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Lipid peroxidation decreases the rotational mobility of cytochrome *P*-450 in rat liver microsomes

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Phenobarbital-induced rat liver microsomes were subjected to NADPH- and Fe^{2+}-catalyzed lipid peroxidation. The formation of approx. 95 nmol malondialdehyde/mg protein during 18 min peroxidation at 37°C was observed. Membrane rigidity measured by means of the steady-state fluorescence anisotropy r^s of diphenylhexatriene increased in parallel with the malondialdehyde formation. Both the amount of malondialdehyde and r^s remained constant thereafter during incubation of the peroxidized membranes for 2 h. The aminopyrine demethylase activity decreased by about 60% upon lipid peroxidation for 18 min, whereas no significant loss of benzphetamine demethylase activity within the same time range was observed. A time-dependent formation of protein complexes of high molecular weight, comprising most of the microsomal polypeptides, upon lipid peroxidation was observed in SDS-polyacrylamide gel electrophoresis. The effect of microsomal lipid peroxidation on protein-protein interactions was examined by measuring the rotational mobility of intact cytochrome *P*-450. Rotational diffusion was measured by observing the decay of flash-induced absorption anisotropy $r(t)$ of the *P*-450 · CO complex. Analysis was based on a 'rotation-about-membrane normal' model with the equation $r(t) = r_1\exp(-t/\phi_1) + r_2\exp(-t/\phi_2)$. In control microsomes, two classes (rapid and slow) of rotating populations of cytochrome *P*-450 were observed with $\phi_1 \approx 150$ μs, fraction $r_1/(r_1 + r_2) \approx 40\%$ and $\phi_2 \approx 2$ ms, fraction $r_2/(r_1 + r_2) \approx 60\%$. A relatively small decrease in the rotational mobility of *P*-450 was observed by a 18-min lipid peroxidation, while a subsequent incubation of peroxidized microsomes for 2 h at 37°C resulted in a dramatic immobilization of *P*-450 by the increase of both $r_2/(r_1 + r_2) \approx 75\%$ and $\phi_2 \approx 10$–25 ms. The decrease in the *P*-450 mobility during 18-min lipid peroxidation would be due to the rigidification of the lipid bilayer. However, because the lipid fluidity remained unchanged thereafter, the significant immobilization of *P*-450 by the subsequent 2-h incubation is deduced to be due to formation of protein aggregates.

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Abbreviations: SDS, sodium dodecyl sulfate; *P*-450, rat liver microsomal cytochrome *P*-450; *L*/*P*, lipid-to-protein weight ratio.

Introduction

Lipid peroxidation in vivo has been identified as a basic deteriorative reaction in oxygen toxicity, in ethanol-induced liver injury, in chlorinated hydrocarbon hepatotoxicity as well as in aging process [1–6]. Rat liver microsomes undergo an enzyme-catalyzed lipid peroxidation in the presence

of NADPH and transition metal ions, concomitant with an increased oxygen uptake and NADPH oxidation [7]. Lipid hydroperoxides which are formed during peroxidation easily decompose to secondary products like malondialdehyde, ketones and ethers [5]. In contact with protein, lipid hydroperoxides cause formation of protein-centered radicals [8,9] which may undergo various ways of decomposition, including protein scission and amino acid damage [10]. An adverse consequence of membrane peroxidation is the formation of high-molecular-weight protein aggregates and lipofuscin-like fluorescent chromolipids, as demonstrated in rat liver microsomes [11] with concomitant loss of specific enzyme content such as microsomal cytochrome *P*-450 [12].

While much had been learned about the biochemical changes concomitant with or due to lipid peroxidation [13,14], little is known about the biophysical consequences of lipid peroxidation in biomembranes. Using pyrene and perylen as fluorescent probes, Dobretsov et al. [15] reported a decreased membrane fluidity after peroxidation of microsomal and liposomal membranes. Spin-label studies showed that γ-radiation-induced lipid peroxidation decreases the degree of order in membrane lipids [16]. Using the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene, we recently have shown that enzyme-catalyzed lipid peroxidation in rat liver microsomes increases the order of the acyl chains of microsomal phospholipids, most probably due to the formation of covalent bonds between adjacent lipid radicals [17].

To date, no direct measurements of protein mobility in peroxidized microsomal membranes have been carried out. Since rotational mobility of cytochrome *P*-450, which is a key enzyme of the microsomal monooxygenase system, has successfully been investigated [18–20], we have extended these studies to investigate changes in the microsomal membrane due to lipid peroxidation at a molecular level. Rotational diffusion of intact cytochrome *P*-450 is examined by observing the flash-induced absorption anisotropy of the heme-CO complex as a function of lipid peroxidation. The present study demonstrates a decreasing rotational mobility of cytochrome *P*-450 in peroxidized membranes subsequent to the increase in order of the acyl chains as probed by fluorescence anisotropy of diphenylhexatriene. The decreased cytochrome mobility appears to be due to slow protein aggregation following lipid peroxidation.

Experimental Procedures

Preparation of microsomes. Male Sprague-Dawley rats weighing 150 g were killed by decapitation after phenobarbital induction (100 mg/kg body wt.) for 3 days. Microsomes were prepared essentially according to the method described by Kornbrust and Mavis [21]. Microsomes were stored in liquid nitrogen. The specific content of cytochrome *P*-450 determined according to Omura and Sato [22] was 2.0–2.2 nmol *P*-450/mg microsomal protein. In the present experiments, microsomes which were stored in liquid nitrogen were used immediately after thawing, unless otherwise stated.

