and Lee (1982) and Gutiérrez-Merino et al. (1987). Briefly, to egg lecithin solubilized with sodium cholate (0.5 mg/mg of lipid) was added the purified protein, and the concentration of the detergent was kept above its cmc, routinely at about 1.2-fold the cmc. After 30-min incubation at room temperature under mild stirring, the solution is largely diluted with 50 mM potassium phosphate (pH 7.2) and 20% v/v glycerol, to achieve a detergent concentration well below its cmc, currently 10–20-fold lower.

RPE was prepared as indicated in Gutiérrez-Merino et al. (1987).

Fluorescence. Fluorescence measurements have been carried out in a Hitachi–Perkin Elmer Model 650-40 spectrofluorometer operated in the ratio mode and at 25 °C in 50 mM potassium phosphate (pH 7.2) buffer plus 20% v/v glycerol, unless otherwise stated. In addition, when dealing with the emission spectra of flavins of diluted reductase solutions, the Raman signal of the buffer has been stored in the microcomputer and then subtracted from the emission spectra of the samples.

Fluorescence energy transfer has been analyzed following the theoretical approach outlined in Gutiérrez-Merino et al. (1987). Briefly, in the presence of acceptors, the fluorescence of the donor can be expressed as

$$(F_0 - F)/F_0 = \langle E \rangle = (\langle k \rangle / k_0)/(1 + \langle k \rangle / k_0) \quad (1)$$

where F_0 and F are fluorescence intensities for the donor in the absence and presence of the acceptor, respectively, and k_0 is the average rate of Forster energy transfer for a donor–acceptor pair separated by the distance R_0 at which the efficiency of energy transfer, $\langle E \rangle$, is 50%.

The average rate of energy transfer, $\langle k \rangle$, in a two-dimensional membrane system can be written as

$$\langle k \rangle = \sum_{i=1}^{n} k_i \quad (2)$$

where the summation extends over all donor–acceptor pairs of the ensemble and where the rate of transfer for pair i separated by a distance r_i is k_i provided that the diffusion rate of the donor and acceptor is much slower than the lifetime of the excited state of the donor. Assuming a random distribution of acceptors in the membrane, with the geometrical constraints imposed by molecular sizes and shapes, the results have been analyzed using the algorithms developed in Gutiérrez-Merino (1981a,b) and Gutiérrez-Merino et al. (1987).

The distance R_0 has been calculated as in Gutiérrez-Merino et al. (1987), using a value for the orientation factor of $K^2 = {}^2/_3$, and with a refractive index, n, set equal to that of a dilute aqueous solution (1.33). When needed, the quantum yield of a given fluorophore not previously reported in the experimental conditions of this study has been calculated by reference to a fluorophore of known quantum yield, e.g., 1 mM quinine sulfate in 1 N sulfuric acid at 25 °C, for which a value of the quantum yield of 0.51 was taken (Lee, 1982).

The more drastic assumption taken in distance estimations using fluorescence energy transfer is the random orientation between donor and acceptor groups, e.g., $K^2 = {}^2/_3$. An estimate of the deviation of K^2 from ${}^2/_3$ can be made from anisotropy measurements, as indicated in Stryer (1978) and Gutiérrez-Merino et al. (1987). Polarization of fluorescence was calculated by using the expression (Weber, 1966):

$$P(\lambda) = \frac{I(\|) - G(\lambda)I(\perp)}{I(\|) + G(\lambda)I(\perp)} \quad (3)$$

where $I(\|)$ and $I(\perp)$ are the fluorescence intensities measured with parallel and perpendicularly oriented polarizers, respectively, and $G(\lambda)$ is the correction factor for polarization characteristics of the emission monochromator. A value of 1.04 ± 0.02 has been obtained for the wavelength pairs 360/430 and 450/525 ($\lambda_{exc}/\lambda_{em}$, in nanometers), averaged from more than 30 measurements, and has been used in our calculations. The steady-state anisotropy of fluorescence, r_s, has been calculated from polarization (P) by using the equation (Weber, 1966):

$$r_s = 2P/(3 - P) \quad (4)$$

The correction of fluorescence quenching in samples of absorbance higher than 0.1 optical density (or in turbid samples) has been carried out as indicated by Birdsall et al. (1983). Briefly

$$F_{exp} = F_{cor}C \quad (5)$$

where F_{exp} and F_{cor} are the intensities of the fluorescence experimentally measured and the corrected value, respectively, and C is the correction factor, defined by the equation:

$$C = \frac{e^{-aL_t d} - e^{-aL_t}}{aL_t(1 - d)} \quad (6)$$

where L_t is the concentration of fluorescent molecules and a and d are parameters related to the molar extinction coefficient and the geometric characteristics of the fluorometer, respectively. Both parameters a and d have to be empirically determined for each fluorophore and fluorometer (Birdsall et al., 1983). In this study, we have applied this approach to

