Enzyme Activity of the Cytochrome P-450 Monooxygenase System in the Presence of Single Chain Lipid Molecules

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The influence of single chain lipids on the 7-ethoxycoumarin O-deethyase activity of the reconstituted binary protein complex of isolated cytochrome P450 and NADPH-cytochrome P450 reductase has been examined. The enzyme activity of this binary enzyme complex has been shown to be influenced by (i) altering the complexation process of both proteins, (ii) by altering the catalytic cycle time of the active binary protein complex and (iii) by altering the fraction of substrate molecules at the catalytic center of the enzyme. Competitive inhibition was measured for all single chain molecules. The following dissociation coefficients of substrate and lipids used for the catalytic center of the protein were obtained: 110 μM 7-ethoxycoumarin (substrate), 1.1 μM MOG (1-monooleoyl-*rac*-glycerol), 0.3 μM SPH (D-sphingosine), 1.5 μM OA (oleic acid), 3.0 μM LPC (L-α-lysophosphatidyl-choline), 15.5 μM MSG (1-monostearoyl-*rac*-glycerol), 9.5 μM AA (arachidonic acid), 9.0 μM PaCar (palmitoyl-L-carnitine), 3.5 μM MPG (2-monopalmitoyl-glycerol), 1.5 μM LPI (L-α-lysophosphatidyl-inositol), 50 μM LA (lauric acid), 60 μM MA (myristic acid), 85 μM PA (palmitic acid), >100 μM SA (stearic acid). Only competitive inhibition with the substrate molecule 7-ethoxycoumarin was observed for the single chain lipids LA, MA, PA, SPH, SA, and OA. Non-competitive effects were observed for MPG ($-0.03\ \mu M^{-1}$), PaCar ($-0.02\ \mu M^{-1}$), MSG ($-0.023\ \mu M^{-1}$), LPC ($-0.03\ \mu M^{-1}$), AA ($-0.03\ \mu M^{-1}$), and MOG ($+0.04\ \mu M^{-1}$). The negative sign indicates that the cycle time of the working binary complex is enlarged. The positive sign indicates that the formation of the binary complex is enhanced by MOG.

Introduction

Double chain lipids are an essential structural element of biological membranes. Here in this paper we are interested how single chain lipids influence the activity of membrane-bound enzymes. Our test system is the microsomal mixed function monooxygenase system consisting of NADPH-cytochrome P450 reductase and a family of cytochrome P450 forms.

We have shown (Müller-Enoch and Gruler, 2000) that the enzyme activity of our test system depends basically on two protein domains of the binary complex: The cytosolic catalytic domain and the membrane-binding domain: (i) The formation of the binary complex depends essentially on the membrane-binding domains and (ii) the catalytic cycle time or turnover number for the catalytic reaction depends on the cytosolic domain.

Double chain phospholipids have been reported to be necessary for optimal catalytic activity of isolated and reconstituted cytochrome P450 enzyme systems (Müller-Enoch *et al.*, 1984; Miwa and Lu, 1984; Taniguchi *et al.*, 1979; Taniguchi and Pyerin, 1988). Both enzymes, the reductase and the P450, are tightly anchored with their binding domains into the lipid bilayer of the endoplasmatic reticulum. The local concentration of the two enzyme species is increased by constraining them into a two-dimensional membrane matrix. Only the formation of the binary enzyme complex leads to a catalyticaly active system (reviewed in Müller-Enoch, 1993). The major effect of double chain phospholipids in P450-based enzyme systems is to facilitate the formation of an active P450:reductase complex. To our knowledge the influence of single chain lipids on the complexation process is unknown.

Double chain phospholipids have been reported to have no influence on the the cycle time or turnover number of the catalytically working enzyme complex (Müller-Enoch and Gruler, 2000; Müller-

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Enoch, 1993). The maximum enzyme activity is reached independent of the phospholipids used when each P450 enzyme is complexed with a reductase and each complex has a substrate molecule. Viner *et al.* (1987) showed that single chain lipids produced by the action of phospholipase A_2 inhibited the enzyme activity of P450 in rat liver microsomes. We will show that the catalytic reaction of reconstituted systems is inhibited by a competitive and a non-competitive process with single chain lipids.

Materials and Methods

Chemicals

7-Ethoxycoumarin and 7-hydroxycoumarin were purchased from EGA-Chemie KG (Steinheim, Germany) and recrystallized from water. L-α-dilauroyl-*sn*-glycerol-3-phosphatitylcholine (DLPC); L-α-lysophosphatidyl-choline; L-α-lysophosphatidic-acid; L-α-lysophosphatidyl-inositol; 1-monooleoyl-*rac*-glycerol; 1-monostearoyl-*rac*-glycerol; 2-monopalmitoyl-glycerol; oleic acid; palmitic acid; stearic acid; arachidonic acid, myristic acid, lauric acid, palmitoyl-L-carnitine; D-sphingosine were purchased from Sigma-Aldrich (Deisenhofen, Germany).