Peroxidation of microsomes. Microsomes were incubated with moderate stirring at 37°C. The incubation medium contained in a final volume of 30 ml 40 mM Tris-maleate buffer (pH 7.4), 200 μg microsomal protein/ml and 20 μM $FeSO_4$ which was added from an acidified stock solution consisting of 0.1 mol HCl/1 mol $FeSO_4$. Lipid peroxidation was started by the addition of 0.2 mM NADPH. Unless otherwise noted, it was stopped by the addition of 1 mM EDTA. The degree of lipid peroxidation was determined by the colorimetric detection of malondialdehyde after complexing with thiobarbituric acid [23].

Substrate hydroxylation by peroxidized microsomes. The *N*-demethylation of benzphetamine and aminopyrine, catalyzed by peroxidized microsomes, was measured as follows. Microsomal membranes were peroxidized as described above. At the indicated time, 500-μl aliquots were removed from the peroxidizing incubation mixture and transferred to 500 μl of 40 mM Tris-maleate buffer (pH 7.4), 2 mM EDTA (to stop peroxidation), 2 mM glucose 6-phosphate and 5 units of glucose-6-phosphate dehydrogenase as a NADPH-regenerating system, 2 mM benzphetamine or 16 mM aminopyrine, and 4 mM NADPH. The final protein content was 100 μg. Hydroxylation was allowed to proceed for 20 min, unless stated otherwise, at 37°C. The reaction was stopped by the addition of 1.5 ml of 12.5% trichloroacetic

acid (w/w), and the formaldehyde formed was measured according to Nash [24].

Steady-state fluorescence anisotropy of diphenylhexatriene in peroxidized microsomes. For labeling peroxidized microsomes with diphenylhexatriene, 1-ml aliquots of the peroxidizing incubation medium were transferred to test-tubes containing 2 ml of 40 mM Tris-maleate (pH 7.4), 1 mM EDTA and 2 μM diphenylhexatriene (from 2 mM stock solution in tetrahydrofuran). The protein concentration thus obtained was 66 μg/ml. Prior to the fluorescence measurement, samples were incubated for 1 h at room temperature in the dark. Steady-state fluorescence anisotropy was measured with an Aminco SPF-500 spectrofluorimeter. The samples were excited by a vertically polarized light at 357 nm and the sample fluorescence was analyzed at 450 nm through optical filters into vertically and horizontally polarized components, I_V and I_H, respectively. The steady-state anisotropy, r^s, is obtained by:

$$r^s = (I_V - SI_H)/(I_V + 2SI_H) \quad (1)$$

where S is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light and was taken as equal to I_V/I_H obtained with horizontally polarized excitation. For each measurement of r^s, I_V and I_H were corrected for contribution of intrinsic fluorescence and light scattering.

Rotational diffusion measurements. For rotational diffusion measurements, microsomes were prepared as described [20], except that sucrose was replaced by 80% (w/w) glycerol. The final heme concentration was about 7 μM for control microsomes and 2–4 μM for peroxidized microsomes.

The flash photolysis apparatus used for rotation measurements is described in detail elsewhere [28]. Briefly, the sample was excited at 532 nm by a vertically polarized flash of duration 10 ns from a Nd/YAG laser (JK-Laser HY 200, second harmonic). Absorbance changes due to photolysis of the heme-CO complex of microsomal cytochrome *P*-450 were measured at 450 nm. About 10% of the *P*-450-CO complex was photo-dissociated with a relatively weak laser flash. The signals were analyzed by calculating the absorption anisotropy, $r(t)$, and the total absorbance change, $A(t)$, given by:

$$r(t) = [A_V(t) - A_H(t)]/[A_V(t) + 2A_H(t)] \quad (2)$$

$$A(t) = A_V(t) + 2A_H(t) \quad (3)$$

where $A_V(t)$ and $A_H(t)$ are, respectively, the absorbance changes for vertical and horizontal polarization at time t after the flash. It should be noted that the anisotropy function $r(t)$ in Eqn. 2 theoretically does not contain any contribution of CO rebinding to the heme. In practice, CO rebinding only decreases the signal-to-noise ratio with increasing time after the flash (for a detailed discussion, see Ref. 26).

In each experiment, 4096 signals were averaged using a Datalab DL 102 A signal averager. A further improvement in the signal-to-noise ratio resulted from averaging the data of several experiments. The absence of a change in the amount of the cytochrome *P*-450-CO complex determined spectrophotometrically excluded degradation of cytochrome *P*-450 during the experiment.

Analysis of $r(t)$ is based on a model of rotation of cytochrome *P*-450 about the membrane normal [26]. When there is a single rotating species of cytochrome *P*-450 with the rotational relaxation time $\phi_{\|}$, $r(t)$ is given by:

$$r(t)/r(0) = \tfrac{3}{4}\sin^4\theta_N \exp(-4t/\phi_{\|}) + 3\sin^2\theta_N \cos^2\theta_N \exp(-t/\phi\|) + \tfrac{1}{4}(3\cos^2\theta_N - 1)^2 \quad (4)$$

where θ_N is the tilt angle of the heme plane from the plane of the membrane. As reported previously [27], the anisotropy decays to zero (i.e., $1/4(3\cos^2\theta_N - 1)^2 = 0$) within 2 ms when all cytochrome *P*-450 is rotating in proteoliposomes with a lipid-to-protein ratio of 30 (w/w), indicating that the heme plane of cytochrome *P*-450 is tilted by about $\theta_N \approx 55°$ from the membrane plane. Multiple rotating species of microsomal cytochrome *P*-450 with different $\phi_{\|}$ are considered by analyzing the data by the following equation:

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) \quad (5)$$

where ϕ_1 and ϕ_2 are rotational time constants, r_1 and r_2 are exponential amplitudes. Note that the

parameters r_j and ϕ_j ($j = 1, 2$), which emerge from the analysis, can be used to compare rotational mobility on a semiquantitative basis under different conditions.