Table I: Stern–Volmer Constants of the Quenching by KI of the Fluorescence (λ_{exc} = 450 nm, λ_{em} = 525 nm) of Native and FMN-Depleted NADPH–Cytochrome P450 Reductase (Temperature 25 °C)

sample	K_{sv}(A) × 10^3 (mM^{-1})	K_{sv}(B) × 10^4 (mM^{-1})	component A (%)[a]	component B (%)[b]
riboflavin	89 ± 1		100	
native reductase in soln	3.7 ± 0.1	7.6 ± 0.1	18.5	81.5
reconstituted[b] at lipid:protein ratio (mol/mol) of				
50:1	2.2 ± 0.1	7.5 ± 0.1	12.8	87.2
200:1	2.7 ± 0.1	8.6 ± 0.1	13.8	86.2
500:1	3.1 ± 0.1	8.2 ± 0.1	19.7	80.3
1000:1	3.0 ± 0.1	7.5 ± 0.1	17.6	82.4
av	2.7 ± 0.4	7.9 ± 0.6	16 ± 3	84 ± 3
aporeductase[c]		7.4		94 ± 2

[a] Expressed as percent of total fluorescence. [b] As indicated under Materials and Methods. [c] FMN-depleted reductase; see the text for details.

fluorescence titrations with relatively high 7-ethoxycoumarin concentrations (up to 0.16 mM), and by numeric simulations, we have found the following values of the parameters: a = 10710 and d = 0.625. Using this approach, we do not obtained a good fit of the data for solutions having an absorbance higher than 1.75 at 330 nm, e.g., for 7-ethoxycoumarin concentrations higher than 0.16 mM, or the turbidity produced by PC liposomes at concentrations higher than 800 μM.

RESULTS

Quenching by Iodine of the Fluorescence of FAD and FMN of NADPH–Cytochrome P450 Reductase. The basic fluorescence properties of NADPH–cytochrome P450 reductase show that in the native form of the enzyme the fluorescence of the flavin coenzymes (FMN and FAD) is largely quenched (Siegel, 1978; results not shown). Most of this quenching is due to interactions with specific amino acids within the protein structure, as revealed by the large increase (more than 20-fold) of the fluorescence centered at 520 pm upon thermal denaturation of the reductase (not shown). Moreover, the excitation and emission spectra of the reductase denatured by heating during 3 min at 100 °C are identical to those of riboflavin in solution. This fact suggests the possibility that FMN, FAD, or both prosthetic groups are deeply located within the 3D structure of the reductase. Fluorescence quenching has been shown to be particularly suitable to experimentally test this type of question (Eftink & Ghiron, 1981).

The quenching by I^- of the fluorescence of FMN + FAD of NADPH–cytochrome P450 reductase clearly shows a nonlinear pattern revealing the presence of two groups of chromophores with different accessibility to the solvent (data not shown). The relevant parameters derived from analysis of these data, e.g., the Stern–Volmer constants and the contribution to the total fluorescence of the two groups of chromophores, are listed in Table I. The data obtained with riboflavin are listed in Table I, for they correspond to the limiting case of a flavin totally exposed to the solvent. Because there is the simple possibility that the two groups of chromophores merely reveal the contribution of FMN and FAD to the overall fluorescence, we decided to experimentally test this hypothesis. FMN-depleted NADPH–cytochrome P450 reductase has been prepared as indicated under Materials and Methods, and the results of quenching by iodine of its fluorescence are shown in Figure 2. Above 50 mM KI, the Stern–Volmer plot of these results fits satisfactorily to a straight line, contrary to the results obtained with the native enzyme. Analysis of the data yields the parameters given in Table I. The presence of a minor component, amounting to 6–8% of the total fluorescence, which does not fit to one straight line is evident in the Stern–Volmer plot of Figure 3, and this observation correlates well with the reductase activity remaining after treatment with Br^-, ca. 8–10% of the activity of native reductase (Calhoun et al., 1987; results not shown). From these results, we conclude that the intensity of the flavin fluorescence of the native NADPH–cytochrome P450 reductase is the sum of the contributions of FAD and FMN, with the contribution of FAD being dominant with 84% of the total fluorescence. Moreover, as the Stern–Volmer constant of component B is not significantly altered in FMN-depleted reductase with respect to the native enzyme, it is unlikely that the microenvironment of FAD bound to the reductase is largely affected upon removal of FMN by treatment with bromine. This is supported as well by the resonance Raman study of Sugiyama et al. (1985), which leads to the conclusion that the interactions of flavin(s) with the protein in the native and FMN-depleted forms are identical.

