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MEMBRANE EFFECTS ON DRUG MONOOXYGENATION ACTIVITY IN HEPATIC MICROSOMES

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SUMMARY

The temperature dependence of drug monooxygenation in phenobarbital-induced rat liver microsomes has been investigated. With 7-ethoxycoumarin as a substrate the activity of the microsomes could be measured down to 0 °C by the increase in fluorescence of the dealkylated reaction product 7-hydroxycoumarin (umbelliferone).

Arrhenius plots of the activities at various temperatures between 0 °C and 45 °C showed a break in the activation energy around 20 °C.

Addition of deoxycholate or high concentrations of glycerol, known to solubilize membrane-bound enzymes, abolished the break of the activation energy. Cholesterol, incorporated into the microsomal membrane in amounts equimolar to the microsomal phospholipid content led to a decrease of the activation energy at low temperatures and to an increase at higher temperatures, resulting in a loss of the break.

The activity of microsomal NADPH-cytochrome *c* reductase with the water-soluble electron acceptor dichlorophenolindophenol showed no discontinuity in the Arrhenius plot. In addition the cumene hydroperoxide-mediated and cytochrome *P*-450-dependent O-dealkylation of 7-ethoxycoumarin proceeded without a break in the activation energy.

It is concluded that phospholipid phase transitions affect the electron transfer from the reductase to cytochrome *P*-450.

INTRODUCTION

The activity of membrane-bound enzymes may be affected in various ways by the lipid environment, one of which is a change of the physical state of the lipid phase [1, 2]. In biological membranes, such a transition from the gel to the liquid-crystalline

Abbreviation: TEMPO, tetramethylpiperidine nitroxide.

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state [3, 4] may lower the activation energies of enzymatic reactions of membrane-bound enzymes above the critical transition temperature [5], provided the phospholipid-protein interaction affects a rate-determining step in the reaction mechanism.

The membranes of the hepatic endoplasmic reticulum contain a high proportion of enzymes associated with electron transport and the oxidative metabolism of drugs [6]. This microsomal monooxygenation system consists of a reductase and cytochrome *P*-450 [7, 8], which in the presence of NADPH and molecular oxygen catalyse the introduction of an oxygen atom into a variety of lipophilic organic compounds. Constant activation energies of various cytochrome *P*-450-catalysed drug monooxygenations have been reported in the range from 16 to 40 °C [9, 10]. Lower temperatures were not measured, mainly because of the insensitivity of the tests.

In contrast, a break in octadecane hydroxylation at 25 °C has been reported by Annayev et al. [11].

Recently we have described a highly sensitive assay for microsomal monooxygenation activity based on the O-dealkylation of 7-ethoxycoumarin and the direct measurement of umbelliferone fluorescence [12]. Using this test we measured the O-dealkylation activity of liver microsomes in the range from 0 to 45 °C and determined the activation energies in an Arrhenius plot after various treatments of the microsomal membrane.

MATERIALS AND METHODS

Chemicals

7-Ethoxycoumarin was prepared according to a published procedure [12]. NADPH was purchased from Boehringer Mannheim (Mannheim, Germany), *p*-nitroanisole, 2,6-dichlorophenolindophenol, glycerol, deoxycholate and cholesterol from Merck (Darmstadt, Germany) and 7-hydroxycoumarin (umbelliferone) from EGA-Chemie (Steinheim, Germany). All other reagents were of highest purity commercially available.

Microsomes

Male Sprague-Dawley rats weighing 100–130 g were pretreated by intraperitoneal injection of phenobarbital (80 mg/kg) once daily for 3 days and liver microsomes were prepared as described previously [13].