Enzyme preparations

Rat cytochrome P4502B1 (CYP2B1) and NADPH-cytochrome P450 reductase (reductase) were purified to electrophoretic homogeneity using microsomal fractions prepared from phenobarbital-treated male Sprague-Dawley rats as described by Guengerich and Martin (1980). The SDS-PAGE pure rat CYP2B1 and reductase had contents of 20.7 and 18.0 nmol/ml, respectively. The specific activity of the reductase was 24 μmol cytochrome *c* oxidized × min^{-1} × mg $protein^{-1}$. The contents of CYP2B1 and reductase were determined by the methods of Omura and Sato (1964) and Yasukochi and Masters (1976). Protein was determined by the method of Lowry *et al.* (1951).

Reconstitution of CYP2B1:reductase systems; lipid solutions

The procedure utilized was a modification of the method described by Müller-Enoch *et al.* (1984). Phospholipid liposomes were prepared by sonication of 4 mg L-α-dilauroyl-*sn*-glycerol-3-phosphatitylcholine in 1 ml of a 0.1 M potassium HEPES (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]) buffer (pH = 7.6) until the solution became completely clear. Then CYP2B1 and reductase, at a molar ratio of 1:2 were added to give a final molar ratio of phospholipid to protein of 200–300. Following the addition of enzymes the systems were allowed to stand for 2–3 h at 25 °C, before measuring the enzyme activity.

Preformed CYP2B1:reductase complexes were prepared by preincubation of CYP2B1 and the reductase in a molar ratio of 1:2 at concentrations of 5 and 10 μM, respectively, in a tube in a final volume of 30 μl for 2–3 h at 25 °C. Following this preincubation, aliquots of these preformed complexes were used for measuring enzyme activity.

Lysophosphatidylcholine and palmitoyl-L-carnitine were microdispersed by sonification in aqueous buffer (0.1 M potassium HEPES (pH = 7.6)) in a concentration of 0.4 mg/ml or 2 mg/ml, respectively. Lysophosphatidic acid was dissolved in DMSO (4 mg/ml). Al other lipids used were dissolved in ethanol in a concentration range of 0.125–3.0 mg/ml.

Enzyme activity measurements

7-Ethoxycoumarin O-deethylase activity of reconstituted vesicular or soluble CYP2B1:reductase systems were assayed using the continuously fluorometric test described by Ullrich and Weber (1972). The test system contained in a total volume of 600 μl: 0.1 mM 7-ethoxycoumarin; 3.3 mM $MgCl_2$; 0.1 M potassium HEPES buffer (pH = 7.6); 5 μl (31.4 pmol CYP2B1 (= 52.3 nM) and 62.7 pmol reductase) of the reconstituted system and 1–4 μl portions of the dispersed or solved lipids, in a quartz cuvette kept for 3 min at 30 °C constant in a sample holder of the spectrofluorometer. All reactions were started by the addition of 0.17 mM NADPH. The formation of 7-hydroxycoumarin was monitored fluorometrically (λ_E = 365 nm; λ_F = 460 nm) as a function of time. To calibrate each assay 10 μl of a 0.1 mM solution of 7-hydroxycoumarin was added twice at the end of each experiment.

Enzyme kinetics

The enzyme activity, A, is defined as the product formation rate, $d[P]/dt$, divided by the initial P450 concentration, $[P450]_0$.

$$A = \frac{\frac{d[P]}{dt}}{[P450]_0}. \quad (1)$$

The enzyme activity, A, can be determined in an experiment if the production formation rate, $d[P]/dt$, is measured for a given initial P450 concentration, $[P450]_0$.

The production rate, $d[P]/dt$, of a working binary protein complex can be predicted:

$$\frac{d[P]}{dt} = [\text{complex}] \cdot k_E^0 \cdot R(S). \quad (2)$$

The production rate is proportional (i) to the concentration of the working protein complexes (first term on the right side of the equation), (ii) to the catalytic rate of one working protein complex (second term), and (iii) to the fraction, R, of occupation of the catalytic center by the substrate molecule, S, (third term). The predicted enzyme activity is then

$$A = \frac{[\text{complex}]}{P450_0} \cdot k_E^0 \cdot R(S). \quad (3)$$

Formation process of a binary complex

The complexation of the P450 enzyme with the reductase can be expressed very generally by the mass action equation where the two enzymes form a binary complex (P450:reductase) (Müller-Enoch *et al.*, 1984; Müller-Enoch, 1993)

$$[P450] + [\text{reductase}] \overset{K_D}{\longleftrightarrow} [\text{complex}] \quad (4)$$

with the dissociation coefficient, K_D

$$K_D = \frac{[P450] \cdot [\text{reductase}]}{[\text{complex}]}. \quad (5)$$

The fraction of binary complex concentration and of initial P450 concentration is obtained from Eqn. (5) and the protein conservation law ($[P450]_0 = [P450] + [\text{complex}]$ and $[Red]_0 = [Red] + [\text{complex}]$).