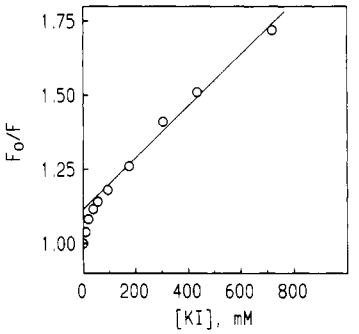

FIGURE 2: Stern–Volmer plot of the quenching by iodine of the fluorescence (λ_{exc} = 450 nm, λ_{em} = 525 nm) of FMN-depleted NADPH–cytochrome P450 reductase in solution (0.1% Tween 20). Temperature, 25 °C.

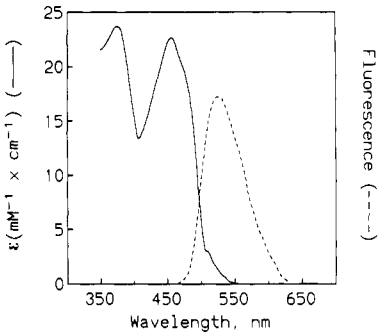

FIGURE 3: Absorption (solid line) and corrected emission (dashed line) spectra of purified NADPH–cytochrome P450 reductase of hepatic microsomes from phenobarbital-induced rats.

In addition, the data suggest that both FMN and FAD are not very accessible to the aqueous solution, for they show Stern–Volmer constants (K_{SV}) at least 1 order of magnitude lower than riboflavin in solution. In order to further assess this point, the collisional quenching constant (k_q) has been calculated from K_{SV} as indicated in Lakowicz (1983), e.g., $K_{SV} = \tau_0 k_q$, where τ_0 is the lifetime of the flavin(s) in the absence of quencher. Taking the average values of τ_0 of 4.88 ± 0.27 and 1.14 ns for free FMN and flavins bound to the reductase, respectively (Bastiaens et al., 1989), from the values of K_{SV} listed in Table I we obtain the following k_q values (in M^{-1} s^{-1}): (1.8 ± 0.1) × 10^{10} (free riboflavin), 3.24 × 10^9 (FMN bound to reductase); 6.67 × 10^8 (FAD bound to reductase). The value of k_q obtained for quenching of the

Table II: Fluorescence Anisotropy (λ_{exc} = 450 nm, λ_{em} = 525 nm) of NADPH–Cytochrome P450 Reductase

sample	lipid:protein ratio (mol/mol)	r_s
riboflavin		0.004
native	0	0.2
	50	0.18
	200	0.19
unfolded	0	0.046
	50	0.045
	200	0.061
aporeductase[a]	0	0.25
	50	0.25
	200	0.24
unfolded aporeductase	0	0.093

[a] FMN-depleted reductase; see the text for details.

Table III: Parameters Used To Calculate Distances between Different Donor/Acceptor Pairs

donor/acceptor pair	J (cm^3 M^{-1})	Q_0	n	K^2	R_0 (nm)
FAD/FMN	0.292×10^{-14}	0.063 (a)	1.33	2/3	1.8
DPH/heme	8.934×10^{-12}	0.5 (b)	1.33	2/3	4.5
4H7HC/heme	0.140×10^{-12}	0.305 (c)	1.33	2/3	4.5
(FAD + FMN)/RPE	0.648×10^{-13}	0.063 (a)	1.33	2/3	3.1

[a] From Bastiaens et al. (1989). [b] From Davenport et al. (1985). [c] Determined as indicated by Lee (1982), using 1 mM quinine sulfate in 1 N sulfuric acid as reference (Q_0 = 0.51 at 25 °C).

fluorescence of free riboflavin by iodine is of the order of magnitude experimentally determined for bimolecular collisional quenching between charged molecules of the size of FMN and iodine in water solutions (Lakowicz, 1983). However, the accessibility to iodine of FMN and FAD bound to the reductase is much lower than that of free FMN, meaning that the flavins are not on the surface of the reductase, FAD being more deeply embedded within the protein structure than FMN.

