Interaction of 7-n-alkoxycoumarins with cytochrome *P*-450₂ and their partitioning into liposomal membranes

Assessment of methods for determination of membrane partition coefficients

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A study was made of the binding of 7-ethoxy-, 7-n-propoxy- and 7-n-pentoxycoumarin to cytochrome *P*-450₂ reconstituted into large unilamellar liposomes composed of a mixture of egg L- α -phosphatidylcholine, egg phosphatidylethanolamine and dipalmitoyl phosphatidic acid (2:1:0.06, by weight). The apparent spectral dissociation constants K_s^{app} increased linearly with increasing proteoliposomal concentration. When both cytochrome *P*-450₂ and NADPH:cytochrome *P*-450 reductase were reconstituted into liposomes, the apparent Michaelis constants K_m^{app} for *O*-dealkylation of 7-methoxy-, 7-ethoxy- and 7-n-propoxycoumarin showed a similar dependence on the proteoliposomal concentration. The results were in accordance with models for kinetic or equilibrium processes in biphasic systems containing membrane-bound catalytic or acceptor sites, in which a linear solute partition in the bilayer membrane is postulated. The methyl, ethyl and n-propyl ether were readily dealkylated. However, the *O*-dealkylation rate of 7-n-butoxycoumarin was low and became very small for longer alkyl ethers. Both the effective dissociation constants and effective Michaelis constants decreased with elongation of the alkyl side chain of the coumarins. From plots of the apparent dissociation constants and apparent Michaelis constants against the lipid volume fraction of the proteoliposomes, the membrane partition coefficients for several homologues were calculated. When protein-free liposomes were added to 7-n-alkoxycoumarin solutions, the fluorescence intensity of the coumarins decreased and eventually became negligible in the presence of an excess of liposomal material. On the assumption that the overall fluorescence can be ascribed exclusively to the fraction of 7-n-alkoxycoumarin molecules present in the aqueous phase, partition coefficients for liposomal accumulation of the test compounds could be determined directly. For several coumarin ethers, comparable values were derived for the membrane partition coefficients from binding, kinetic and fluorescence intensity measurements. The change in free energy per methylene group of the 7-n-alkoxycoumarins for partitioning between n-octanol and buffer was significantly different from the value for liposome partitioning.

INTRODUCTION

Metabolism of amphipathic and hydrophobic compounds by the liver microsomal mono-oxygenase system does not follow enzyme-kinetic laws applicable to homogeneous solutions. For example, the rate of hydroxylation of benzpyrene increases hyperbolically and its K_m^{app} linearly with the enzyme concentration (Nebert & Gelboin, 1968; Lu *et al.*, 1977). A similar dependence on enzyme concentration has been observed for the apparent dissociation constants K_s^{app} of the interaction of liver microsomal cytochrome *P*-450 with a number of amphipathic ligands (Waterman *et al.*, 1973; Ebel *et al.*, 1978; Backes *et al.*, 1982, 1984) and of the steroid binding to testicular microsomal cytochrome *P*-450 (Barbieri *et al.*, 1981; Kühn-Velten *et al.*, 1989) and adrenal cytochrome *P*-450 reconstituted in liposomal membranes (Kominami *et al.*, 1986, 1988). Emphasizing the biphasic nature of membrane suspensions and assuming that accumulation of ligands and substrates into the membranes can be considered as a partition equilibrium, Parry *et al.* (1976) developed a formalism for the description of ligand and substrate binding to membrane-bound proteins. In this model, the sizes of the aqueous and lipid phases are defined in terms of their volume fractions. Heirwegh *et al.* (1988) formulated a similar model in terms of mole fraction concentrations.

7-Ethoxycoumarin is a classic substrate of the mono-oxygenase system. Komori *et al.* (1984) purified a cytochrome *P*-450

isoenzyme, called cytochrome *P*-450₂, with high affinity for 7-n-alkoxycoumarins. The present paper reports on the determination of the membrane partition coefficients of several 7-n-alkoxycoumarins based on application of existing models (Parry *et al.*, 1976; Heirwegh *et al.*, 1988) to binding and kinetic studies performed with cytochrome *P*-450₂ reconstituted into liposomal material. The validity of these methods was assessed by direct determination of liposomal partition coefficients based on quenching of the fluorescence of the ligands by the liposomal material. For comparison, values of the n-octanol/buffer partition coefficients, which are often used to characterize the effects of membrane partitioning were also obtained.

THEORY

The modelling of ligand binding or substrate transformation in biphasic systems containing membrane-bound acceptors or catalytic sites is based on the assumptions that the membrane forms a single homogeneous bulk phase and that the accumulation of ligands or substrates in the membrane is governed by a constant membrane partition coefficient. Moreover, the luminal compartment of the vesicles and the surrounding aqueous medium will be considered as a single aqueous phase with identical local concentrations of ligands and substrates.

In the following model, kinetic expressions are derived for reactions involving membrane-bound enzyme system. The mem-

Abbreviations used: PC, egg L- α -phosphatidylcholine; PE, egg phosphatidylethanolamine; DPPA, dipalmitoyl phosphatidic acid.

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brane partition coefficient K_p of the substrate will be defined in terms of molar concentrations. Further it will be assumed that mass transport is fast compared with enzymic conversion.