$$\frac{[\text{complex}]}{[P450]_0} = \frac{1}{2}\left(\left[1 + \frac{[Red]_0}{[P450]_0} + \frac{K_D}{[P450]_0}\right] - \sqrt{\left[1 + \frac{[Red]_0}{[P450]_0} + \frac{K_D}{[P450]_0}\right]^2 - \frac{4[Red]_0}{[P450]_0}}\right). \quad (6)$$

The dissociation coefficients, K_D, depends on the physical and chemical environment of the membrane-binding domains. Small dissociation coefficients are obtained if the two types of enzymes are anchored in lipid membranes (Experiments from Müller-Enoch *et al.*, 1984) were evaluated by Eqn. (6) (Müller-Enoch and Gruler, 2000): $K_D = (1.4 \pm 0.1) \cdot [P450]_0$ for lipids extracted from liver microsomes ($[P450]_0 = 33$ nM) and $(2.4 \pm 0.2) \cdot [P450]_0$ for DLPC (dilauryl phosphatidyl cholin). Large dissociation coefficients are obtained if the two types of enzymes are dissolved in buffer containing small amounts of glycerol: $K_D = (13 \pm 2) \cdot [P450]_0$ for Tris-buffer and $K_D = (4 \pm 1) \cdot [P450]_0$ for HEPES-buffer. Here, the two types of proteins ($[Red]_0/[P450]_0 = 2$) were dissolved in HEPES-buffer containing a small amount of glycerol. Under such a condition, 30% of the P450 enzyme molecules form working protein complexes.

If a further type of molecule like a single chain lipid, I, is in the reaction volume then the dissociation coefficient, K_D, can be altered and the dissociation coefficient is a function of the concentration of the single chain lipid, $K_D(I)$.

Catalytic rate coefficient of the working enzyme complex

The action of one working enzyme complex can be characterized by its catalytic rate coefficient, k_E^0. It can be obtained under optimal conditions (Müller-Enoch *et al.*, 1984; Gruler and Müller-Enoch, 1991): (i) $[Red]_0 \to \infty$, where every P450-enzyme forms a working binary complex and (ii) $[S] \to \infty$ where every working binary complex is loaded with a substrate molecule. The cycle time, τ, of the cyclic working enzyme complex is given by the inverse of the catalytic rate coefficient ($1/k_E^0 = \tau$). The catalytic reaction rate, k_E^0, for the binary complex formed with rat P450 (CYP2B1) and rat reductase is $40 \pm 2\ \text{min}^{-1}$. This value was obtained for binary complexes in buffer containing glycerol as well as for binary complexes incorporated into membranes composed of two-

chain lipids (Müller-Enoch *et al.*, 1984). The catalytic rate coefficient slightly increases if light is absorbed in the reaction centers of the proteins (Müller-Enoch and Gruler, 1986; Gruler and Müller-Enoch, 1991). The catalytic rate coefficient, k_E^0, could be altered in the case of a non-competitive inhibition by a single chain lipid, I.

Competition for the catalytic reaction center

The enzyme activity is proportional to the fraction of enzyme complexes loaded with substrate molecules.

$$\frac{\frac{[S]}{K_S}}{1+\frac{[S]}{K_S}} \tag{7}$$

with the dissociation coefficient K_S for the substrate S.

If a further type of molecule like a single chain lipid is in the reaction volume then the two types of molecules compete for the catalytic reaction center. The fraction of enzyme complexes loaded with the substrate molecules is

$$\frac{\frac{[S]}{K_S}}{1+\frac{[S]}{K_S}+\frac{[I]}{K_I^{(c)}}} \tag{8}$$

with the dissociation coefficient $K_I^{(c)}$ of the further type of molecule (*c* stands for competitive inhibition).

Modified Michaelis-Menten Equation

Summing up: The enzyme activity (Eqn. (3)) has now the following form

$$A = K(I)^{(nc)} \cdot \frac{\frac{[S]}{K_S}}{1+\frac{[S]}{K_S}+\frac{[I]}{K_I^{(c)}}} \tag{9}$$

with the following abbreviation

$$K(I)^{(nc)} = \frac{k_E^0(I)}{2}\left(\left[1+\frac{[Red]_0}{[P450]_0}+\frac{K_D(I)}{[P450]_0}\right] - \sqrt{\left[1+\frac{[Red]_0}{[P450]_0}+\frac{K_D(I)}{[P450]_0}\right]^2 - \frac{4[Red]_0}{[P450]_0}}\right). \tag{10}$$

The influence of the additional type of molecule can be factorized as shown in Eqn. (9): (i) in a non-competitive effect (nc) (first term on the right side) and (ii) a competitive effect (c) (second term). For the competitive effect one has an analytic expression and hence there is no problem to compare the measured data with the theoretical prediction. But in case of the non-competitive effect one has the coefficient, $K(I)^{(nc)}$, but not its concentration dependence. To proceed further one needs an analytic expression to compare the measured data with the theoretical prediction.