Other methods. NADPH-cytochrome *P*-450 reductase was assayed according to Strobel and Dignam [28] at room temperature with cytochrome *c* as electron acceptor in 1 ml of 0.3 M potassium phosphate buffer (pH 7.7) and 0.1 mM EDTA. SDS-polyacrylamide gel electrophoresis was performed in the discontinuous (10–15%) Laemmli system [29]. The sample buffer contained 10% SDS and 1% mercaptoethanol. Protein concentration was determined by the method of Lowry et al. [30] with bovine serum albumin as standard.

Materials. 2-Thiobarbituric acid and diphenylhexatriene were purchased from Fluka (Buchs). Cytochrome *c* from horse heart, type III, and glucose-6-phosphate dehydrogenase were obtained from Sigma (St. Louis, MO). Benzphetamine was from Applied Science. Aminopyrine was obtained from Aldrich Europe. All other reagents were from Merck (Darmstadt).

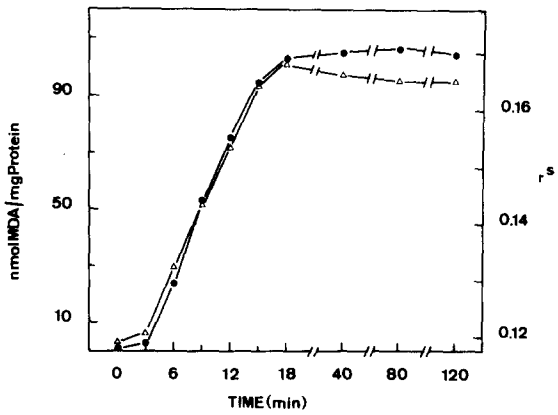

Fig. 1. Formation of malondialdehyde (MDA) and increase of the steady-state fluorescence anisotropy r^s of diphenylhexatriene in peroxidized microsomes. Phenobarbital-induced rat liver microsomes were peroxidized and the formation of malondialdehyde (●———●) was followed. 1 mM EDTA was added after 18 min to stop lipid peroxidation. r^s of diphenylhexatriene (△———△) was measured at room temperature. Excitation was at 357 nm, emission at 450 nm. The test cuvette contained 66 μg microsomal protein/ml. Diphenylhexatriene was 2 μM. r^s was calculated according to Eqn. 1. The values are the average of three different experiments.

Results and Analysis

Effect of lipid peroxidation on membrane fluidity

Fig. 1 shows the time-course of malondialdehyde production during peroxidation of rat liver microsomes, and the concomitant increase in steady-state fluorescence anisotropy r^s of diphenylhexatriene incorporated into these membranes. Peroxidation for 18 min resulted in the formation of about 100 nmol malondialdehyde/mg microsomal protein. At this time, peroxidation was stopped by addition of 1 mM EDTA to the incubation mixture. Incubation was then continued for 2 h at 37°C. No further malondialdehyde formation was observed during this period. Parallel to the production of malondialdehyde, r^s of diphenylhexatriene increased from approx. 0.12 to approx. 0.17 by a 18-min peroxidation. Values of r^s remained at this level thereafter during the incubation of the membranes for 2 h. In other experiments not shown here, no increase of r^s was detected upon incubation of microsomes in a non-peroxidizing medium (NADPH omitted) for 18 min and an additional 2 h. Under these conditions, malondialdehyde formation was maximally 5 nmol/mg protein after 2 h. As already shown previously [17], addition of malondialdehyde (100 nmol/mg protein) to control microsomes does not result in an increase of r^s, indicating that the increase is not simply due to the presence of the potential cross-linking compound malondialdehyde.

Enzymatic activities of peroxidized microsomes

Table I summarizes the substrate hydroxylation activities and the activity of NADPH-cytochrome *c* reductase of peroxidized microsomes. Although microsomes were peroxidized up to approx. 100 nmol malondialdehyde/mg protein, benzphetamine demethylase activity remained close to the control level, while aminopyrine demethylase activity was decreased by about 60%. Further incubation of the peroxidized membranes for an additional 2 h (see Experimental procedure) resulted in an apparent increase of the aminopyrine demethylase activity to the control level, as measured by the Nash test. We also measured the time-course of benzphetamine-*N*-demethylation in peroxidized microsomes. Similar to control micro-

TABLE I

SUBSTRATE HYDROXYLATION AND NADPH-CYTOCHROME *c* REDUCTASE ACTIVITY OF PEROXIDIZED MICROSOMES

The *N*-demethylation of benzphetamine and of aminopyrine by peroxidized microsomes was measured at 37°C for 20 min. The amount of formaldehyde formed was determined according to Nash [24]. NADPH-cytochrome *P*-450 reductase activity was measured according to Strobel and Dignam [28] at room temperature. Values are the means of two experiments. MDA, malondialdehyde.