Consider a membrane-bound enzyme with active site located in phase i , catalysing the reaction:

S^i and P^i represent substrate and product, and E^i , $(ES)^i$ and $(EP)^i$ are free enzyme, enzyme-substrate and enzyme-product complex respectively. Under steady-state conditions, the initial reaction rate v , measured in the absence of product, is given by:

$$v = k_{\text{cat}} \frac{[E_0][S]^i}{K'_m + [S]^i} \quad (1)$$

where k_{cat} is the catalytic constant (Fersht, 1985), $[E_0]$ the total concentration of enzymic sites, K'_m the effective Michaelis constant (Heirwegh *et al.*, 1988) and $[S]^i$ the concentration of substrate in phase i . Neglecting enzyme-bound substrate, the total mass of substrate can be expressed as:

$$V_T[S_0] = V_A[S]_A + V_L[S]_L \quad (2)$$

where $[S_0]$, $[S]_A$ and $[S]_L$ are the substrate concentration in the total system (with volume V_T), in the aqueous phase (with volume V_A) and in the lipid phase (with volume V_L) respectively. Defining the membrane partition coefficient as:

$$K_p = [S]_L/[S]_A \quad (3)$$

and the lipid volume fraction of the liposomal suspension, α_L , as:

$$\alpha_L = V_L/V_T \quad (4)$$

eqn. (2) can be rearranged to:

$$[S]_A = [S_0]/(1 - \alpha_L + K_p \alpha_L) \quad (5)$$

and

$$[S]_L = K_p[S_0]/(1 - \alpha_L + K_p \alpha_L) \quad (6)$$

The factor $(1 - \alpha_L + K_p \alpha_L)$ is equivalent to the distribution function defined in the model of Heirwegh *et al.* (1988). The substrate concentration relevant for the rate equation [eqn. (1)] will be dependent on the nature and the orientation of the enzymic site. Beside aqueous-faced hydrophilic and lipid-faced sites, hydrophobic aqueous-faced and mixed sites have been considered (Heirwegh *et al.*, 1988). Rearranging in eqn. (1) the local concentration of free substrate $[S]^i$ by the expressions of eqn. (5) and (6) gives:

$$v = k_{\text{cat}} \frac{[E_0][S_0]}{K_m^{\text{app}} + [S_0]} \quad (7)$$

where the apparent Michaelis constant K_m^{app} is given by:

$$K_m^{\text{app}} = K'_m(1 - \alpha_L + K_p \alpha_L) \quad (8)$$

For aqueous-faced hydrophilic sites:

$$K'_m = K_m \quad (9)$$

where K_m is the true Michaelis constant applicable to a corresponding single-phase system. For lipid-faced sites and for mixed sites:

$$K'_m = K_m/K_p \quad (10)$$

where K_p is the membrane partition coefficient. For aqueous-faced hydrophobic sites, K_p can be replaced by a related quantity.

By analogy with the kinetic model, expressions for ligand binding to membrane-bound acceptors can be formulated (Parry *et al.*, 1976; Heirwegh *et al.*, 1988). For the equilibrium reaction:

the predicted dependence of the apparent dissociation constant K_s^{app} on the volume fraction of the membrane lipid compartment is given by:

$$K_s^{\text{app}} = K'_s(1 - \alpha_L + K_p \alpha_L) \quad (11)$$

The effective dissociation constant K'_s has a similar meaning for the equilibrium reaction to K'_m for the enzyme reaction (Heirwegh *et al.*, 1988). According to eqns. (8) and (11), the membrane partition coefficient K_p of a substrate or a ligand can be derived from the slope of a plot of apparent Michaelis or apparent dissociation constants against the lipid volume fraction α_L of the liposomes.

MATERIALS AND METHODS

Materials

Egg L- α -phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). Dipalmitoyl phosphatidic acid (DPPA) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The phospholipids showed a single spot on t.l.c. on silica (solvent, chloroform/methanol/water, 65:25:4, by vol.; I_2 staining). 7-Ethoxycoumarin was from Aldrich Chemie (Brussels, Belgium), 7-hydroxycoumarin from Merck (Darmstadt, Germany) and the n-alkyl halogenides were products from Janssen Chimica (Beerse, Belgium). Sodium cholate was purchased from Serva Feinbiochemica G.m.b.H. (Heidelberg, Germany) and NADPH from Boehringer G.m.b.H. (Mannheim, Germany). The liquid-chromatography media for the purification of the microsomal enzymes, DEAE-Sepharose Fast Flow, 2',5'-ADP-Sepharose 4B, CM-Sepharose Fast Flow and Sepharose 4B-CL, were obtained from Pharmacia (Uppsala, Sweden). n-Octylamino-Sepharose 4B was prepared as described by Guengerich & Martin (1980). Hydroxyapatite was from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were of analytical reagent grade. The Lipoprep instrument, used to prepare the liposomes and proteoliposomes, was from Diachema (Langnau a.A./Zürich, Switzerland). Titrations were performed using Agla micrometer syringes (Wellcome Research Laboratories, Beckenham, Kent, U.K.).

Isolation of the microsomal enzymes

Cytochrome $P-450_2$ was isolated from liver microsomes of phenobarbital-pretreated rabbits as described by Komori *et al.* (1984). The purified enzyme was homogeneous on SDS/PAGE and had a specific content of 16.5 nmol of cytochrome $P-450$ /mg of protein. The overall yield was about 0.55% of total cytochrome $P-450$. NADPH-cytochrome $P-450$ reductase was purified according to the method of Vermilion & Coon (1978). The concentration of the reductase was calculated using $\epsilon = 21.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 456 nm (French & Coon, 1979). The specific activity, expressed as μmol of cytochrome c reduced/min per mg of protein in 0.3 M-potassium phosphate buffer, pH 7.7, at 30 °C, varied between 55 and 60 for different preparations. Cytochrome $P-450$ was quantified by the method of Omura & Sato (1964). Protein concentrations were estimated by the method of Lowry *et al.* (1951) with modifications for interfering substances (Wessel & Flügge, 1984).