The non-competitive effect can be expressed by a Taylor series up to the first non-trivial term

$$K(I)^{(nc)} = K(0)\cdot(1+a_1^{(nc)}\cdot[I]+\cdots)\cdot \tag{11}$$

The value, K(0), can be obtained by measuring the enzyme activity, A, at different substrate concentration but without the additional type of molecule: $K(0) = A_{max}$. The non-competitive effect is characterized by the coefficient $a_1^{(nc)}$. As described above the non-competitive effect has two sources and, therefore, $a_1^{(nc)}$ can be expressed as

$$a_1^{(nc)} = \alpha_{rate} + \alpha_{complex}. \tag{12}$$

The disturbance of the catalytic cycle by the additional type of molecule is described by α_{rate}. The catalytic cycle time is presumably enlarged and in this case α_{rate} is negative. The influence of the additional type of molecule on the complex formation is described by $\alpha_{complex}$. The additional type of molecules like the one-chain lipids presumably favor the membrane binding domain and in this case $\alpha_{complex}$ is positive. This consideration shows that the coefficient $a_1^{(nc)}$ for the complete non-competitive effect can be positive where the enzyme activity is enhanced or negative where it is inhibited.

The following measurements were performed to quantify the effect of a second type of molecule on the enzyme kinetics:

(i) The enzyme activity was measured for different substrate concentrations but at fixed concentration of the second type of molecule I ([S] = variable and [I] = constant). The inverse of the enzyme activity, A^{-1}, is plotted versus the inverse of the substrate concentration, S^{-1}. A straight line is predicted from Eqn. (9)

$$\frac{1}{A} = \frac{1}{K(\mathrm{I})^{(nc)}} + \frac{1}{[\mathrm{S}]} \cdot \frac{K_\mathrm{m}}{K(\mathrm{I})^{(nc)}} \tag{13}$$

with the Michaelis-Menten coefficient

$$K_\mathrm{m} = K_\mathrm{S}\left(1 + \frac{[\mathrm{I}]}{K_\mathrm{I}^{(c)}}\right). \tag{14}$$

The Michaelis-Menten coefficient, K_m, is a function of the dissociation constant K_S of the substrate S and of the dissociation constant, $K_\mathrm{I}^{(c)}$, of the second type of molecule I with the catalytic reaction center of the protein P450. The maximum enzyme activity

$$A_\mathrm{max} = K(\mathrm{I})^{(nc)} \tag{15}$$

is constant in this type of experiment since the concentration of the second type of molecule I is kept constant. The half-maximum enzyme activity is achieve if the substrate concentration equals the K_m-value.

From the measurement without the additional type of molecule ([I] = 0) one obtains the maximum enzyme activity A_max (=$K(0)$) and the dissociation coefficient, K_S, for the substrate molecule S by fitting Eqn. (13) to the experimentally determined points. In a further experiment, the measurement were repeated with a fixed concentration of the second type of molecule. The dissociation constant, $K_\mathrm{I}^{(c)}$, is obtained by fitting Eqn. (13) to the experimentally determined points. The competitive effect of the second type of molecule on the enzyme kinetics is quantified by this coefficient $K_\mathrm{I}^{(c)}$.

(ii) In the second type of experiment, the enzyme activity was measured for different concentrations [I] of the second type of molecule but at fixed substrate concentration [S] ([I] = variable and [S] = constant). The inverse of the enzyme activity, A^{-1}, is plotted versus the concentration of the second type of molecule, [I]. One obtains from Eqn. (9).

$$\frac{1}{A} = \frac{1}{K(\mathrm{I})^{(nc)}}\left(1 + \frac{K_\mathrm{S}}{[\mathrm{S}]} + \frac{K_\mathrm{S}}{[\mathrm{S}]} \cdot \frac{[\mathrm{I}]}{K_\mathrm{I}^{(c)}}\right). \tag{16}$$

The unknown function $\mathrm{K(I)}^{(nc)}$ is approximated by a linear function (Eqn. (9)–(11))

$$\frac{1}{A} = \frac{1}{K(0)} \cdot \frac{1 + \frac{K_\mathrm{S}}{[\mathrm{S}]} + \frac{K_\mathrm{S}}{[\mathrm{S}]} \cdot \frac{[\mathrm{I}]}{K_\mathrm{I}^{(c)}}}{1 + a_\mathrm{I}^{(nc)} \cdot [\mathrm{I}]} \tag{17}$$

The coefficient $a_\mathrm{I}^{(nc)}$ is obtained by fitting Eqn. (17) to the experimentally determined points. The non-competitive effect of the second type of molecule on the enzyme kinetics is quantified by this coefficient.