Location of the FAD and FMN Groups of NADPH–Cytochrome P450 Reductase with Respect to the Lipid/Water Interphase. The basic properties of the fluorescence of flavins of the NADPH–cytochrome P450 reductase (intensity, excitation, and emission maxima, and anisotropy of fluorescence) are unaffected upon reconstitution with egg PC. This result by itself suggests that the prosthetic groups of the reductase are far from the lipid/water interphase, or in a protein domain that is not significantly altered upon insertion of the protein in the lipid bilayer. In addition, since the aggregation state of the reductase is altered in the presence of lipids (French et al., 1980), these results also suggest that neither FAD nor FMN is close to the domains involved in protein–protein interactions in the aggregated form(s).

The anisotropy values of the fluorescence of flavins in the reductase under different experimental conditions are listed in Table II. The value of the anisotropy of fluorescence of the native reductase is lower than this value in the FMN-depleted reductase. This result can be rationalized in terms of energy transfer between FAD and FMN, or in terms of a significant distortion of the FAD binding loci (Bastiaens et al., 1989). This latter possibility seems unlikely on the basis of the lack of effect of removal of FMN on the Stern–Volmer constant for quenching by iodine. Figure 3 shows that there is significant spectral overlap between FAD and FMN and the relevant parameters to quantify energy transfer have been determined and are listed in Table III. Consistent with the hypothesis of energy transfer between FAD and FMN, the intensity of the fluorescence of the FMN-depleted reductase is approximately 15% higher than that of native reductase.

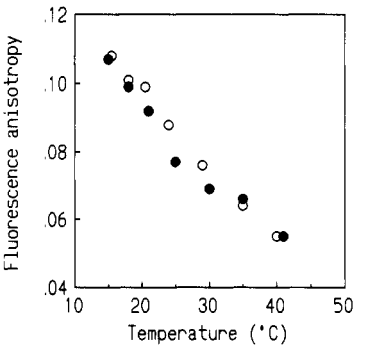

FIGURE 4: Temperature dependence of the steady-state fluorescence anisotropy of DPH (λ_{exc} = 360 nm; λ_{em} = 430 nm) in PC-reconstituted vesicles. The symbols correspond top (○) DPH in reconstituted vesicles without cytochrome P450 and (●) with cytochrome P450. [Cytochrome P450] = 0.2 mg/mL. Lipid:DPH molar ratio = 500:1.

When both FMN and FAD are present in the reductase, the major contribution to the overall fluorescence emission comes from FAD (see above), and energy transfer will flow mostly from FAD to FMN, as indicated earlier by Bastiaens et al. (1989). Considering that in native reductase about 16% of the intensity of the fluorescence emission of flavins comes from FMN, the total energy transfer from FAD to FMN can be estimated to be (15 + 16)%, i.e., approximately 31% of the total FAD fluorescence in the absence of FMN. Using eq 1 under Materials and Methods, with the parameters listed in Table III for the FAD/FMN donor/acceptor pair, we can estimate that both groups are at a distance of about 2 nm, a value which is in good agreement with the one estimated from time-resolved measurements by Bastiaens et al. (1989).

To assess further that FAD and FMN groups of the reductase are far from the lipid/water interphase, we have reconstituted the reductase in vesicles with PC/RPE at a total lipid:protein ratio of 200:1, for the rhodamine group of RPE could be the acceptor of the fluorescence of the reductase. There is not any significant quenching (<5%) of the fluorescence of the reductase, even at acceptor concentrations as high as 20 RPE molecules per molecule of reductase. Using eq 1 and 2 under Materials and Methods with the parameters listed in Table III for the FAD/FMN–rhodamine pair, we can conclude that the flavins of the reductase must be at a distance >5 nm from the rhodamine group of RPE.

Distance from the Heme Group of Cytochrome P450 to the Lipid/Water Interphase. We have used DPH/heme and 4H7HC/heme as donor/acceptor pairs. Cytochrome P450 was purified and reconstituted in PC as indicated under Materials and Methods. In both cases, because the heme group is nonfluorescent, energy transfer has been monitored as the quenching of the fluorescence of the donor group.