Lipid peroxidation		Benzphetamine (1 mM) (nmol CH_2O formed/ 0.1 mg protein per 20 min)	Aminopyrine (8 mM) (nmol CH_2O formed/ 0.1 mg protein per 20 min)	Reductase activity (μmol Cyt *c* reduced/ mg protein per min)
time (min)	extent (nmol MDA formed/ mg protein)			
0	–	40.4	24.8	0.72
6	43	41.2	12.2	0.65
12	72	38.8	11.2	0.52
18	102	36.6	11.2	0.58
18 + 120 [a]	105	36.6	30.2	0.61

[a] Peroxidation for 18 min, further incubation for 120 min in the presence of 1 mM EDTA.

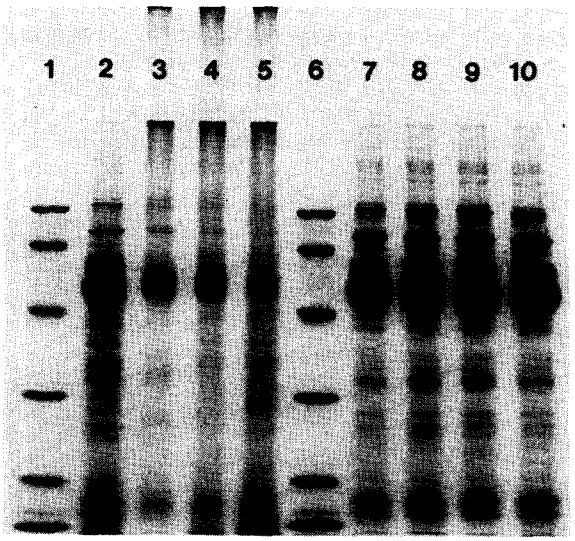

Fig. 2. SDS-polyacrylamide gel electrophoresis of peroxidized microsomes. Microsomes were peroxidized as described in Experimental procedures. Migration in this 10–15% SDS-polyacrylamide gel was from top to bottom. The lanes were loaded as follows: (1) and (6) molecular weight standards, from top: phosphorylase B, 92500; bovine serum albumin, 66200; ovalbumin, 45000; carboanhydrase, 31000; soybean trypsin inhibitor, 21500 and lysozyme, 14400; total amount = 20 μg; (2) control microsomes, 40 μg; (3) peroxidized microsomes (approx. 95 nmol malondialdehyde/mg, 18 min peroxidation), 40 μg; (4) peroxidized microsomes (approx. 95 mol malondialdehyde/mg, 2-h incubation after termination of the lipid peroxidation with 1 mM EDTA), 40 μg; (5) peroxidized microsomes (approx. 95 nmol malondialdehyde/mg) after 2-h incubation in 40 mM Tris-maleate buffer (pH 7.4), 40 μg; (7) and (8) control microsomes after incubation in 40 mM Tris-maleate buffer (pH 7.4), containing 20 μM $FeSO_4$, for 18 min (7) or 2 h (8), 40 μg each; (9) and (10) control microsomes after incubation with water-soluble products of lipid peroxidation (approx. 95 nmol malondialdehyde/mg protein) for 18 min (9) or 2 h (10).

somes, there was an initial high rate of demethylation which slowed down after about 5 min and remained linear between 10 and 40 min. Neither control nor peroxidized microsomes produced Nash test-reactive material when incubated in the complete hydroxylating medium omitting NADPH. In control microsomes, 10 mM metyrapone caused a 60% inhibition of the benzphetamine demethylase activity. In contrast, at most 30% inhibition of the activity by metyrapone was observed in peroxidized microsomes. NADPH-cytochrome *P*-450 reductase activity in microsomes was decreased upon lipid peroxidation to about 80% of the control (Table I).

Analysis of peroxidized microsomes by SDS-polyacrylamide gel electrophoresis

In rat liver microsomes, lipid peroxidation can lead to the appearance of protein complexes of high molecular weight [11]. Fig. 2 (lanes 2–5) shows a time-dependent formation of high-molecular-weight proteins upon lipid peroxidation in the present system. As compared to the control (lane

2), a significant amount of proteins in all molecular weight regions has disappeared after 18 min of lipid peroxidation (95 nmol malondialdehyde/mg protein), and a large amount of high-molecular-weight-protein aggregates has been formed, which do not penetrate into the gel (lane 3). Incubation of the membranes after termination of lipid peroxidation (95 nmol malondialdehyde/mg protein) by the addition of 1 mM EDTA for 2 h resulted in further disappearance of proteins (lane 4). Similar results were obtained when peroxidized microsomes were incubated for 2 h in a non-peroxidizing medium, i.e., 40 mM Tris-maleate (pH 7.4) without $FeSO_4$ and NADPH (lane 5).

The following observations indicate that in the present peroxidizing system water-soluble products containing malondialdehyde are not responsible for a significant formation of protein aggregates: incubation of non-peroxidized microsomes in 40 mM Tris-maleate (pH 7.4), containing 20 μM $FeSO_4$, for 18 min and an additional 2 h, respectively, had no effect on the microsomal protein pattern in SDS-polyacrylamide gel electrophoresis (lanes 7 and 8) when compared to control microsomes. In an additional experiment, microsomes were peroxidized to 95 nmol malondialdehyde/mg protein, peroxidation was stopped and the incubation mixture was centrifuged at 13000 $\times g$ for 15 min. The resulting supernatant was added to control microsomes to a concentration of 200 μg protein/ml supernatant. Incubation was carried out for 18 min or 2 h. No formation of high-molecular-weight-protein aggregates was observed under these conditions (lanes 9 and 10).