Enzyme assays

O-dealkylation activities for 7-ethoxycoumarin as substrate were determined in 1 ml assays by the fluorescence method, as described by Ullrich and Weber [12]. To calibrate the fluorescence response at different temperatures and turbidities, a known amount of umbelliferone (7-hydroxycoumarin) was added about 2 min after the start of the enzymatic reaction. The individual activities at the certain temperatures were reproducible with an accuracy of better than 5 %. The specific activity of 7-ethoxycoumarin dealkylation, obtained after phenobarbital pretreatment, was 2.0–2.5 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of microsomal protein at 25 °C. The K_m of 7-ethoxycoumarin in phenobarbital-induced microsomes is $2.7 \cdot 10^{-5} \pm 0.5 \cdot 10^{-5}$ M [13]. A second binding constant with decreased affinity has been found ($K_m = 1.1 \cdot 10^{-4} \pm 0.3 \cdot 10^{-4}$ M). Both affinity constants are unaffected by the various treatments

reported in this paper. When cumene hydroperoxide was used in the dealkylation assay, NADPH was omitted and the reaction was started by the addition of 1 μl of cumene hydroperoxide (1 M in methanol) to 1 ml of the assay mixture. In control experiments with NADPH this concentration of methanol did not affect the monooxygenation reaction.

The temperature dependence of the de-ethylation activity was measured from 0 °C up to about 45 °C in intervals of 2–5 °C. The photometer was equipped with thermostated cuvette holders connected to a cooling thermostat or a waterbath for low or high temperatures, respectively.

The actual temperature during the reaction was measured in the cuvette with a precision thermistor (Knauer, Berlin). Its resistance was displayed by a digital voltmeter and used to calculate the temperature by means of a calibration curve.

Incorporation of cholesterol into the microsomal membranes was achieved by addition of a suspension of cholesterol, sonicated for 10 min in Tris buffer (pH 7.6) to microsomes and subsequent stirring for 20 min at 30 °C under an atmosphere of nitrogen. The cholesterol concentration in the experiment described was chosen equimolar to the phospholipid content, which was calculated on the assumption of a phospholipid/protein ratio of 0.4 [14].

The O-demethylation of *p*-nitroanisole was measured in an Eppendorf photometer with a 405 nm filter, as described by Netter et al. [15].

The cytochrome *P*-450 content in microsomes was determined in an Aminco DW-2 dual-wavelength spectrophotometer (Aminco, Silversprings, Md., USA) in cuvettes with 1 cm light path. An extinction coefficient of 91 $cm^{-1} \cdot mM^{-1}$ [16] was used to calculate the cytochrome *P*-450 concentration for the extinction difference at 450–490 nm.

NADPH-cytochrome *c* reductase activity was measured with dichlorophenolindophenol as electron acceptor according to Omura et al. [17].

Protein was determined by the biuret method as described by Gornall et al. [18] with bovine serum albumin as a standard.

RESULTS

The monooxygenation activity of phenobarbital-induced rat liver microsomes was measured with 7-ethoxycoumarin as a substrate by following the increase in fluorescence of the dealkylation product, umbelliferone (7-hydroxycoumarin), at various temperatures from 0 to 45 °C. Because of the sensitivity of the assay, the activity could be measured down to temperatures near freezing point. When the results were plotted according to Arrhenius a break in the slope was observed (Fig. 1).

The temperature range of the break and the activation energies obtained from these plots were reproducible within one microsomal preparation, but varied from one batch to the other. The average value for the temperature of the break was 21.3 ± 5.1 °C ($\pm$S.E.M., $n = 15$), while values for the activation energies of 18.5 ± 4.8 kcal/mol and 8.4 ± 2.7 kcal/mol were obtained below and above the break, respectively. Because of the variations in different microsomal preparations we shall show the results of a typical experiment in the following figures together with control assays, which were always performed with the same microsomal suspension.