Synthesis of 7-n-alkoxycoumarins

The potassium salt of 7-hydroxycoumarin was prepared by dissolving 0.3 mol of 7-hydroxycoumarin and 0.31 mol of KOH

in 500 ml of methanol. The solvent was evaporated and the salt stored in a desiccator. For preparation of a 7-n-alkoxycoumarin, 0.03 mol of the potassium salt of 7-hydroxycoumarin was refluxed for several hours with the corresponding n-alkyl bromide (0.06 mol) in 50 ml of dimethylformamide, dried previously on a molecular sieve. For 7-methoxycoumarin, methyl iodide was used as the alkylating agent. The 7-n-alkoxycoumarins were extracted from the reaction mixture with dichloromethane and washed extensively with 2 M-NaOH and water. After evaporation of the dichloromethane, the n-butyl to n-octyl ethers were dissolved in n-hexane and then purified by column (2 cm × 20 cm) chromatography on silica using n-hexane/ethyl acetate (22:3, v/v) as eluent. 7-Methoxy- and 7-n-propoxy-coumarin were dissolved in n-hexane/ethyl acetate (3:1, v/v) and the eluent composition was modified to (3:2, v/v) and (3:1, v/v) respectively. The 7-n-alkoxycoumarins were monitored photometrically at 280 nm during chromatography. The compounds were recrystallized from aqueous methanol. Their purity was assessed by t.l.c. on silica (solvent, benzene/ethanol/acetic acid, 179:20:1, by vol.; I₂ staining).

Preparation of liposomes and proteoliposomes

The buffer referred to as 'standard buffer' had the following composition: 0.1 M-Tris/HCl, pH 7.5, 20 % (v/v) glycerol and 0.2 mM-EDTA. Liposomes and proteoliposomes were prepared at 4 °C using a detergent-dialysis method (Bösterling *et al.*, 1979). For the preparation of the liposomes, 76.5 mg of phospholipid (PC/PE/DPPA, 2:1:0.06, by weight), dissolved in chloroform, was evaporated to dryness under argon. Remaining solvent was removed *in vacuo* for 1 h. Phospholipid was dissolved with sodium cholate at a detergent/phospholipid ratio of 3 (w/w) in 7.65 ml of standard buffer. The mixture was dialysed for 16 h against 3 l of standard buffer using a Lipoprep flow-through dialyser (Zumbühl & Weder, 1981). When proteoliposomes were prepared, either cytochrome *P*-450₂ (binding assays) or cytochrome *P*-450₂/NADP-cytochrome *P*-450 reductase in a molar ratio 1/4 (kinetic assays) was added to the phospholipid/detergent/buffer mixture at a lipid/protein molar ratio of 550 (binding studies), 1100 (kinetic assays at fixed substrate concentration, 25 μM) or 1750 (determination of K_m^{app}). The final mixtures (total volume 6.375 ml; phospholipid concentration, 6 mg/ml) were dialysed against standard buffer as indicated above. Non-incorporated enzymes were removed by gel chromatography on Sepharose 4B-CL.

Phospholipid concentration was determined by quantification of P_i (Fiske & Subbarow, 1925; Folch *et al.*, 1957). The average M_r of the phospholipids was taken as 770. The density of the phospholipid vesicles was assumed to be 1 g/ml (Newman & Huang, 1975).

Incubation mixtures

The *O*-dealkylation measurements and binding studies with proteoliposomes and the partitioning studies with liposomes were performed in standard buffer at 30 °C.

O-Dealkylation of 7-n-alkoxycoumarins

***O*-Dealkylating activity of the mono-oxygenase system as a function of the alkyl chain length of 7-n-alkoxycoumarin homologues.** The incubation mixtures contained proteoliposomal material at a final concentration of cytochrome *P*-450₂ and NADPH:cytochrome *P*-450 reductase of 0.22 and 0.88 μM respectively. The 7-n-alkoxycoumarins were assayed at 25 μM final concentration. Stock solutions of the coumarin ethers were prepared in methanol/water (1:1, v/v), the final methanol concentration in the incubation mixture being 1.25 % (v/v). This concentration proved not to be inhibitory. After preincubation

of each reaction mixture for 5 min, *O*-dealkylation was initiated by adding NADPH to a final concentration of 50 μM, in a total incubation volume of 2 ml. The reaction was monitored fluorimetrically (Ullrich & Weber, 1972) (excitation at 380 nm, emission at 460 nm) with an Aminco SPF-500 ratio spectrofluorimeter (SLM Instruments, Urbana, IL, U.S.A.).

Kinetic determination of K_m^{app} and of the membrane partition coefficients. Using the incubation procedure described above, initial-rate measurements of the *O*-dealkylating activity were performed at, at least, seven substrate concentrations for 7-methoxy-, 7-ethoxy- and 7-n-propoxy-coumarin. K_m^{app} was obtained for each saturation curve by application of the computer program of Cleland (1979). The procedure was repeated for several dilutions of the cytochrome *P*-450₂/NADPH:cytochrome *P*-450 reductase proteoliposomes in standard buffer (final concentration of cytochrome *P*-450₂ 0.03, 0.06, 0.09, 0.12 and 0.15 μM). For each substrate, eqn. (8) was then fitted to (α_L , K_m^{app})-data pairs to obtain the corresponding membrane partition coefficient.