Results and Discussion

Viner *et al.* (1987) reported that the 7-ethoxycoumarin 0-deethylation is inhibited by the products of the hydrolysis of phospholipids by phospholipase A_2. They found that lysolecithins but not oleic acid inhibits the P450-dependent enzyme activity. They used in their studies liver microsomes from phenobarbital-induced rats. In our study we used isolated cytochrome P450 and reductase in a reconstituted system to investigate the influence of single chain lipids on the 7-ethoxycoumarin 0-deethylase activity. This well defined system made it possible to examine the inhibition in details. We can distinguish between competitive and non-competitive inhibition. The chemical structure of the single chain lipids are shown in Fig. 1.

First, we measured the enzyme activity of the reconstituted system for the substrate 7-ethoxycoumarin with and without an inhibitor. A typical result is shown for monooleoyl-glycerol in Fig. 2a where the inverse enzyme activity is plotted versus the inverse substrate concentration. In case without an inhibitor (full square) the experimental dots are approximated by a straight line as predicted by Eqn. (13). The maximum enzyme activity, A_max, (= $K(0)$= const.) is determined from the ordinate section, A_max = 14.5 min^{-1}. The dissociation coefficient, K_S, for the substrate 7-ethoxycoumarin is determined from the slope, K_S = 110, μM. The substrate concentration at half-maximum enzyme activity yields the K_m-value. In case without inhibitor K_m=K_S.

In a second experiment, the enzyme activity was measured with a fixed concentration of monooleoyl-glycerol for different substrate concentrations 7-ethoxycoumarin (Fig. 2a). The results are shown in the same figure. The experimental dots (full circle) are approximated by a straight line as predicted by Eqn. (13) with $K(\mathrm{I})^{(c)}$=A_max= const.. The maximum enzyme activity determined from the ordinate section is the same as without monooleoyl-glycerol (A_max = 14.5 min^{-1}). This indicates a competitive inhibition at low concentration of monooleoyl-glycerol. The dissociation coefficient, $K_\mathrm{I}^{(c)}$, for monooleoyl-glycerol can be determined

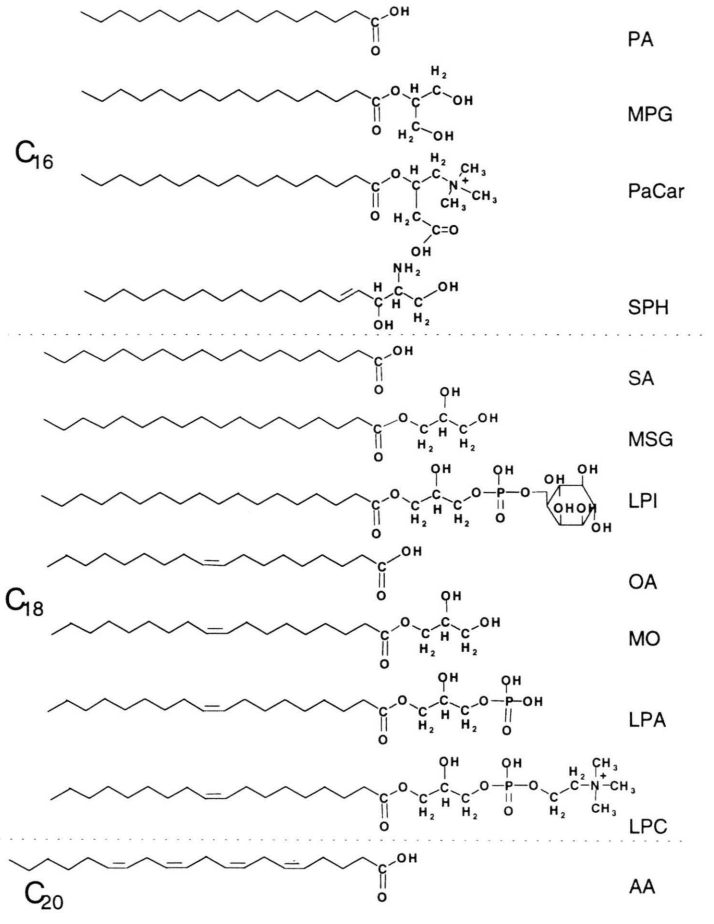

Fig. 1. The chemical structure of most of the used single chain lipids.