Rotational mobility of cytochrome P-450 in peroxidized microsomal membranes

Heterogeneous rotating populations: Examination of the experimental $r(t)$ in Figs. 3 and 4 showed that there was more than one rotating population of *P*-450. When the $r(t)$ curve was analyzed by Eqn. 5, the ratio of the time constants ϕ_2/ϕ_1 was very much greater than the factor 4 predicted by Eqn. 4 with $\theta_N = 55°$ (i.e., $1/4(3\cos^2\theta_N - 1)^2 = 0$). This is inconsistent with a single rotating species but compatible with multiple rotating components. In fact, an excellent fit to the data was obtained with the double-exponential equation (Eqn. 5). No improvement in fit was

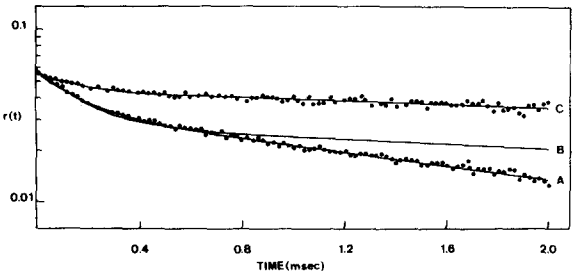

Fig. 3. Time-dependent absorption anisotropy of cytochrome *P*-450 in peroxidized microsomal membranes. Samples were photolyzed by a vertically polarized laser flash at 532 nm, and $r(t)$ was recorded at 450 nm. The measurements were carried out in 80% (w/w) glycerol (approx. 0.6 poise at 20°C). The concentration of cytochrome *P*-450 is 7 μM in A, 5 μM in B, and 3 μM in C. (A) Control microsomes; (B) peroxidized microsomes without any additional incubation (approx. 95 nmol malondialdehyde/mg protein); (C) peroxidized microsomes (approx. 95 nmol malondialdehyde/mg protein after 18 min peroxidation) with additional 2-h incubation at 37°C after terminating the peroxidation with EDTA. Solid lines were obtained by fitting the data to Eqn. 5. The curves have been slightly normalized to the same $r(0)$ in order to facilitate the comparison. This is justified by the fact that although $r(0)$ is dependent on the laser flash intensity, the normalized anisotropy $r(t)/r(0)$ is not affected by the different flash intensity [26]. For clarity, the data points of curve B have been omitted.

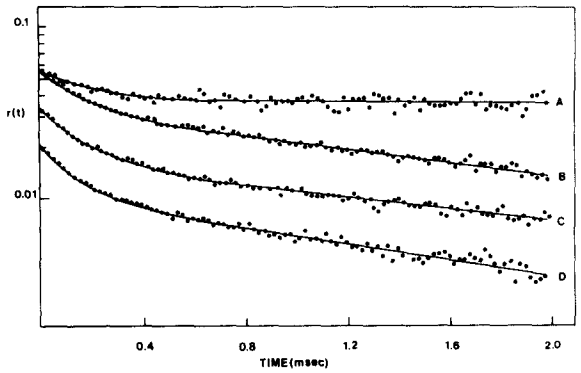

Fig. 4. Time-dependent absorption anisotropy of cytochrome *P*-450 in differently treated microsomes. Experimental conditions are the same as described for Fig. 3. The concentration of *P*-450 is 3.3 μM in A and 7–7.5 μM in B, C and D. (A) Microsomes were peroxidized for 18 min, washed, resuspended in non-peroxidizing medium, and incubated for 2 h at 37°C. (B) Control microsomes. (C) Non-peroxidized microsomes were incubated for 18 min plus 2 h at 37°C with the water-soluble products (containing approx. 95 nmol malondialdehyde/mg protein) obtained by centrifugation of the peroxidized microsomes. (D) Non-peroxidized microsomes were incubated for 18 min plus 2 h at 37°C in non-peroxidizing medium. Solid lines were obtained by fitting the data to Eqn. 5. Curve A has been vertically displaced to the same $r(0)$ as that of curve B for illustrative purposes. Curves C and D have been vertically displaced for clarity.

obtained by adding further terms. Therefore, we have tentatively adopted the double-exponential analysis for $r(t)$ curves. However, since there could be many (unknown) numbers of rotating components, each of which contributes two exponential terms, the numbers ϕ_1 and ϕ_2 in Eqn. 5 do not have precise physical significance. They may be regarded as approximate average relaxation times for, respectively, rapidly and slowly rotating populations of *P*-450 in the microsomal membrane. In this explanation $r_1/(r_1+r_2)$ and $r_2/(r_1+r_2)$ in Eqn. 5 correspond to approximate average fractions for, respectively, the rapidly and slowly rotating components of *P*-450.