Binding studies

The 7-n-alkoxycoumarins have an end absorbance situated at about 380–390 nm. Difference spectra due to the interaction of those compounds with cytochrome *P*-450₂, therefore, have to be corrected for the proper absorbance of the ligands. The proteoliposomes were present in the sample cuvette and the reference cuvette contained liposomes at identical phospholipid concentration. First, the spectrum of ligand-free reconstituted cytochrome *P*-450₂ was recorded and stored in the computer memory of the instruments. Identical portions of a 7-n-alkoxycoumarin homologue were then added to both cuvettes, the difference spectrum was measured and the reference spectrum of cytochrome *P*-450₂ was subtracted. The same procedure was performed at several ligand concentrations. The final volume change did not exceed 0.8 %. The ligand saturation curves obtained in this way, are represented by rectangular hyperbolae (see the Appendix). For each ligand, titration curves were established at proteoliposomal *P*-450₂ concentrations between 0.8 and 4.0 nmol/ml. Difference spectra were registered between 350 and 450 nm with an Aminco/SLM DW-2000 UV/VIS spectrophotometer (SLM Instruments, Urbana, IL, U.S.A.). For each titration curve, an apparent dissociation constant K_s^{app} was calculated, using the computer program of Cleland (1979). The membrane partition coefficients were obtained by fitting eqn. (11) to (α_L , K_s^{app}) data pairs.

Fluorescence measurements for the partitioning of the 7-n-alkoxycoumarins into liposomes

Solutions (1 μM) of 7-n-alkoxycoumarins (methoxy- to n-hexoxy-coumarin) were prepared in standard buffer. The solutions were stable for at least 2 weeks. To 2 ml of each solution, microlitre quantities of a liposomal suspension (phospholipid concentration, 10 mg/ml) were added. Fluorescence emission spectra were recorded after each addition, the fluorescence intensities being calculated from the integrated spectra. The excitation wavelength was set at 320 nm. For the determination of the quantum yield of 7-n-hexoxycoumarin in different solvents, a value of 0.55 was used for the quantum yield of the standard reference quinine sulphate in 0.1 M-H₂SO₄. Fluorescence spectra were recorded with a computerized Spex Fluorolog 212/Datamate (Spex Industries GmbH, München, Germany).

Bulk phase partition coefficients

7-n-Alkoxycoumarins were equilibrated at 30 °C between standard buffer and n-octanol in Erlenmeyer flasks provided with separate sampling tubes for the aqueous and the organic

phase, as described by Brooke *et al.* (1986). Partition coefficients were determined at three different concentrations of the compounds. The equilibrium concentrations in the *n*-octanol and the aqueous phase were measured fluorimetrically or spectrophotometrically. The *n*-octanol and the standard buffer were saturated previously with the other phase.

RESULTS

O-Dealkylation of 7-*n*-alkoxycoumarins

Relationship between the alkyl chain length and the *O*-dealkylation rate of 7-*n*-alkoxycoumarins. The influence of the alkyl chain length of the compounds on the *O*-dealkylation rate is shown in Table 1. The methyl, ethyl and *n*-propyl ethers were easily converted into 7-hydroxycoumarin and the corresponding

Table 1. *O*-Dealkylation activity of cytochrome *P*-450₂ and NADPH:cytochrome *P*-450 reductase (molar ratio *P*-450₂/reductase, 1:4) reconstituted in PC/PE/DPPA (2:1:0.06, by weight) liposomes, as a function of the alkyl-chain length of 7-*n*-alkoxycoumarins

The cytochrome *P*-450₂ and reductase concentrations were 0.22 and 0.88 μ M respectively. The concentration of the coumarin ethers in the incubation mixtures was 25 μ M. *O*-Dealkylation measurements were performed in 0.1 M-Tris/HCl buffer, pH 7.5, containing 20% (v/v) glycerol and 0.2 mM-EDTA. Results are means $\pm$ 1 S.D. for three proteoliposomal preparations.

7- <i>n</i> -Alkoxy-coumarin	Specific activity (nmol of 7-hydroxycoumarin formed/min per nmol of cytochrome <i>P</i> -450 ₂)
7-Methoxy-	0.917 $\pm$ 0.195
7-Ethoxy-	0.977 $\pm$ 0.140
7- <i>n</i> -Propoxy-	0.691 $\pm$ 0.100
7- <i>n</i> -Butoxy-	0.134 $\pm$ 0.022
7- <i>n</i> -Pentoxy-	0.044 $\pm$ 0.020
7- <i>n</i> -Hexoxy-	0.024 $\pm$ 0.010
7- <i>n</i> -Heptoxy-	0.020 $\pm$ 0.007
7- <i>n</i> -Octoxy-	0.014 $\pm$ 0.005

Fig. 1. Dependence of the apparent Michaelis constant for *O*-dealkylation of 7-*n*-alkoxycoumarins on the lipid volume fraction of the proteoliposomes

The symbols refer to: 7-methoxycoumarin (■), 7-ethoxycoumarin (▲) and 7-*n*-propoxycoumarin (●). Results are apparent Michaelis constants $\pm$ 1 S.D.

aldehydes. From 7-*n*-butoxycoumarin onwards, the *O*-dealkylation rate decreased and became very small for longer alkyl chains. 7-Ethoxycoumarin was the best substrate; its *O*-deethylation rate was about 70-fold higher than the *O*-dealkylation rate of the *n*-octyl ether. An identical pattern was observed when cytochrome *P*-450₂ and NADPH:cytochrome *P*-450 reductase were in solubilized forms (results not shown).

Kinetic measurements for the determination of partition coefficients. Since the *O*-dealkylation rate decreased substantially for 7-*n*-butyl and longer-alkyl-chain coumarin ethers, kinetic measurements with variable proteoliposomal concentration were only performed with 7-methoxy-, 7-ethoxy- and 7-*n*-propoxycoumarin. The substrate saturation could be represented accurately by rectangular hyperbolae. The apparent Michaelis constants increased linearly with increasing concentration of proteoliposomes (Fig. 1). Values of K'_m obtained by extrapolation to zero lipid volume fraction are given in Table 2, and the values of the membrane partition coefficients in Table 3.

