ROTATIONAL DIFFUSION OF CYTOCHROME *P*-450 IN RAT LIVER MICROSOMES

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1. Introduction

According to current concepts, cell membranes exist in a dynamic state in which at least some membrane components are free to diffuse in the plane of the membrane [1]. Experiments which demonstrate the diffusion of lipids and proteins in membranes have been reviewed in [2,3]. Diffusion measurements provide a powerful method for investigating the interaction of membrane components. For example, rhodopsin apparently undergoes free diffusion in the rod outer segment disc membrane [4,5], while bacteriorhodopsin is immobilised in the purple membrane by self-association into a crystalline lattice [6,7].

The mode of interaction of the two main components of the liver microsomal hydroxylating system, i.e., NADPH–cytochrome *P*-450-reductase and cytochrome *P*-450, is an unresolved and important question. For each reductase molecule there are ~20–30 molecules of cytochrome *P*-450. Two models have been proposed to account for the observed stoichiometry and for inhibitory studies. The 'cluster model' [8] assumes that each reductase is tightly surrounded by 8–10 molecules of cytochrome *P*-450, whereas the remaining cytochromes are reduced following collisions resulting from lateral diffusion in the membrane. The model in [9], on the other hand, assumes that all cytochromes react with the reductase by a collision process.

It is clear that the measurement of protein diffusion in the microsomal membrane is relevant to both the above models. Rotational diffusion may be measured by observing flash-induced transient dichroism of either intrinsic or extrinsic chromophores [4,6,10–13]. Here we have utilized the photo-dissociation of the heme–CO complex to investigate rotation of cytochrome *P*-450 in the rat liver microsomal membrane. We demonstrate that rotational motion does occur and discuss the implications of this finding for the mechanism of the hydroxylating system. Photolysis of heme–CO was also used [13] to study rotation of cytochrome oxidase in the inner mitochondrial membrane, although no decay of dichroism was detected.

2. Materials and methods

Microsomes were isolated from phenobarbital-induced male Wistar rats (150–200 g) by the method in [14]. For the flash experiments, microsomes were diluted into buffer containing 150 mM KCl, 50 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$ and varying amounts of sucrose to 1.5–2 mg protein/ml final conc. Microsomal pigments were reduced by the addition of a few grains of dithionite and then slowly bubbled for 1 min with CO. In fixation experiments, glutaraldehyde was added to 0.1% final conc. to a dilute suspension of microsomes (1.5 mg protein/ml). After incubation for 5 min at 0°C the sample was concentrated by centrifugation and used for flash photolysis measurements as described above.

For negative contrast electron microscopy, samples were stained with 2% uranyl acetate (pH 7) on Formvar coated grids and examined with a Philips 200 electron microscope.

The flash photolysis apparatus used for rotational diffusion measurements is detailed in [10,12]. Briefly, the sample was excited by a linearly polarized light pulse of 1–2 μs duration and wavelength 540 nm

from a dye laser Intensity changes due to photolysis of the heme–CO complex were measured at 450 nm using a continuous 100 W tungsten–halide source The signals were collected with a Datalab DL 102A signal averager and processed on a Hewlett-Packard 9825A desktop computer The data were analysed by calculation of the absorption anisotropy $r(t)$ given by [15]

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

where $A_{\parallel}(t), A_{\perp}(t)$ are, respectively, the absorbance changes at time t after the flash for light polarized parallel and perpendicular with respect to the polarization of excitation

3 Results and discussion

Figure 1 shows the time dependence of the absorption anisotropy r measured following photolysis of the cytochrome *P*-450–CO complex in microsomal membranes in 60% sucrose at different temperatures The initial decay of r at the higher temperatures implies rotational motion in the sample Before this can be assigned to rotation of the protein, it is necessary to consider whether tumbling of the microsomal vesicles themselves could be responsible

Several observations indicate that vesicle tumbling is insignificant in 60% sucrose solutions We estimated the vesicle size distribution from electron micrographs of negatively stained preparations Most vesicles were > 800 Å diam , although some smaller vesicles were also present Calculations based on the Stokes-Einstein equation indicate that the decay of anisotropy due to vesicle tumbling is negligibly small over 2 ms in 60% sucrose This is supported experimentally by results obtained with microsomes in 40% sucrose at −8°C (fig 2, lower curve) The horizontal plot obtained for the absorption anisotropy against time demonstrates that no significant vesicle tumbling occurs under these conditions Since the viscosity of 40% sucrose at −80°C is less than that of 60% sucrose at 23°C, vesicle rotation should also be insignificant under the latter conditions Finally, a relatively light fixation with glutaraldehyde (0 1%) abolishes most of the rotational motion in the 60% sucrose sample (fig 2, upper curve) Electron micrographs showed that little change in vesicle size or aggregation was produced by fixation, implying that immobilisation was due to protein crosslinking in the membrane