Effect of lipid peroxidation on cytochrome P-450 mobility. Here we investigated the consequences of lipid peroxidation on the rotational mobility of cytochrome *P*-450. It should be noted that any denatured *P*-450, including *P*-420 and the apocytochrome, is silent in the present flash photolysis and only intact *P*-450 contributes to $r(t)$. The decay parameters of $r(t)$ analyzed by Eqn. 5 are summarized in Table II. In control microsomes, a rapidly rotating population ($r_1/(r_1+r_2)\approx 40\%$) with the rotational time constant of $\phi_1 \approx 150$ μs and a slowly rotating population $r_2/(r_1+r_2)\approx 60\%$) with $\phi_2 \approx 2$ ms were observed. Fig. 3 (curve B) shows that after 18 min of peroxidation the mobility of *P*-450 is diminished but not to so large an extent. Although ϕ_2 increases to approx. 5 ms, $r_2/(r_1+r_2)$ decreases to approx. 45% and ϕ_1 remains unchanged. The amount of intact *P*-450 in the peroxidized microsomes was 60–70% of the control non-peroxidized membranes. As shown in Fig. 3, curve C, and in Table II, the subsequent incubation of the peroxidized microsomal membrane in the complete peroxidizing medium in the presence of 1 mM EDTA for 2 h at 37°C results in a dramatic increase of the very slowly rotating population of *P*-450 ($r_2/(r_1+r_2)\approx 75\%$, $\phi_2 \approx$ 10–20 ms). A further loss of 10–20% of the heme · CO complex was observed during this 2-h incubation. When membranes were peroxidized for 18 min to approx. 95 nmol malondialdehyde/mg protein, separated from the peroxidizing medium by centrifugation and further incubated in a non-peroxidizing medium (40 mM Tris-maleate (pH 7.4) for 2 h at 37°C, *P*-450 was also largely immobilized to a similar extent as when incubated with the peroxidizing medium.

The role of water-soluble products of lipid peroxidation in immobilization of cytochrome P-450. A possible role of water-soluble degradation products of peroxidized lipids in cross-linking of proteins was also investigated. As shown in Fig. 4, incubation of non-peroxidized microsomes for 18 min and an additional 2 h in the presence of the supernatant (containing approx. 90 nmol malondialdehyde/mg protein) obtained by centrifugation of the peroxidized microsomes did not affect the rotational mobility of *P*-450. A significant immobilization of *P*-450 was observed after 2-h incubation of peroxidized microsomes at 37°C, regardless of whether water-soluble products including malondialdehyde were removed or not by washing the peroxidized microsomes. These results rule out the possibility that the products of lipid peroxidation once liberated from the membrane induce the cross-linking and immobilization of *P*-450.

Effect of storage of microsomes. Rotational mobility of cytochrome *P*-450 was observed to be dependent on aging of microsomes (see Fig. 5). Incubation of microsomes at 0°C for 1 day, which is often used to store microsomes after preparation for biochemical experiments, significantly im-

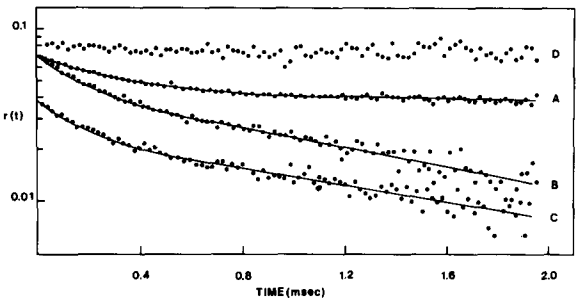

Fig. 5. Effect of storage of microsomes at different temperatures on the rotational mobility of cytochrome *P*-450. Experimental conditions are the same as described for Fig. 3. The concentration of *P*-450 is 7–10 μM in A, B and C. (A) Microsomes used after storage at 0°C for 1 day. (B) Microsomes used immediately after isolation. (C) Microsomes used after storage in liquid nitrogen for 4 days. (D) Microsomes (1.6 mg protein/ml) labeled with 10 μM eosin maleimide. $r(t)$ of eosin was measured after cross-linking by irradiation as described under Results and Analysis. Solid lines were obtained by fitting the data to Eqn. 5. Curve B has been vertically displaced to the same $r(0)$ as that of curve A in order to facilitate the comparison. Curve C has been vertically displaced for clarity.

TABLE II

DECAY PARAMETERS OF THE ABSORPTION ANISOTROPY $r(t)$ OF CYTOCHROME *P*-450 IN PEROXIDIZED MICROSOMES

The measurements were carried out in 80% (w/w) glycerol at 20°C. Analysis of data was according to Eqn. 5. Lipid peroxidation was stopped after 18 min by addition of 1 mM EDTA. MDA, malondialdehyde. Values are presented as means ± S.D.

Sample	$\frac{r_1}{r_1+r_2}$ (%) [a]	$\frac{r_2}{r_1+r_2}$ (%)	$\phi_1(\mu s)$ [b]	$\phi_2(\mu s)$
Control microsomes, 8.5 μM *P*-450	42 ± 5	58 ± 5	148 ± 36	1883 ± 823
Microsomes, lipid peroxidation for 18 min, approx. 95 nmol MDA/mg protein, 5 μM *P*-450	57 ± 5	43 ± 5	162 ± 2	5140 ± 569
Microsomes, lipid peroxidation for 18 min, approx. 95 nmol MDA/mg protein, additional 2-h incubation [c], 3 μM *P*-450	25 ± 4	75 ± 4	231 ± 31	10465 ± 1107
Microsomes, lipid peroxidation for 18 min, approx. 95 nmol MDA/mg protein, additional 2-h incubation with vigorous stirring, 3 μM *P*-450	30 ± 3	70 ± 3	229 ± 51	24071 ± 1659
Microsomes, lipid peroxidation for 18 min, approx. 95 nmol MDA/mg protein, additional 2-h incubation [c] without water-soluble products of lipid peroxidation [d]	35 ± 3	65 ± 3	176 ± 25	21602 ± 757
Control microsomes, without lipid peroxidation [e], with additional 2-h incubation, 8 μM *P*-450	48 ± 2	52 ± 2	143 ± 7	2696 ± 794
Control microsomes, without lipid peroxidation [e], 2-h incubation with water-soluble products of lipid peroxidation [f]	52	48	173	2484