Binding studies

Interaction of 7-*n*-alkoxycoumarins with reconstituted cytochrome *P*-450₂ caused a difference spectrum, with a minimum around 417 nm, similar to a type-I difference spectrum (Schenkman *et al.*, 1967). Since the usual type-I peak around 385 nm was absent, apparent spectral dissociation constants were determined from the absorbance difference between 417 and 450 nm. Difference spectra were recorded with 7-ethoxy-, 7-*n*-propoxy- and 7-*n*-pentoxy-coumarin as ligands. In Fig. 2, 7-*n*-pentoxy-coumarin-induced difference spectra are shown. The ligand saturation also fitted accurately to rectangular hyperbolae. In analogy with the apparent Michaelis constants, the apparent spectral dissociation constants increased linearly with the proteoliposomal concentration (Fig. 3). The effective dissociation constants, obtained by extrapolation to zero lipid volume fraction, are given in Table 2 and the membrane partition coefficients in Table 3.

Determination of liposomal membrane partition coefficients by fluorescence measurements

The fluorescence of 7-*n*-alkoxycoumarins is influenced by the solvent. The fluorescence quantum yield of e.g. 7-*n*-hexoxycoumarin in standard buffer was about 10-fold and about 60-fold higher than the quantum yield in *n*-octanol and toluene respectively. Incremental amounts of liposomes, added to solutions of 7-*n*-alkoxycoumarins, caused a decrease in their fluorescence intensity. This is illustrated in Fig. 4 for 7-*n*-pentoxy-coumarin. The quenching was greater when the alkyl chain of the coumarin ethers increased. Since the fluorescence intensity decreased continuously on further addition of liposomes, it was assumed that only the 7-*n*-alkoxycoumarin molecules present in the aqueous phase contribute to the fluorescence. If so, one can write the following equation for the partition coefficient K_p :

$$K_p = \frac{[1 - (I/I_{\max})]V_A}{(I/I_{\max})V_L} \quad (12)$$

$I_{\max}$ and I represent the fluorescence intensity in the absence and the presence of liposomes respectively; V_A and V_L are the aqueous and the membrane volume respectively. Eqn. (12) can be rearranged to:

$$I_{\max}/I = 1 + K_p (V_L/V_A) \quad (13)$$

or with $V_A \approx V_T$, the total volume, and $\alpha_L = V_L/V_T$:

$$I_{\max}/I = 1 + K_p \alpha_L \quad (14)$$

The partition coefficient K_p is obtained as the slope of this linear equation. The curves in Fig. 5 show the fluorescence ratio

Table 2. Effective Michaelis constants K'_m and effective dissociation constants K'_s for the binding and kinetic interaction of 7-n-alkoxycoumarins with cytochrome *P*-450₂ reconstituted in PC/PE/DPPA (2:1:0.06, by weight) liposomes

The K'_m and K'_s values were obtained by extrapolation of values of K_m^{app} and K_s^{app} (Figs. 1 and 3) to zero liposomal lipid volume fraction. Results are y -intercepts $\pm$ 1 s.d.

7-n-Alkoxy-coumarin	K'_m (μ M)	K'_s (μ M)
7-Methoxy-	2.82 ± 0.02	—
7-Ethoxy-	1.14 ± 0.02	29.5 ± 0.8
7-n-Propoxy-	0.60 ± 0.02	12.0 ± 0.4
7-n-Pentoxo-	—	2.4 ± 0.6

Table 3. Liposomal partition coefficients of 7-n-alkoxycoumarins, as determined from the kinetic, binding and fluorescence intensity measurements and n-octanol/standard buffer partition coefficients for some homologues

Liposomal partition coefficients were derived from (K_m^{app} versus α_L) plots (eqn. 8), (K_s^{app} versus α_L) plots (eqn. 11) and (I_{max}/I versus α_L) plots (eqn. 14). Results are calculated values $\pm$ 1 s.d.

7-n-Alkoxy-coumarin	K_p (liposomes)			K_p (n-octanol/buffer)
	Kinetic	Binding	Fluorescence	
7-Methoxy-	98 ± 11	—	104 ± 4	55 ± 1
7-Ethoxy-	224 ± 21	201 ± 25	195 ± 7	—
7-n-Propoxy-	481 ± 54	446 ± 37	455 ± 4	724 ± 19
7-n-Butoxy-	—	—	1190 ± 20	—
7-n-Pentoxo-	—	2320 ± 240	—	8240 ± 290

Fig. 2. Difference spectra resulting from the binding of 7-n-pentoxycoumarin to reconstituted cytochrome *P*-450₂

Increasing displacements from the baseline correspond to 1.50, 3.00, 4.49, 5.99, 8.97, 13.44, 19.37 and 26.76 μ M 7-n-pentoxycoumarin (total concentration). The phospholipid and cytochrome *P*-450₂ concentration of the proteoliposomes was 1.6 mg/ml and 3.12 nmol/ml respectively.

I_{max}/I as a function of the membrane volume fraction α_L . A linear relationship was indeed found for the methyl to n-butyl ethers. From 7-n-pentoxycoumarin onwards, the curves clearly were non-linear. Liposomal partition coefficients were determined according to the proposed model for 7-methoxycoumarin up to 7-n-butoxycoumarin. Their values are given in Table 3.

Fig. 3. Effect of the proteoliposomal lipid volume fraction on the apparent dissociation constant of the interaction of 7-n-alkoxycoumarins with cytochrome *P*-450₂

The symbols refer to: 7-ethoxycoumarin (■), 7-n-propoxycoumarin (▲) and 7-n-pentoxycoumarin (●). Results are apparent dissociation constants $\pm$ s.d.