Monooxygenase activities were measured up to temperatures at which thermal

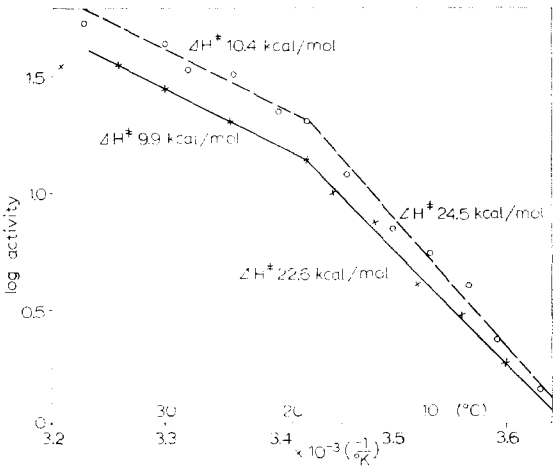

Fig. 1. Arrhenius plots of microsomal 7-ethoxycoumarin and *p*-nitroanisole dealkylation reactions. The 7-ethoxycoumarin assay contained in 1 ml 0.1 M Tris buffer (pH 7.6), 10^{-3} M 7-ethoxycoumarin, $5 \cdot 10^{-4}$ M NADPH and 1.0 mg of microsomal protein. In the *p*-nitroanisole assay 2 ml of 0.1 M Tris buffer (pH 7.6) contained $5 \cdot 10^{-4}$ M *p*-nitroanisole, $2.5 \cdot 10^{-4}$ M NADPH and 1.4 mg of protein. The reactions were started by the addition of NADPH. From the increase in fluorescence above 430 nm (×) and the initial increase in absorption at 405 nm (○), respectively, specific activities were calculated by means of calibration values and plotted in an arbitrary activity scale (as in the following figures). The activation energies are calculated from the slope of the lines in kcal/mol.

inactivation of the enzyme started. A decrease in activity could usually be observed above 40 °C.

Studies on the temperature-dependence of the O-demethylation of *p*-nitroanisole resulted in almost identical values of the break temperature, as well as of the activation energy above and below the transition (Fig. 1).

Glycerol is known to solubilize and stabilize membrane-bound enzymes [19]. When it was added to the assay mixture in higher concentrations, the turbidity of the

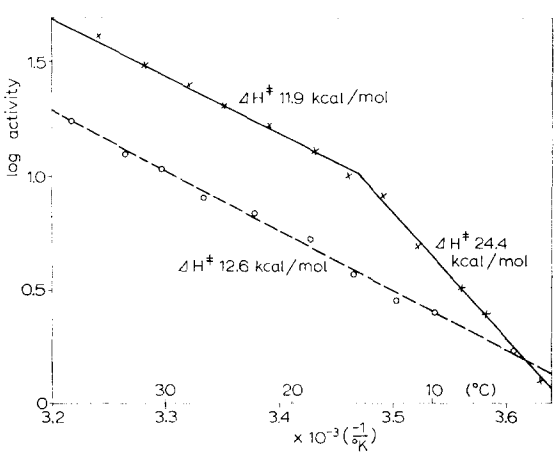

Fig. 2. Arrhenius plots of 7-ethoxycoumarin dealkylation in the absence (×) and presence (○) of 30 % glycerol. Assays as described in Fig. 1, but with 1.2 mg of microsomal protein.

solution decreased and a pronounced change in the Arrhenius plot could be observed. At a concentration of 30 % glycerol, the break in the Arrhenius plot could no longer be observed and over the whole temperature range a constant activation energy was measured, which came close to the value usually found above the break in control experiments (Fig. 2).

The same phenomena could be observed when deoxycholate was added to the reaction mixture. At a concentration of 1.5 mM (0.062 %), which partially inhibited the activity, the break of the activation energy in an Arrhenius plot disappeared (data not shown). Again the value of the activation energy is about that of control experiments above the break point. Rapid inactivation of the enzyme at higher temperatures was observed after this treatment. A cytochrome *P*-450 system solubilized according to Lu et al. [8] by means of deoxycholate and glycerol also exhibited a straight line in an Arrhenius plot (data not shown).

Cholesterol can be incorporated into the microsomal membrane in amounts equimolar to the phospholipids, when incubated as a sonicated suspension with microsomes above the transition point. This has been reported in other membrane systems [20, 21] and can be controlled by the disappearance of the additional cholesterol-induced turbidity and a subsequent cholesterol analysis of the microsomal pellet. A continuous activation energy is found after this treatment throughout the whole temperature range, with an intermediate value between the two activation energies found in the control experiment, which had also been preincubated at 30 °C for 20 min.