[a] $r_1/(r_1+r_2)$ (%) and $r_2/(r_1+r_2)$ (%) are percentages of rapidly and slowly rotating populations of *P*-450, respectively.
[b] ϕ_1 and ϕ_2 are rotational time constants of rapidly and slowly rotating populations of *P*-450, respectively.
[c] Samples were very mildly stirred during a 2-h incubation.
[d] Water-soluble products of lipid peroxidation were removed by washing, and microsomes were incubated in a non-peroxidizing medium.
[e] This sample was not supplemented with 20 μM $FeSO_4$; 0.2 mM NADPH was added at time zero, and 1 mM EDTA at 18 min.
[f] Samples were incubated with water-soluble products of lipid peroxidation containing approx. 90 nmol malondialdehyde/mg protein.

mobilized *P*-450. On the other hand, no significant change in the $r(t)$ curve was observed when microsomes were stored in liquid nitrogen up to 2 months. Concomitantly, the time-course of microsomal lipid peroxidation was not significantly affected by storing microsomes in liquid nitrogen up to half a year. Therefore, in the present study we used freshly thawed microsomes which had been stored in liquid nitrogen.

Absence of microsome tumbling. A significant contribution of microsome tumbling to the observed decay in $r(t)$ has been experimentally ruled

out in previous [20,41] and the present investigations. For this, we labeled microsomes by eosin maleimide, and rotational mobility of eosin-labeled microsomal proteins was measured by observing absorption anisotropy of the eosin probe at 520 nm. In 80% glycerol solution, a decrease of $r(t)$ and a time-independent residual anisotropy $r(\infty)/r(0)$ was observed. Furthermore, no decay in $r(t)$ curves was observed after cross-linking of eosin-labeled proteins by irradiation from a tungsten-halide lamp [20] (see Fig. 5). If microsomes were tumbling isotropically within the time range of 2 ms, $r(t)$ should continuously decay to zero. Therefore, the above results rule out any contribution of microsomes tumbling to the observed decay in $r(t)$ of cytochrome *P*-450.

Discussion

Multiple rotating populations of cytochrome P-450 in liver microsomes

We consider the possible structural implications of the presence of differently rotating populations of *P*-450 in the membrane. In general terms, the existence of a slowly rotating fraction of *P*-450 implies that the mobility of this fraction is restricted, either by protein-protein interactions or by a lipid phase of high viscosity. If the slow component of *P*-450 would be restricted in a lipid region of extremely high viscosity, a gel-phase lipid would have to be envisaged to explain such a slow component. However, a variety of physical studies show that there are negligible gel-phase lipids present in microsomal membranes above 0°C [17,31]. Therefore, it appears to be most likely that protein aggregates represent the slow component of *P*-450. Indeed, heterogeneous rotating populations of *P*-450 are formed when purified *P*-450 is incorporated into proteoliposomes at $L/P = 1$–5 [20].

Generally, rotational diffusion of membrane proteins is very sensitive to protein-protein interactions. Extensive studies have been performed with cytochrome oxidase. When analyzed over a 2-ms time range, cytochrome oxidase has several rotating populations including an immobile population in the inner membrane of mitochondria with $L/P \approx 0.3$. Moreover, it was demonstrated that the immobile oxidase is included in nonspecific protein aggregates with relatively weak affinity and that these nonspecific protein aggregates can easily be disaggregated by incorporating lipids into the inner mitochondrial membranes [32]. Similarly, multiple rotating populations and an immobile population of the ADP/ATP translocator were also observed in the inner membrane of mitochondria [33].

Thus, when the membrane is rich in proteins, many different types of protein-protein interactions (e.g., stable specific protein complexes, nonspecific protein aggregates, transient protein complexes, etc.) are expected to be present, resulting in multiple decay phases of the $r(t)$ curve. Rat liver microsomes have a L/P value of approx. 0.5, being crowded with integral membrane proteins. Therefore, the two decay phases observed in the $r(t)$ curve of *P*-450 most likely indicate heterogeneous rotating populations of *P*-450. These populations cannot correspond in a one-to-one fashion to individual *P*-450 species, since even a single purified *P*-450 isozyme shows multiple rotating populations in liposomes [20], and several distinct species of *P*-450 exist in phenobarbital-induced microsomes [34,35].

Immobilization of cytochrome P-450 in microsomal membranes due to lipid peroxidation

In this report, two significant observations related to microsomal lipid peroxidation are made. First, the rotational mobility of *P*-450 is largely decreased upon lipid peroxidation in microsomal membranes. Secondly, the time-course of the change in the order of fatty acid chains of phospholipids, which increases during and is remained unchanged after lipid peroxidation, is not paralleled by the decrease in the mobility of *P*-450.

For a quantitative expression of the rotational mobility of *P*-450 molecules, not only rotational time constants ϕ_j but also fractions $r_j/(r_1 + r_2)$ or rapidly ($j = 1$) and slowly ($j = 2$) rotating populations of *P*-450 have to be considered. When phonobarbital-induced rat liver microsomes were peroxidized for 18 min to approx. 95 nmol malondialdehyde/mg protein, the lipid fluidity decreased drastically (Fig. 1), while the *P*-450 mobility decreased only slightly: although the rotational time constant ϕ_2 of the slowly rotating *P*-450 was increased 2-fold, the fraction $r_2/(r_1 + r_2)$ even de-