Fig. 4. Decrease in the fluorescence intensity of 7-n-pentoxycoumarin on addition of incremental amounts of PC/PE/DPPA (2:1:0.06, by weight) vesicles

At the top is the emission spectrum in the absence of liposomes. The other curves, in order of decreasing fluorescence intensity, were recorded in the presence of 0.074, 0.148, 0.476, 0.909, 1.667 and 2.857 mg liposomal phospholipid/ml. Excitation was at 320 nm.

Bulk phase partition coefficients

For comparison, n-octanol/buffer partition coefficients determined for 7-methoxy-, 7-n-propoxy- and 7-n-pentoxycoumarin are shown in Table 3.

Size-dependence of free energy of partition and binding

The free energy of liposomal partitioning [$-\Delta G^\circ(K_p)$] of the 7-n-alkoxycoumarins was calculated from the liposomal partition coefficients. Linear relationships were obtained for the free energy of partitioning versus the number of carbon atoms of the alkyl chain. Values of -2.06 ± 0.02 and -2.05 ± 0.13 kJ/mol per methylene group were determined for the size-dependence of free energy of partitioning from the binding and fluorescence studies respectively. The kinetic measurements gave an identical value of

Fig. 5. Ratio of the fluorescence intensity of the 7-n-alkoxycoumarins in the absence ($I_{\max}$) and the presence (I) of liposomes, as a function of the lipid volume fraction of the liposomes

The concentration of the 7-n-alkoxycoumarins in the cuvette amounted to $1 \mu\text{M}$. The symbols refer to: $\square$, 7-n-hexoxycoumarin; $\blacktriangle$, 7-n-pentoxycoumarin; $\circ$, 7-n-butoxycoumarin; $\bullet$, 7-n-propoxycoumarin; $\triangle$, 7-ethoxycoumarin; $\blacksquare$, 7-methoxycoumarin.

-2.00 ± 0.05 kJ/mol per methylene group. In contrast, the size-dependence of free energy determined from the n-octanol/buffer partition coefficients was -3.16 ± 0.06 kJ/mol per methylene group, which is significantly different from the values of liposomal partitioning. The change in free energy of binding per methylene group, calculated from the effective association constants, amounted to -2.09 ± 0.06 kJ/mol per methylene group.

DISCUSSION

Our results on the influence of the alkyl chain length of the 7-n-alkoxycoumarins on the *O*-dealkylating activity of the reconstituted mono-oxygenase system (Table 1) are in accord with the results of Komori *et al.* (1984), who also found a high dealkylating activity for the methyl, ethyl and n-propyl ethers and a substantial decrease in activity for 7-n-butoxycoumarin. Our measurements show that the rate of *O*-dealkylation further decreases for longer-alkyl-chain homologues. This decreased substrate transformation can hardly be rationalized by a decline in affinity for cytochrome *P*-450₂. Indeed, the effective dissociation constant of 7-n-pentoxycoumarin is smaller than the K'_s value of 7-n-propoxycoumarin, although the *O*-dealkylation of the n-propyl ether is about 15-fold higher than that of the n-pentyl ether. Possibly, a decrease in the catalytic constant k_{cat} of the longer-alkyl-chain coumarin ethers is responsible for their decreased metabolism. Similar observations have also been reported for homologous series of n-alkoxyresorufins (Burke & Mayer, 1983; Burke *et al.*, 1985) and naphthyl alkyl ethers (Hunter & Wilson, 1981). For the latter compounds, however, side-chain hydroxylation has been observed which can compete for *O*-dealkylation (Hunter & Wilson, 1981). In contrast, increasing metabolic rates with increasing alkyl-chain length have been observed for alcohols (Morgan *et al.*, 1982) and *p*-nitrophenylalkyl ethers (Al-Gailany *et al.*, 1975).

For several homologous series of compounds, a good correlation has been found between the lipophilicity of the homologues and their interaction (binding, kinetic or inhibitory) with cytochrome *P*-450 (Cohen & Mannering, 1973; Al-Gailany *et al.*, 1975, 1978; Yih & Van Rossum, 1977; Backes *et al.*, 1982; Morgan *et al.*, 1982; Van De Straat *et al.*, 1986). In general, for the short-chain homologues, $-\log(K^{\text{app}})$ increases linearly with

the logarithm of the partition coefficient. A linear plot of $-\log(K'_m)$ versus $\log(K_p)$ had a slope of 1.04 ± 0.15 (S.D.) with $r = 0.99$. The slope of $-\log(K'_s)$ versus $\log K_p$ was 1.03 ± 0.03 (S.D.) with $r = 1.00$. The results with the 7-n-alkoxycoumarins thus seem to confirm the linear relationship. It has been reported that, on further lengthening of the alkyl chain of certain homologous series, the relationship deviates from linearity (Yih & Van Rossum, 1977; Born & Hadley, 1980). This could be due to sterically hindered binding at a size-limiting binding site (Yih & Van Rossum, 1977). However, this was not investigated for the 7-n-alkoxycoumarins. Figs. 1 and 3 show that the Michaelis constants are lower than the dissociation constants of the 7-n-alkoxycoumarins. Likewise, K_m is less than K'_s for the *O*-dealkylation of *p*-nitrophenylalkyl ethers (Al-Gailany *et al.*, 1975). These observations are compatible with the occurrence of additional intermediates in the reaction pathway after the formation of the enzyme-substrate complex. In this case, K_m is always less than or equal to K'_s (Fersht, 1985). The involvement of hemiacetal intermediates has been suggested for the *O*-dealkylation of aromatic ethers (Al-Gailany *et al.*, 1975; Hunter & Wilson, 1981).