from the slope of the straight line, $K_I^{(c)}$= 1.1 μM. The apparent K_m-value, K_m^{app} (measured as [S] required for $A_{max}/2$) will increase in the presence of a competitive inhibitor ($K_m^{app}=K_S(1+[I]/K_I^{(c)})$). In a third experiment the enzyme activity was measured with a fixed substrate concentration 7-ethoxycoumarin for different concentrations of monooleoyl-glycerol. The results are shown in Fig. 2b. A straight line is predicted in the case of non-competitive inhibition (Eqn. (17) with $a_1^{(nc)} = 0$). The straight dashed line is calculated by using the values (A_{max} = 14.5 min^{-1}, K_S = 110 μM), and $K_I^{(c)}$= 1.1 μM) which were obtained from Fig. 2a. The experimentally determined dots deviate from the straight line behavior. This deviation is used to quantify the non-competitive inhibition coefficient, $a_I^{(nc)}$, of monooleoyl-glycerol by fitting Eqn. (17) to the experimental data. The fit yields $a_I^{(nc)}$ = +0.04 μM^{-1}. The positive sign means that the maximum enzyme activity, A_{max}, (=$K(I)^{(nc)}$), increases with increasing concentration of monooleoyl-glycerol.

In Fig. 3 the results of monostearoyl-glycerol are shown. The same values were obtained for the enzyme activity and the substrate binding as for monooleoyl-glycerol (A_{max} = 14.5 min^{-1} and K_S = 110 μM). The dissociation coefficient, $K_I^{(c)}$, for monostearoyl-glycerol is determined to $K_I^{(c)}$= 15.5 μM. The non-competitive inhibition coefficient is negative, $a_I^{(nc)}$ = −0.023 μM^{-1}. The negative sign denotes that the maximum enzyme activity, A_{max} (= $K(I)^{(nc)}$), decreases with increasing concentration of monostearoyl-glycerol.

The results for all single chain lipids used as inhibitors for the 7-ethoxycoumarin O-deethylase activities are summarized in Table I.

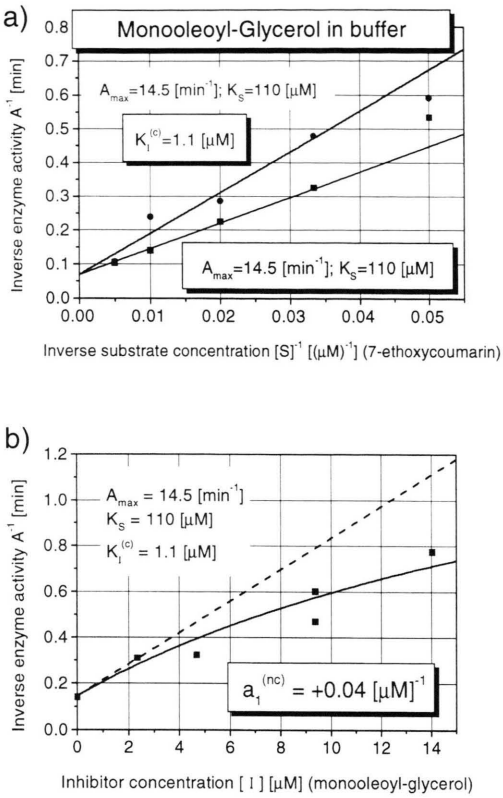

Fig. 2. The 7-ethoxycoumarin O-deethylase activity of cytochrome P450:reductase complexes dissolved in buffer is shown with and without monooleyl-glycerol. a) The inverse enzyme activity, A^{-1}, is plotted versus inverse substrate concentration, $[S]^{-1}$: (i) Full squares (only substrate) and (ii) full circles (substrate and [I] = 0.58 μM monooleyl-glycerol). The straight lines are theoretical predictions (Eqn. (17)): A_{max} = 14.5 min^{-1}, K_S = 110 μM (only substrate) and A_{max} = 14.5 min^{-1}, K_S = 110 μM, $K_I^{(c)}$ = 1.1 μM (substrate and monooleyl-glycerol). b) The inverse enzyme activity, A^{-1}, is plotted versus the monooleyl-glycerol concentration [I]. The straight dashed line is a prediction with the values obtained from Fig. a (A_{max} = 14.5 min^{-1}, K_S = 110 μM), and $K_I^{(c)}$= 1.1 μM). The bent line is fitted (Eqn. (17)) to the experimental data where the only fitting parameter is the coefficient $a_1^{(nc)}$ for the non-competitive effect $a_1^{(nc)}$ = +0.04 μM^{-1}.