For the case of the DPH/heme pair, the heme group quenches about 6% of the fluorescence of DPH in the range of DPH:cytochrome P450 molar ratios between 0.2 and 1, at a fixed molar ratio of DPH/lipid of 1:500. Using the spectral parameters given in Table III, we obtain a distance between the heme and DPH groups of 7.1 nm. Our measurements of the fluorescence anisotropy of DPH give values of 0.08–0.09, and as indicated in Gutierrez-Merino et al. (1987), we have estimated that the distance between both groups ranges between 6.3 and 8 nm, with about 90% probability of occurrence. It is to be noted that the fluorescence anisotropy of DPH is identical within experimental error in PC vesicles and in cytochrome P450 systems in the temperature range 15–40 °C (Figure 4) and that abrupt discontinuities are not observed in this temperature range.

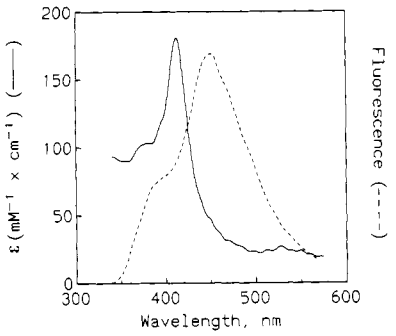

FIGURE 5: Absorption (solid line) spectra of cytochrome P450 reconstituted in PC vesicles with a lipid:protein molar ratio of 100:1 and corrected emission spectra of 4H7HC (dashed line).

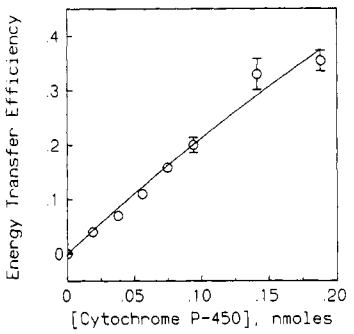

FIGURE 6: Efficiency of energy transfer from 7-ethoxycoumarin to purified cytochrome P450 reconstituted in PC vesicles with a lipid: protein molar ratio of 200:1. [7-Ethoxycoumarin] = 0.1 μM. Temperature, 25 °C.

The coumarin group of 4H7HC bound to lipid bilayers localizes close to the polar headgroups (Pal et al., 1985), whereas DPH is more deeply embedded within this lipid bilayer, likely close to the bottom of the fatty acyl chains (Davenport et al., 1985). Figure 5 illustrates that the emission spectra of 4H7HC overlap with the absorption spectra of the heme group. Thus, they can be used as a donor/acceptor pair, and the relevant parameters of this pair are listed in Table III. Using molar ratios of 4H7HC/cytochrome P450 between 0.1 and 0.2:1, we have obtained about 40% quenching of the fluorescence of 4H7HC by the heme groups. From these data, and using the approach outlined under Materials and Methods, we have estimated a distance of approximately 4.8 nm from the heme group to the lipid/water interphase.

Distance between the Heme Group and the 7-Ethoxycoumarin Binding Site in Cytochrome P450. Because in the absence of NADPH–cytochrome P450 reductase and the reduced form of cytochrome b_5 cytochrome P450 does not metabolize the substrate bound to the catalytic site, we have used purified cytochrome P450 isoenzymes reconstituted in vesicles of PC. The cytochrome P450 isoenzymes were purified from phenobarbital-treated rats as indicated under Materials and Methods, and the substrate used in these studies was 7-ethoxycoumarin, known to be a good substrate of these isoenzymes (Ullrich & Weber, 1972; Prough et al., 1978).

Figure 6 shows the quenching of 7-ethoxycoumarin fluorescence by cytochrome P450 reconstituted in PC. At a molar ratio of approximately 0.5:1 (ethoxycoumarin/heme group), the quenching reaches a maximum of 40%. Using eq 1 under Materials and Methods with the parameters listed in Table III for this donor/acceptor pair, we obtain a distance between the heme group and 7-ethoxycoumarin bound to cytochrome P450 of ca. 5.3 nm. Because this distance is quite similar to that estimated between the heme group and the lipid/water interphase, we have considered the possibility that 7-ethoxycoumarin would significantly bind to the lipid bilayer under these experimental conditions. Therefore, we have titrated 7-ethoxycoumarin with PC vesicles, for binding to the lipid/water interphase often increases the quantum yield of fluorescent dyes (Waggoner, 1979; Lakowicz, 1983). Upon correction of the apparent filtering effects, as indicated under Materials and Methods, we have obtained the titration curve shown in Figure 7. Because the corrections of inner filter effects above 800 μM PC (e.g., absorbance at 330 nm of 1.7 ODU) are not good enough, we could not complete the saturation of the titration curve. Nonlinear curve fitting of the data of Figure 7 to a single type of binding sites yields $K_{0.5}$ values ranging from 1.5 to 2.5 mM. Therefore, we can safely exclude significant binding of 1 μM 7-ethoxycoumarin to the lipid bilayer in reconstituted cytochrome P450 systems.