Evidence has been provided that cytochrome *P*-450 forms oligomers in lipid membranes (Schwarz *et al.*, 1982; Greinert *et al.*, 1982). Hence, the possibility of 7-n-alkoxycoumarin interaction with protein aggregates, eventually giving rise to allosteric processes, should be considered. The kinetic and binding saturation curves, however, did not exhibit any sigmoidal shape, but fitted accurately to rectangular hyperbolae. Moreover, analysis of the equilibrium and kinetic data according to the Hill equation yielded plots with a slope 1 within the experimental error, indicating that no allosteric effects occurred.

Assuming that the distribution of 7-n-alkoxycoumarins between an aqueous phase and a liposomal membrane is governed by a constant membrane partition coefficient, partition coefficients were calculated using eqns. (8) and (11). Moreover, a model was proposed to determine partition coefficients directly, without the use of reconstituted protein probes. A similar model has been used to determine the partition of parinaric acid between aqueous and lipid phases (Sklar, 1980). From Table 3, it can be seen that the values obtained with kinetic and binding measurements are almost identical with those determined by application of eqn. (14) of the fluorescence model. The assumption that the overall fluorescence can be ascribed exclusively to the fraction of 7-n-alkoxycoumarin molecules present in the aqueous phase is probably not completely correct. There may still be a minor fluorescence in the liposomal membrane. However, this can be neglected when the fraction of 7-n-alkoxycoumarin molecules, present in the lipid phase, is relatively small. This fraction increases with increasing partition coefficients, resulting in smaller values for the ratio of fluorescence intensities $I_{\max}/I$ than those expected from eqn. (14). This could explain the non-linear curve of $I_{\max}/I$ versus the lipid volume fraction for 7-n-pentoxycoumarin and 7-n-hexoxycoumarin. For the shorter-alkyl-chain coumarin ethers, however, the fluorescence model is a valuable alternative model for the determination of their liposomal partition coefficients.

Parry *et al.* (1976) distinguished between aqueous- and lipid-faced binding sites of membrane-bound proteins. Backes *et al.* (1982, 1984) used the mathematical model of Parry to derive the free energy of binding at microsomal cytochrome *P*-450 from apparent association constants, extrapolated to zero microsomal concentration, for series of aromatic hydrocarbons and barbiturates. This extrapolation eliminates the effect of ligand partitioning on the apparent association constants. If the binding site is aqueous-faced, the extrapolated association constants are independent of the partition coefficients of ligands (Parry *et al.*,

1975; Backes *et al.*, 1982). However, Heirwegh *et al.* (1988) pointed out that this is true only for an aqueous-faced fully hydrophilic binding site, which can be excluded for cytochrome *P*-450. The latter authors stated that cytochrome *P*-450 may have a lipid-faced or a hydrophobic aqueous-faced binding site or even a mixed binding site, which overlaps both the aqueous and the phospholipid compartment. They further argued that a fully lipid-faced binding site is unlikely because the wide variety of substrates with variable polarities would render an active site situated at a fixed depth somewhat inefficient. So, the two most probably orientations are an aqueous-faced hydrophobic site or a mixed site with the haem moiety in the contact zone of the aqueous phase and the polar part of the membrane. Probably, the orientation of the active site may differ for different cytochromes *P*-450. For several species of cytochrome *P*-450, functioning in the biosynthesis of steroid hormones, it was suggested that the binding site is embedded in the lipid membrane (Seybert *et al.*, 1979; Kominami *et al.*, 1986, 1988). From studies of the amino acid sequence of liver microsomal cytochrome *P*-450 forms, models were proposed for the membrane topology, based on the arrangement of hydrophobic segments in cytochrome *P*-450 (Tarr *et al.*, 1983; Ozols *et al.*, 1985; Hudecek & Anzenbacher, 1988). In these studies, a multitransmembrane insertion of hydrophobic segments was suggested with the haem-binding cysteine situated in an aqueous-exposed domain, near to the membrane-immersed part of the protein. Recent investigations on the membrane topology of rabbit liver microsomal cytochrome *P*-450 LM2 and of rat liver microsomal cytochrome *P*-450IIB1 revealed that the proteins are bound to the endoplasmic reticulum membrane by only one or two transmembrane segments, located at the *N*-terminal residue of the polypeptide chain (De Lemos-Chiarandini *et al.*, 1987; Sakaguchi *et al.*, 1987; Brown & Black, 1989; Uvarov *et al.*, 1989; Vergères *et al.*, 1989). The main mass of the liver microsomal cytochromes *P*-450 is exposed to the cytosolic side of the membrane with the active centre located outside or on the membrane border (Brown & Black, 1989; Uvarov *et al.*, 1989). Brown & Black (1989) suggested that substrates might have access to the active site of cytochrome *P*-450 LM2 either by diffusion from the cytosol or from within the lipid membrane. The size-dependences of free energy of partitioning and of binding of the 7-n-alkoxycoumarins do not allow one to give a decisive answer about the exact orientation of the binding site of cytochrome *P*-450₂. The similarity between $-\Delta G^\circ$ (binding) and $-\Delta G^\circ$ (partition) could indicate that the site is a lipid-faced or a mixed binding site if the transfer of a methylene unit from the apolar lipid membrane environment to the binding site occurs without change in free energy. However, it cannot exclude the possibility that the binding site is actually aqueous-faced, and that the transfer of a methylene unit from the aqueous environment to the cytochrome *P*-450₂ binding site resembles the liposomal partitioning. A conclusive answer to the exact orientation of the binding site will need further investigations.