In view of these results we conclude that the decay of r observed in 60% sucrose solution arises from

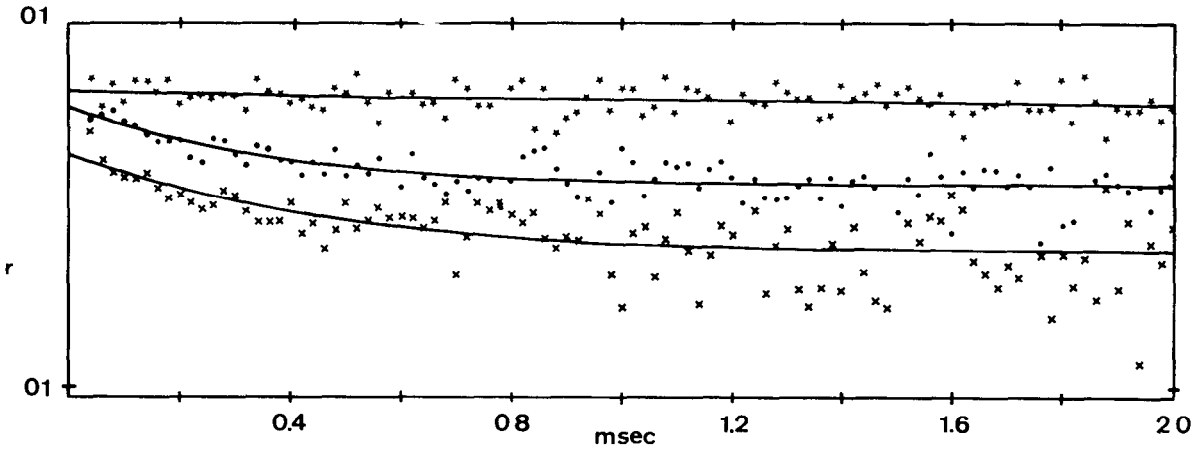

Fig 1 Time dependence of absorption anisotropy r after flash photolysis of microsomal cytochrome *P*-450–CO in 60% sucrose Rat liver microsomes (1 5 mg/ml, 2 7 μM cytochrome *P*-450–CO) were photolysed with a pulse of polarised laser light (540 nm, 1–2 μs pulse duration), and the absorption anisotropy r was determined as described in section 2 Sample temperatures −2°C (⋆), 23°C (•), 37°C (×)

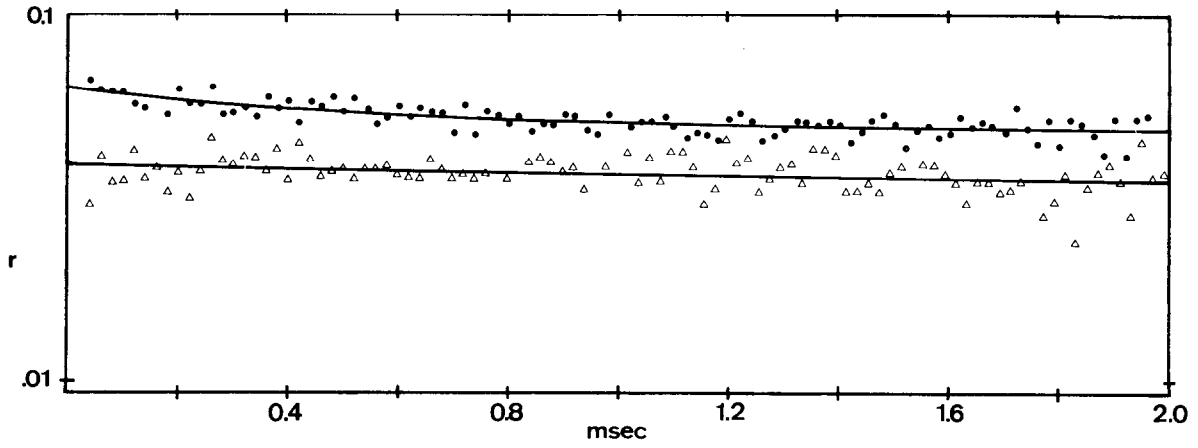

Fig.2. Time dependence of absorption anisotropy r after flash photolysis of microsomal cytochrome *P*-450–CO at low temperature or after fixation with glutaraldehyde. Upper curve (•): Glutaraldehyde was added to a final concentration of 0.1% to a dilute suspension of microsomes (1.5 mg protein/ml). After incubation for 5 min at 0°C the sample was concentrated, and flash photolysis was performed in 60% sucrose at 23°C as described in fig.1. Lower curve (△): Experimental conditions as in fig.1 except that the sucrose concentration was 40% and the temperature −8°C. The curve is arbitrarily lowered by 0.025 to avoid overlapping the upper curve.