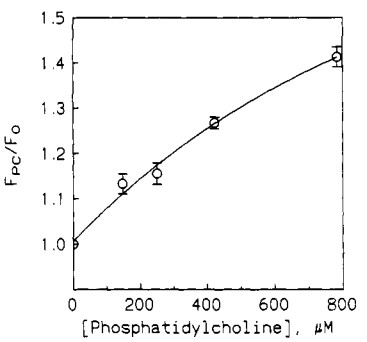

FIGURE 7: Dependence of the fluorescence emission intensity of 7-ethoxycoumarin upon the concentration of PC vesicles. On the y axis is plotted the ratio between the corrected fluorescence intensity of 7-ethoxycoumarin at different concentraions of PC vesicles (F_{PC}) and the corrected fluorescence intensity of 7-ethoxycoumarin in the absence of PC vesicles (F_0). [7-Ethoxycoumarin] = 3–7 μM; λ_{exc} = 330 nm, λ_{em} = 392 nm.

DISCUSSION

Our results show that the two prosthetic groups of cytochrome P450 reductase (FMN and FAD) are not exposed to the surface of this enzyme. In addition, FAD seems to be more buried than FMN in the 3D structure of this enzyme. On the other hand, keeping in mind the properties of the fluorescence of flavins in solution, the conclusion that most of the fluorescence emission of the reductase comes from FAD (i.e., approximately 84% of the total fluorescence) deserves to be further analyzed. In solution, the fluorescence of the flavin group is largely quenched by the adenylyl group in FAD, resulting in a much lower quantum yield of fluorescence of FAD than of FMN (Koziol, 1971; Lakowicz, 1983). Therefore, the results obtained might suggest that, as shown earlier for other flavoproteins (for example, glutathione reductase; Schulz et al., 1978), FAD bound to the reductase adopts a fully extended conformation, so that intramolecular quenching does not significantly occur. Alternatively, differences in quenching of FAD and of FMN groups by interactions with side chains of amino acids of the reductase, likely aromatic amino acids (Nisimoto & Shibata, 1982), could account for the higher quantum yield of FAD in the reductase. Because the quantum yield of the flavin fluorescence in reductase is lower than 0.01 (Bastiaens et al., 1989; data not shown), while that of flavins in solution is 0.25–0.26 (Weber & Teale, 1957; data not shown), this latter possibility appears most likely. Consistent with this hypothesis, our results show that upon denaturation the fluorescence of flavins increases more than 20-fold. Additionally, in our experimental conditions, the reductase is likely to be in the semiquinoid form (Otvos et al., 1986), e.g., with FAD in the oxidized form and

FMN mostly in the semireduced state, and this should account for the lowered fluorescence emission of FMN as well.

In addition, several results point out that both flavins are bound to a *loci* in the reductase far from the lipid/water interphase. First, there is no significant energy transfer to the rhodamine groups of RPE in reconstituted membranes. Second, there is no large alteration of the flavin fluorescence upon reconstitution of solubilized reductase. A functional consequence of this is that the activity of the reductase can be predicted to show little sensitivity to changes of the membrane potential, a basic bioenergetic parameter of primary importance in any membrane showing high oxidoreduction and electron-transport activity. Moreover, since the overall basic fluorescence properties (intensity of fluorescence emission, anisotropy values, and the Stern–Volmer constant derived from iodine quenching) of the flavins of the reductase are not dependent upon the lipid to protein ratio, it is unlikely that the prosthetic groups are close to the polypeptidic domains involved in protein/protein interactions stabilizing oligomeric states of the reductase, likely the state present in the absence of added lipids (French et al., 1980). As a side result of our studies with reductase reconstituted in PC/RPE lipid bilayers, we have noticed that the fluorescence of rhodamine increases as the protein:lipid ratio increases, without a parallel decrease of the fluorescence of FAD + FMN (results not shown). As indicated above, this latter result excludes the existence of significant energy transfer from FAD + FMN to the rhodamine group of RPE and strongly suggests that the increase of fluorescence of rhodamine is attributable to an altered environment of the dye as the protein:lipid ratio increases. It is likely that this increase is reflecting partition of the RPE in the annular lipids of cytochrome P450 reductase, e.g., binding of RPE to the protein at the lipid/protein interface.