Since the microsomal monooxygenation cycle involves the activity of two enzymes, the NADPH-dependent flavoprotein and cytochrome *P*-450, both activities were tested separately with regard to their temperature dependence. In the case of the reductase, the water-soluble dye, dichlorophenolindophenol, replaced the natural

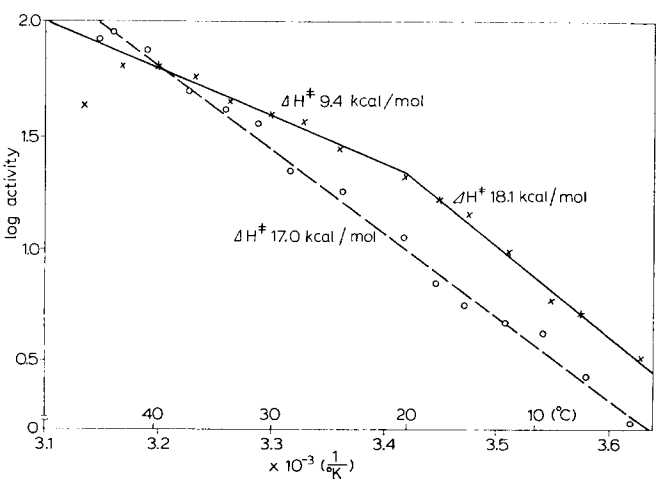

Fig. 3. Arrhenius plots of 7-ethoxycoumarin dealkylation in the presence of NADPH (×) and cumene hydroperoxide (1 mM) (○). The assays with NADPH as electron don orwere performed as described in Fig. 1, but with 0.9 mg of microsomal protein. The same amount of protein was used, but NADPH was omitted, when the reactions were started by the addition of 1 µl of cumene hydroperoxide in methanol (1 M).

acceptor cytochrome *P*-450. As a result the reductase activity showed a constant activation energy of 12–14 kcal/mol over the whole temperature range.

A possibility to test the catalytic activity of cytochrome *P*-450 in the absence of NADPH and the reductase was opened up recently by the experiments of Kadlubar et al. [22] and Rahimtula and O'Brien [23]. These authors presented evidence that the formation of the active oxygen at cytochrome *P*-450 can also be mediated by reacting cumene hydroperoxide with the oxidized cytochrome. We could confirm that the O-dealkylation of 7-ethoxycoumarin was also initiated in the presence of cumene hydroperoxide at an optimum concentration $5 \cdot 10^{-4}$–10^{-3} M. The response to the inhibitor metyrapone was also very similar to the NADPH-supported O-dealkylation. The results on the activation energy for the cumene hydroperoxide catalysed O-dealkylation are shown in Fig. 3. The activity with the hydroperoxide was slightly lower at room temperature, but no break occurred in the Arrhenius plot compared to the control with NADPH and oxygen. The numerical value of the activation energy was about 17 kcal/mol and thus resembled that of controls below the transition point.

DISCUSSION

Phase transitions from the gel to the liquid-crystalline phase have been shown to change the permeability [24], fluidity [25, 26] and enzymatic activity [27–31] of membrane-bound enzymes, as well as the properties of various optical [32, 33], fluorimetric [27, 34] and magnetic probes [32, 35–37]. The temperature of the phase transition is dependent mainly on the fatty acid composition of the phospholipids and the cholesterol content of the membrane [24, 38].