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APPENDIX

Mathematical analysis of difference spectra

The mathematical formalism is given for analysis of the difference spectra obtained from the binding of a ligand B to a membrane-bound acceptor E occurring in phase i of the biphasic system. The formulae are applicable to ligands which absorb at the wavelength at which the difference spectra are registered. The sample cuvette contains the liposomes with incorporated acceptor molecules. The absorption spectrum of ligand-free acceptor is recorded against a liposomal suspension with identical phospholipid concentration and stored in the instrument's memory. Identical samples of ligand are added to both cuvettes and the difference spectrum is recorded. Afterwards, the absorption spectrum of ligand-free acceptor is subtracted from each difference spectrum. The binding of ligand B to the acceptor E in phase i can be represented by:

K' is the effective dissociation constant (Heirwegh *et al.*, 1988) and $[E]^i$, $[B]^i$ and $[EB]^i$ represent the concentration of free acceptor site, of unbound and bound ligand in phase i respectively. Eqn. (1A) can be rearranged to:

$$[B]^i = K' n_{EB}^i / n_E^i \quad (2A)$$

where n_{EB}^i and n_E^i are the number of moles of occupied and free acceptor sites respectively. The total amount of acceptor sites $n_{E,t}^i$ is given by:

$$n_{E,t}^i = n_{EB}^i + n_E^i \quad (3A)$$

Combining eqns. (2A) and (3A) gives:

$$n_{EB}^i = n_{E,t}^i [B]^i / (K' + [B]^i) \quad (4A)$$

The mass balance on ligand in the reference cuvette (r) is:

$$n_{B,t} = n_{B(r)}^L + n_{B(r)}^A = n_{B(r)}^A (1 + K_p \alpha_L) \quad (5A)$$

where K_p is the membrane partition coefficient and α_L the lipid volume fraction. $n_{B,t}$ is the total amount of ligand, and $n_{B(r)}^L$ and $n_{B(r)}^A$ are the amounts present in the lipid and the aqueous phase respectively. The ligand absorbance in the reference cuvette, $A_{B(r)}$, can be expressed as:

$$A_{B(r)} = (\epsilon_B^L n_{B(r)}^L + \epsilon_B^A n_{B(r)}^A) d / V_T \quad (6A)$$

where d is the path length of the radiation through the cuvette's contents, V_T is the total volume of the liposomal suspension, and ϵ_B^L and ϵ_B^A represent the absorption coefficients of the ligand in the lipid and the aqueous phase respectively. Eqn. (6A) can be rearranged to:

$$A_{B(r)} = n_{B(r)}^A (\epsilon_B^L K_p \alpha_L + \epsilon_B^A) d / V_T \quad (7A)$$

The mass balance on ligand in the sample cuvette (s) is:

$$n_{B,t} = n_{B(s)}^A (1 + K_p \alpha_L) + n_{EB}^i \quad (8A)$$

The absorbance in the sample cuvette in the absence of ligand, $A_{0(s)}$, and in the presence of ligand, $A_{B(s)}$, is given by:

$$A_{0(s)} = \epsilon_E n_{E,t}^i d / V_T \quad (9A)$$

and

$$A_{B(s)} = (\epsilon_B^L n_{B(s)}^A K_p \alpha_L + \epsilon_B^A n_{B(s)}^A + \epsilon_{BE}^i n_{EB}^i + \epsilon_E n_E^i + \epsilon_{EB} n_{EB}^i) d / V_T \quad (10A)$$

where ϵ_{BE}^i is the molar absorption coefficient of acceptor-bound ligand present in phase i, and ϵ_E and ϵ_{EB} represent the absorption coefficient of ligand-free and occupied acceptor respectively. Taking account of eqns. (5A), (7A) and (8A), the difference $\Delta A_B = A_{B(s)} - A_{B(r)}$ between the absorbance in the sample cuvette in the presence of ligand and the reference cuvette becomes:

$$\Delta A_B = \left\{ n_{EB}^i \left[\epsilon_{BE}^i - \left(\frac{\epsilon_B^A + \epsilon_B^L K_p \alpha_L}{1 + K_p \alpha_L} \right) + \epsilon_{EB} \right] + \epsilon_E n_E^i \right\} d / V_T \quad (11A)$$

Subtraction of the acceptor absorbance $A_{0(s)}$ and introduction of eqn. (4A) gives:

$$\Delta A = \Delta A_B - A_{0(s)} = \Delta A_{\max.} [B]^i / (K' + [B]^i) \quad (12A)$$

with

$$\Delta A_{\max.} = n_{E,t}^i \left[\epsilon_{BE}^i - \left(\frac{\epsilon_B^A + \epsilon_B^L K_p \alpha_L}{1 + K_p \alpha_L} \right) + \epsilon_{EB} - \epsilon_E \right] d / V_T \quad (13A)$$

Replacement in eqn. (12A) of $[B]^i$ by the formulae for aqueous (eqn. 5) or lipidic (eqn. 6) ligand concentrations, in either case, yields a rectangular hyperbola. For a ligand that does not absorb light at the wavelengths used for obtaining the absorbance difference, eqn. (13A) reduces to:

$$\Delta A_{\max.} = n_{E,t} (\epsilon_{EB} - \epsilon_E) d / V_T \quad (14A)$$

The absence of the usual type-I peak around 385 nm in 7-n-alkoxycoumarin-induced difference spectra is explained as follows. The experimental procedure corrects for the major fraction of the light absorbed by the ligand. However, modelling of this procedure of necessity leads to a correction term $[\epsilon_{BE}^i - (\epsilon_B^A + \epsilon_B^L K_p \alpha_L) / (1 + K_p \alpha_L)]$ which depends on the wavelength, resulting in a distortion of the difference spectrum. Similarly, no typical type-I peak was present in *p*-nitrotoluene-induced difference spectra of cytochrome *P*-450 LM2, corrected for the proper absorbance of the solute (White & McCarthy, 1986).

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