From anisotropy and fluorescence intensity data, we have concluded that the distance between the flavins FAD and FMN in the reductase is approximately 2 nm (see above). The range of distances with 90% probability of occurrence has been estimated as in Gutierrez-Merino et al. (1987), using a value of 0.26 for the anisotropy of riboflavin in glycerol (experimentally determined at 25 °C), and to be 1–2.7 nm. Keeping in mind the molecular dimensions of the extended conformations of FAD and FMN, this result points out that both molecules must be quite close in the 3D structure of the reductase.

The distance from the heme group of cytochrome P450 to the lipid bilayers is an important parameter to appropriately establish structure–function relationships of this protein in rat hepatic microsomes. Kunz et al. (1985) have shown that the distance between DPH and the heme group of cytochrome P450 is larger than 6 nm, using time-resolved anisotropy measurements of DPH in reconstituted proteoliposomes of PC/PE/PS and cytochrome P450. From the results reported in this paper, we have estimated a distance of 7.1 ± 0.8 nm between both groups (DPH and the heme group), with approximately 90% probability of occurrence. To assess this point further, we have used 4H7HC, which localizes near the lipid/water interphase (Pal et al., 1985). Using this donor/acceptor pair, we have estimated a distance between the coumarin and heme groups of 4.8 nm, or in the range of 3.7–6.1 nm, with 90% probability of occurrence. Considering that DPH localizes close to the central part of the lipid bilayer (Davenport et al., 1985), and a bilayer thickness of 4.2 nm (Parsegian et al., 1979; Rand et al., 1980; Lis et al., 1982; Herbette et al., 1984), we conclude that the distance between the heme group and the lipid/water interphase is ca. 5 nm,

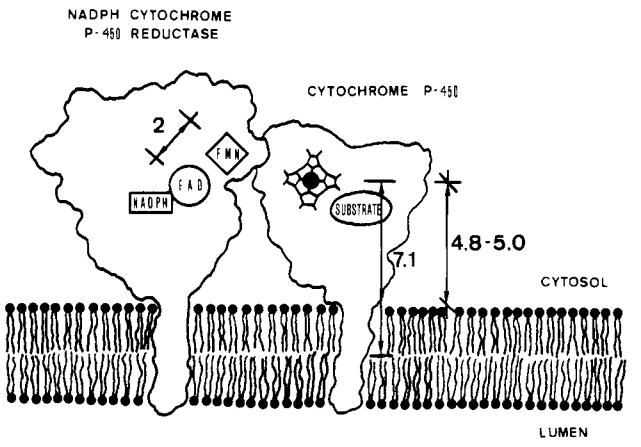

FIGURE 8: Proposed model for the localization of the functional centers in cytochrome P450 and NADPH–cytochrome P450 reductase in the microsomal membrane, on the basis of the results obtained in this study. The distances are given in nanometers. The overall shape of cytochrome P450 has been taken from Edwards et al. (1991), and the overall size of the cytochrome P450 reductase has been produced from that of cytochrome P450 enlarged considering the molecular weight ratio between both proteins, and assuming roughly the same shape for both proteins. The iron atom position is indicated by a filled circle.

in good agreement with the estimation made from the results obtained with 4H7HC as the donor group. These distance estimations thus give support to the structural model of cytochrome P450 proposed by Edwards et al. (1991), e.g., with a heme group quite far from the lipid/water interphase, and perpendicularly oriented to the surface of the lipid bilayer.