Our studies with the microsomal membrane-bound cytochrome *P*-450-dependent dealkylation reaction of rat liver showed a break of the activation energy in Arrhenius plots. Since this break is found not only with the substrate 7-ethoxycoumarin, but also with *p*-nitroanisole at the same temperature, it can be excluded that specific enzyme substrate interactions cause this change in the activation energy. The membrane-disintegrating agents glycerol and deoxycholate eliminate the break. The activation energies observed after these treatments indicate that the enzyme system exhibits properties usually observed above the phase transition. The intermediate activation energy observed after cholesterol incorporation indicates a more rigid behaviour above and a more fluid behaviour below the phase transition. This corresponds to the effect of cholesterol on other membrane parameters described by Chapman and coworkers [38, 39]. Therefore, the experiments described in this paper indicate that the change in activation energy is due to a phase transition of membrane phospholipids.

This assumption is supported by the reports of Annayev et al. [11] on the change of the rotatory diffusion parameter of a fatty acid amide spin label with a concomitant break in the activation energy of octadecane hydroxylation. Other laboratories also reported breaks in the temperature dependence of spin label parameters in hepatic microsomes [40–42], in contrast to results found by Stier and Sackmann [43] and in our laboratory [44]. We did not find a break in the order parameters of lipophilic spin labels in the very heterogeneous microsomal membrane; we did find, however, a discontinuity in the partitioning of the TEMPO (tetra-

methylpiperidine nitroxide) spin label at a certain temperature, which corresponded to the break observed in our O-dealkylation experiments (Ruf and Duppel [44], manuscript in preparation).

Although there is little doubt about a phospholipid phase transition as a cause for the break in the activation energy, it is more difficult to localize the process which is affected by this transition in the reaction cycle. According to our experiments neither the membrane-bound reductase nor cytochrome *P*-450 show discontinuities in their activities, when tested separately. Therefore, a direct regulatory effect of the microsomal membrane on the kinetic properties of the enzyme-substrate interaction, as has been described recently for UDPglucuronyltransferase [45], can be excluded from our experiments with cumene hydroperoxide and dichlorophenolindophenol for cytochrome *P*-450 and the reductase, respectively. It seems likely, therefore, that the lateral diffusion process in the membrane of the reductase and the cytochrome *P*-450 is affected by the phase transition.

This would be in accord with the proposed electron transfer to cytochrome *P*-450 as the rate-limiting step of the monooxygenation cycle [46, 47]. Cytochrome *P*-450 is closely associated with certain phospholipids which have a stimulating effect on its activity. Phosphatidylcholine is required for maximum activity in a solubilized microsomal cytochrome *P*-450 system [48], lysophosphatidylethanolamine in a similar system in yeast [49] and in the adrenal mitochondrial steroid monooxygenation system phosphatidylcholine and phosphatidylethanolamine have been shown to be closely associated with cytochrome *P*-450 [50]. In addition a specific concomitant increase in phosphatidylcholine and phosphatidylethanolamine was reported with the phenobarbital-induced increase in drug monooxygenation in rat liver microsomes [51]. These findings support our results, which indicate a defined lipid environment, surrounding the *P*-450 molecule in the membrane, which is able to undergo a phase transition. Such a model has been suggested from spin label experiments in microsomes [43] and has recently been shown to occur associated with Ca^{2+}-ATPase in sarcoplasmic reticulum [52]. The nonstoichiometry of one reductase to about 10 or more cytochrome *P*-450 molecules [53] led to the proposal of a rigid [53] and a nonrigid complex [54] of these two components in the membrane. For enzymatic activity of these membrane-bound proteins a lateral diffusion is required. This diffusion in such a defined lipid environment could well be rate limiting in the gel phase at lower temperatures.

At temperatures above the transition point, the influence of the lipid phase will become smaller and may be dominated by the protein-protein interaction, required for the electron transfer from the reductase to cytochrome *P*-450. Some support for this assumption could be derived from the same activation energies found in solubilized microsomal membranes and in intact microsomes, and a similar conclusion can be drawn from the results of the following paper [55]. The high continuous activation energy observed in cumene hydroperoxide-mediated monooxygenation indicates that another rate-limiting step is involved in this process, which is not affected by the phase transition.

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and break temperatures were obtained for the microsomal monooxygenation of 7-ethoxycoumarin after various pretreatments of mature and immature rats. This work has been supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 38, Membranforschung.

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