rotation of cytochrome *P*-450 in the microsomal membrane. The expected form of $r(t)$ for a protein which rotates only about an axis normal to the membrane is given by [11,12]:

$$r(t) = A_1\exp(-D_{\parallel}t) + A_2\exp(-4D_{\parallel}t) + A_3 \qquad (2)$$

where A_1, A_2, A_3 are constants which depend on the orientations of the transition dipole moments of the absorption bands used for excitation and measurement, t is the time after the flash and $D_{\parallel}$ is the rotational diffusion coefficient. For the present case, eq. (2) should be regarded as an approximation, since the derivation does not take into account the circular degeneracy of the heme–CO chromophore. It nevertheless illustrates two important features of the analysis which are of general applicability. These are, firstly, the existence of a residual time independent anisotropy and secondly, two exponential components in the decaying part of the curve.

The experimental curves for cytochrome *P*-450 do decay to a constant value in agreement with eq. (2). This is therefore consistent with rotation of the protein occurring only about the membrane normal as has been found with all other proteins so far investigated [4,11,16]. However, it cannot be ruled out that an immobile fraction of the protein contributes to the residual anisotropy.

It is of interest to note that the heme group in bovine adrenal cortex mitochondria cytochrome *P*-450 was recently reported to be oriented with its plane parallel to the plane of the membrane [17]. The present experiments indicate that the heme of cytochrome *P*-450–CO in rat liver microsomes does not have such an orientation. Due to the circular degeneracy of the heme–CO absorption band, rotation about the membrane normal would not lead to any decay in the anisotropy when the heme plane is in the plane of the membrane. Conceivably, such a decay could arise from a large rocking motion about axes lying in the plane of the membrane. However, model system studies suggest that this is unlikely [18] and we consider it much more probable that the heme plane is inclined at an appreciable angle to the plane of the membrane.

The experimental data are not sufficiently accurate to justify curve fitting to the multiple exponential decay indicated by eq. (2). We therefore fitted the data to the simpler equation:

$$r(t) = B_1\exp(-t/\alpha) + B_2 \qquad (3)$$

using an iterative non-linear least squares procedure This yields $\alpha = 270 \pm 90$ μs at 21°C (mean and standard deviation of 10 measurements) It should be noted that α is not identical with the relaxation time (defined as $1/D_{\parallel}$), since the two expected components of the decay are not resolved It can, however, be regarded as a reasonable order of magnitude estimate

The value of α varies only little with temperature at > 13°C ($<$ 2-fold over 13–37°C) At −2°C, however, the protein is virtually immobilized (fig 1) This finding is consistent with calorimetric studies which show that the bulk of the membrane lipids undergo a broad gel to liquid–crystalline phase transition centred at about 0°C [19]

The present results demonstrate that cytochrome *P*-450 is capable of relatively rapid diffusion in the microsomal membrane at physiological temperature The translational diffusion coefficient D_T is related to $D_{\parallel}$ by the approximate expression [20]

$$D_T = 4a^2 D_{\parallel} \quad (4)$$

where a is the radius of the protein (assumed to be cylindrical) Taking $a = 2$ nm, the present rotation measurements imply that D_T is in the order of 10^{-9}–10^{-10} cm^{2}s^{-1} Of course, long range diffusion could be slower than this due to restrictions by other membrane components, as may be the case in the human erythrocyte membrane [11] Nevertheless, the present observations support the idea that lateral diffusion of cytochrome *P*-450 is sufficiently rapid to permit a reaction mechanism involving random collision between membrane components

At the present time the experimental $r(t)$ curves are not sufficiently accurate to decide whether they are attributable to a single rotating species or to multiple components Thus a distinction between the two models for interaction between cytochrome *P*-450 and NADPH–cytochrome *P*-450-reductase cited in the first paragraph is not yet possible Further advance can be expected from refinement of the present experimental technique In addition, recent studies have demonstrated how rotational diffusion measurements, when coupled with chemical crosslinking, provide a powerful method of detecting specific associations between protein components [21] Thus a continuation of the studies reported here will provide further insight into the details of molecular interactions in the microsomal membrane

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