creased, and ϕ_1 remained unchanged. In contrast, the prolonged incubation of these peroxidized membranes for 2 h resulted in no further change in the lipid fluidity but in a large decrease in the rotational mobility of *P*-450, as shown by the remarkable increase in both $r_2/(r_1 + r_2)$ and ϕ_2 (see curve C in Fig. 3, and Table II). The decrease in the *P*-450 mobility during 18-min lipid peroxidation may be due to the rigidification of the lipid bilayer. However, because the lipid fluidity remained unchanged thereafter, the significant immobilization of *P*-450 during the subsequent 2-h incubation cannot be caused by the lipid phase but might be induced by formation of nonspecific protein aggregates caused by lipid peroxidation. As visualized on the polyacrylamide gel, microsomal lipid peroxidation indeed induces formation of protein aggregates which may include cytochrome *P*-450. Rotational diffusion of integral membrane proteins is particularly sensitive to the size of the protein, that is, the rotational relaxation time $\phi_{||}$ in Eqn. 4 is proportional to the square of the diameter of the membrane-immersed part of the protein [36]. Therefore, the protein mobility strongly depends on the degree of protein aggregation [37,38].

On polyacrylamide gels, the formation of high-molecular-weight-protein aggregates is observed by both 18-min lipid peroxidation and the following 2-h incubation, and the simultaneous decrease of polypeptide bands of 45–58 kDa, including *P*-450 bands, is observed. However, we cannot directly determine when and to what an extent *P*-450 bands are moved into protein aggregates, because not only *P*-450 but also many other polypeptide bands are present in the region of 45–58 kDa. On the other hand, since only *P*-450 molecules are selectively measured in our rotation measurements, the observed immobilization of *P*-450 strongly suggests that a majority of *P*-450 molecules are trapped in protein aggregates during the 2-h incubation after lipid peroxidation. During 18-min lipid peroxidation, a relatively small number of *P*-450 molecules might form protein aggregates which may also decrease the *P*-450 mobility in addition to the mobility decrease induced by the rigidification of the lpid bilayer.

Here, it should be noted that in our rotation measurements only intact *P*-450 reactable with CO contributes to the $r(t)$ curve, and that no information about denatured silent *P*-450 is extractable by analysis of the $r(t)$ curve. An understanding of the dynamic behavior of denatured *P*-450 (e.g., *P*-420 and apocytochrome *P*-450) which is formed during lipid peroxidation may be desirable for a better understanding of the effect of microsomal lipid peroxidation on the *P*-450 mobility.

The mobility of *P*-450 is not significantly dependent on the presence or the absence of water-soluble products of peroxidation during an additional 2 h incubation. Moreover, a 2-h incubation of control microsomes with water-soluble products obtained from freshly peroxidized microsomes containing approx. 90 nmol malondialdehyde/mg protein decreases neither the mobility nor the amount of *P*-450. This incubation also does not change the protein pattern on SDS-polyacrylamide gel electrophoresis. Therefore, it can be concluded that water-soluble products of lipid peroxidation, containing the potent cross-linking agent malondialdehyde, are not responsible for the observed protein aggregation. Although a broad variety of breakdown products other than malondialdehyde is formed during lipid peroxidation [39], a possible cross-linking agent, if indeed formed upon lipid peroxidation, must be of lipophilic nature remaining in the membrane even after repetitive washing.

Possible other factors affecting the rotational mobility of cytochrome P-450

Several other factors could be responsible for the observed immobilization of cytochrome *P*-450 by microsomal lipid peroxidation. Nielsen [40] reported the covalent binding of peroxidized phospholipids to bovine serum albumin concomitant with an apparent increase in protein molecular size. However, an increase in protein molecular size due to lipid binding would hardly be sufficient to account for the observed immobilization of *P*-450. Binding of peroxidized lipids to the *P*-450 molecules could not be expected to increase dramatically the diameter of the membrane-immersed part of the cytochrome and thereby would not largely decrease the rotational time constant. Cytochrome *P*-450 could be immobilized, however, when lipid peroxides bound to it act as

cross-linking reagents which induce protein aggregates, because such protein aggregates could easily be much larger in size compared with *P*-450.

Physiological significance of lipid peroxidation

A significant rotational and/or lateral mobility of protein components of microsomal membranes is probably necessary for some of the proteins' functions. Although phenobarbital-induced rat liver cytochrome *P*-450 can form a heterodimeric complex with NADPH-cytochrome *P*-450 reductase [27,41], not all *P*-450 can form such a complex with the reductase at a given time, because the liver microsomal membrane contains 20–30 cytochrome *P*-450 molecules per reductase molecule. One individual reductase molecule would therefore interact with many different individual cytochrome *P*-450 molecules in a lateral collision-controlled manner in order to transfer electrons. A large decrease in the protein mobility and the membrane fluidity by microsomal lipid peroxidation would significantly decrease such lateral collision-controlled electron transfer, because the rotational mobility of membrane proteins has a close relation to the lateral mobility [36].

Another consequence of lipid peroxidation in microsomal membranes is a significant breakdown of cytochrome *P*-450 heme. Since *P*-450 is the key enzyme of the microsomal monooxygenase system, an inactivation of this protein may have dramatic consequences in vivo and in vitro for the metabolism of endogenous and exogenous substrates. Interestingly, the demethylation of benzphetamine in our in vitro study is only slightly affected by lipid peroxidation, while that of aminopyrine decreased drastically during a 18-min peroxidation. This may reflect different sensitivity towards peroxidation of different isozymes of *P*-450. The apparent increase of aminopyrine demethylase activity during the post-peroxidative 2-h incubation remains puzzling. It may reflect the peroxidative activity of *P*-450 [42] with endogenous lipid peroxides as substrates.

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