From the analysis of fluorescence energy transfer, we obtain an average distance of 5.3 nm between 7-ethoxycoumarin and the heme group. This result would imply that the substrate, 7-ethoxycoumarin, binds quite far from the heme group, and considering the overall shape and dimensions of cytochrome P450 (see the diagram of Figure 8), to accommodate the substrate in this structure its binding *loci* must be located near the lipid/water interphase. This is a rather forced conclusion, and we decided to critically reevaluate it by explicitly considering the possibility that self-aggregation of 7-ethoxycoumarin could introduce an important source of error in the distance estimation, for it should produce an extensive self-quenching of 7-ethoxycoumarin. Self-aggregation of lipophilic molecules led to the formation of micelles (Tanford, 1980), and micellization produces a marked increase of light scattering of the solution. Therefore, we have measured the light scattering of 7-ethoxycoumarin solutions (measured from the Rayleigh peak) as a function of the concentration at 400 nm (a wavelength where the absorption of 7-ethoxycoumarin is negligible; see Figure 9). A noticeable increase of light scattering is already observed at concentrations of 7-ethoxycoumarin as low as 1 μM. It follows that the free 7-ethoxycoumarin concentration is lower than the total concentration used in our fluorescence energy-transfer experiments and that the distance between the substrate and the heme group is likely overestimated. Data reported by Omata et al. (1986) are consistent with this hypothesis. These investigators have shown that the emission of fluorescence of 7-ethoxycoumarin is linearly related to the substrate:enzyme ratio, even at ratios higher than 2. However, when other substrates are used (e.g., benzo[*a*]pyrene), there is a clear discontinuity at a substrate:enzyme ratio of 1. These results were interpreted to mean that the transition dipole moments of the substrate bound to the protein and of the heme group are perpendicularly oriented, resulting in a negligible energy-transfer efficiency. Alter-

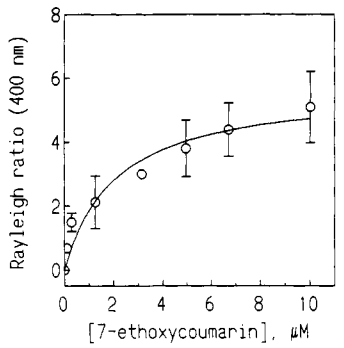

FIGURE 9: Dependence of the Rayleigh scattering intensity at 400 nm upon the concentration of 7-ethoxycoumarin. On the *y* axis is plotted the ratio between the intensity of scattering at 400 nm at a given concentration of 7-ethoxycoumarin and the intensity in the absence of this compound.

natively, significant substrate aggregation (micellization) in the saturation range of cytochrome P450 isoenzymes can also account for these results.

The main conclusions about the localization of the functional sites of cytochrome P450 and of the reductase derived from the studied reported herein are presented in the form of a 3D structural diagram in Figure 8. The 3D organization of the complex cytochrome P450/reductase is likely to set FAD and heme groups closer than illustrated in the diagram. This suggestion is based mostly in the results of modulation by paraquat of cytochrome P450 microsomal electron transport (Centeno, 1991). Briefly, we have shown that paraquat directly interacts with cytochrome P450, resulting in alteration of the spectral properties of the heme group, and with a similar $K_{0.5}$ modulates the activity (activates) of the reductase. This latter effect on the reductase has been proposed to be due to a synergistic redox cycle between paraquat and NADPH in a process that likely involves the reduction of the FAD prosthetic group (Clejan & Cederbaum, 1989). Therefore, it is likely that paraquat binds to a site of the reductase which is close to, or partially overlaps with, the FAD binding *loci*. Obviously, the relative location of FMN and FAD with respect to the heme group in the microsomal electron-transport system shall require further studies to be rigorously established.

Finally, although the use of reconstituted systems of membrane proteins to obtain 3D structural details should always be taken cautiously as the protein structure in itself may be altered during reconstitution with respect to the native membrane, it is to be noted that (i) both hepatic cytochrome P450 reductase and cytochrome P450 isoenzymes induced by phenobarbital have only a small transmembrane domain (Black & Coon, 1982; Brown & Black, 1989); (ii) the reconstituted systems used in this study were functionally active, and most notably the reductase and cytochrome P450 were functionally coupled in co-reconstituted systems as evidenced by their efficiency to hydroxylate 7-ethoxycoumarin using NADPH as the primary electron donor; and (iii) even for membrane proteins more deeply embedded in the lipid bilayer, such as the sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase, the approaches to obtain 3D structural details used in this study lead to conclusions fully consistent with the shape of this protein in the native membrane obtained from electron microscopy images or X-ray diffraction of bidimensional crystals (Gutiérrez-Merino et al., 1987; Cuenda et al., 1990).

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