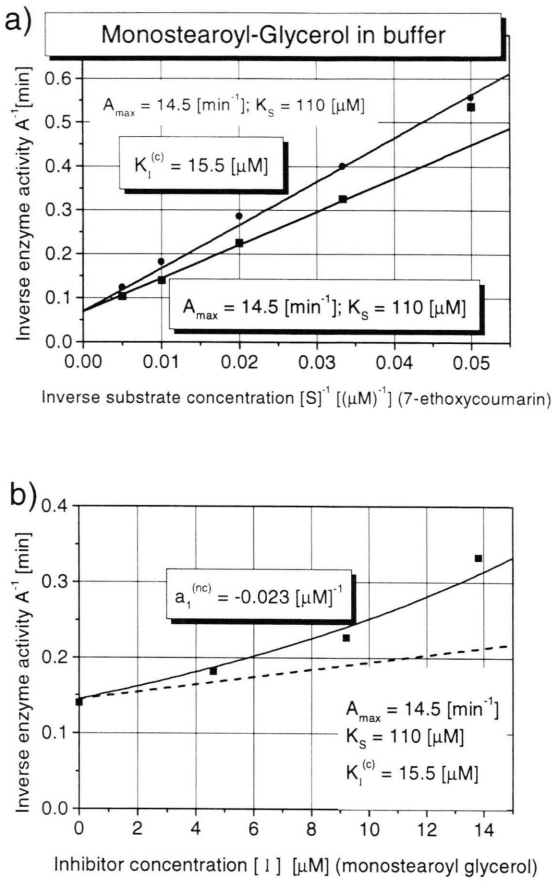

Fig. 3. The 7-ethoxycoumarin O-deethylase activity of human cytochrome P450:reductase complexes is shown with and without monostearoyl-glycerol. a) The inverse enzyme activity, A^{-1}, is plotted versus inverse substrate concentration, $[S]^{-1}$: full squares (only substrate) and ful circles ([I] = 4.64 μM monostearoyl-glycerol). The straight lines are theoretical predictions (Eqn. (13)): A_{max} = 14.5 min^{-1}, K_S = 110 μM (only substrate) and A_{max} = 14.5 min^{-1}, K_S = 110 μM, $K_I^{(c)}$ = 15.5 μM (substrate and monostearoyl-glycerol). b) The inverse enzyme activity, A^{-1}, is plotted versus the monostearoyl-glycerol concentration [I]. The straight dashed line is a prediction with the values obtained from Fig. a (A_{max} = 14.5 min^{-1}, K_S = 110 μM, and $K_I^{(c)}$= 15.5 μM). The bent line is fitted (Eqn. (17)) to the experimental data where the only fitting parameter is the coefficient $a_1^{(nc)}$ for the non-competitive effect $a_1^{(nc)}$ = −0.023 μM^{-1}.

The effect of the single chain lipids used on the maximal enzyme activity, A_{max} and the dissociation coefficient, K_S, of the enzyme-substrate complex, [ES], are 14.5 min^{-1} and 110 μM, respectively. One exception is for the vesicular reconstituted system where the P4502B1 and the reductase are incorporated into the DLPC vesicles. In this case the maximal enzyme activity is increased to 33 min^{-1} and the dissociation, K_S, of the enzyme-substrate complex to 200 μM.

The competitive inhibition of the single chain lipids exhibit a strong structure-action relationship. The competitive inhibition is quantified by the dissociation coefficient, $K_I^{(c)}$, between the in-

Table I: Influence of single chain lipids on the 7-ethoxycoumarin O-deethylase activity of the reconstituted CYP2B1:NADPH-P450 reductase system.

Single chain lipid	A_{max} [min^{-1}]	K_S [μM]	$K_I^{(c)}$ [μM]	$a_I^{(nc)}$ [μM^{-1}]
Lauric acid (LA)	10*	110	50	0
Myristic acid (MA)	10*	110	60	0
Palmitic acid (PA)	14.5	110	85	0
2-Monopalmitoyl-glycerol (MPG)	14.5	110	3.5	−0.03
Palmitoyl-L-carnitine (PaCar)	14.5	110	9	−0.02
D-Sphingosine (SPH)	14.5	110	0.3	0
Stearic acid (SA)	14.5	110	> 100	0
1-Monostearoyl-*rac*-glycerol (MSG)	14.5	110	15.5	−0.023
L-α-Lysophosphatidyl-inositol (LPI)	14.5	110	1.5	–
Oleic acid (OA)	14.5	110	1.5	0
1-Monooleoyl-*rac*-glycerol (MOG)	14.5	110	1.1	+0.04
L-α-Lysophosphatidic acid (LPA) in DMSO	14.5	110	(270)	0
L-α-Lysophosphatidyl-choline (LPC) in buffer	13	120	3.0	−0.03
L-α-Lysophosphatidyl-choline (LPC) in DLPC** vesicles	33	200	3.0	−0.11
Arachidonic acid (AA)	14.5	110	9.5	−0.03

* A different P4502B1 enzyme preparation with a lower specific activity was used.
** L-α-dilauroyl-*sn*-glycerol-3-phosphatitylcholine (DLPC).

hibitor molecule and the enzyme complex. The lower the $K_I^{(c)}$-value the greater is the degree of inhibition.

(i) The highest $K_I^{(c)}$-values were found for the saturated free fatty acids. The gradual difference depends on their chain lengths: 50 μM for C_{12}, 60 μM for C_{14}, 85 μM for C_{16}, and a very large $K_I^{(c)}$ > 100 μM for C_{18}.

(ii) The unsaturated fatty acids have a higher degree of inhibition compared with the saturated fatty acids with the same chain length: 1.5 μM for oleic acid (1:18) and a very large $K_I^{(c)}$ > 100 μM for stearic acid (0:18).

(iii) The head groups of the single chain lipid esters play an import role on the degree of inhibition. In the case of the same chain length of the monoacyl esters we found: (a) for C(0:16): 85 μM for palmitic acid; 3.5 μM for monopalmitoyl-glycerol; and 9 μM for palmitoyl-carnitine; (b) for C(0:18): a very large $K_I^{(c)}$> 100 μM for stearic acid and 15.5 μM for monostearyl-glycerol; (c) for C(1:16): 1.5 μM for oleic acid and 1.1 μM for monooleoyl-glycerol, and 3.0 μM for lysophosphatidyl-choline.

(iv) In the case of lysophosphatidyl-choline inhibition we found no difference in the $K_I^{(c)}$-value whether we used the soluble or the vesicular reconstituted system.

The last point is the non-competitive inhibition of the single chain lipids on the 7-ethoxycoumarin O-deethylase. It is quantified by the coefficient $a_I^{(nc)}$. Only a competitive inhibition was found ($a_I^{(nc)}$ = 0) for (i) the free fatty acids (lauric acid, myristic acid, palmitic acid, stearic acid and oleic

acid) and (ii) D-sphingosine and L-α-lysophosphatitic acid. The non-competitive inhibition can have a negative or positive effect. In case of a negative sign the maximal enzyme activity decreases at high inhibitor concentrations. This effect was found for 2-monopalmitoyl-glycerol, palmitoyl carnitine, 1-monostearoyl-*rac*-glycerol, L-α-lysophosphatidylcholine and arachidonic acid. In case of a positive sign the maximal enzyme activity increases at high inhibitor concentration. This effect was only observed for 1-monooleoyl-*rac*-glycerol.

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Gruler H. and Müller-Enoch D. (1991), Slaving the cytochrome P450 dependent monooxygenase system by periodically applied light pulses. Eur. Biophys. J. **19**, 217–219.

Guengerich F. P. and Martin M. V. (1980), Purification of cytochrome P450, NADPH-cytochrome P450 reductase, and epoxide hydratase from a single preparation of rat liver microsomes. Arch. Biochem. Biophys. **205** 365 –379

Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951), Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**, 265 –275.

Miwa G. T. and Lu A. Y. H. (1984), The association of cytochrome P450 and NADPH-cytochrome P450 reductase in phospholipid membranes. Arch. Biochem. Biophys. **234**, 161–166.

Müller-Enoch D., Churchil P., Fleischer S., and Guengerich F. P. (1984), Interaction of liver microsomal cytochrome P450 and NADPH-cytochrome P450 reductase in the presence and absence of lipid. J. Biol. Chem. **259**, 8174 –8182.

Müller-Enoch D. and Gruler H. (1986), The activation of the cytochrome P450-dependent monooxygenase system by light. Z. Naturforsch. **41c**, 604 –612

Müller-Enoch D. (1993), Localization of cytochrome P450 in membranes: Reconstituted systems. In: Handbook of Exp. Pharm., Vol. 105, Cytochrome P450. (J. B. Schenkman, H. Greim, eds.). Springer Publ., Berlin, pp. 71–83.

Müller-Enoch D. and Gruler H. (2000), Complexation of membrane-bound enzyme systems. Z. Naturforsch. **55c**, 747–752

Omura T. and Sato R., (1964), The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. **239** 2370–2378

Taniguchi H., Imai Y., Iyanagi T., and Sato R. (1979), Interaction between NADPH-cytochrome P450 reductase and cytochrome P450 in the membrane of phosphatidylcholine vesicles. Biochim. Biophys. Acta **550**, 341–356.

Taniguchi H. and Pyerin W. (1988), Phospholipid bilayer membranes play decisive roles in the cytochrome P450-dependent monooxygenase system. J. Cancer Res. Clin. Oncol. **114**, 335–340

Ullrich V. and Weber P. (1972), The O-dealkylation of 7-ethoxycoumarin by liver microsomes. A direct fluorometric test. Hoppe-Seylers Z. Physiol. Chem. **353**, 1171–1177

Viner R. I., Novikov K. N., Kozlov Yu.P., and Kagan V. E. (1987) Inhibition of the dealkylating activity of isoforms of cytochrome P450 in rat liver microsomes by products of the hydrolysis of phospholipids by phospholipase A_2. Biokhimiya **52**: 459–468

Yasukochi Y. and Masters B. S. S., (1976), Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P450) reductase purified by biospecific affinity chromatography. J. Biol. Chem. **251** 5337–5344.