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EVIDENCE FROM CROSS-LINKING AND ROTATIONAL DIFFUSION STUDIES THAT CYTOCHROME P450 CAN FORM MOLECULAR AGGREGATES IN RABBIT-LIVER MICROSOMAL MEMBRANES

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1. Introduction

The monooxygenase system of the endoplasmic reticulum of liver cells plays a major role in the metabolism of xenobiotics and of steriods. An important question of current debate is how the components of the system (various distinct molecular forms of cytochrome P450 and the flavoprotein, cytochrome P450 reductase) are organised in the membrane [1–3]. Do long-lived complexes (e.g., 'clusters' [3]) of components occur, or do random and transient collisions between components fully account for enzymic activity?

Chemical cross-linking is a technique that is used to determine which components of a system of several macromolecules can be in close physical association with each other. We have developed a cross-linking method involving cupric ions that brings about the cross-linking of two particular forms (viz. form 4 and form 6) of rabbit-liver cytochrome P450 into homodimers (form 4 + form 4; form 6 + form 6) and heterodimers (form 4 + form 6) [4–6]. Oligomers larger than dimers have not been seen. The reaction is remarkably specific to these two proteins and can be used in intact microsomes. These forms of cytochrome P450 are induced by treatment of rabbits with β-naphthoflavone. For this reason cross-linking is largely restricted to microsomes prepared from rabbits so treated, and is not seen to great extent in microsomes prepared from untreated or phenobarbitone-induced rabbits. Additional techniques are required in order to determine whether the interacting cytochromes pre-exist as dimers (e.g., as part of a cluster of cytochromes P450) or whether dimers are formed after random collisions.

We have attempted to resolve this question by observation of the rotational diffusion characteristics of microsomal cytochromes P450 before and after cross-linking of forms 4 and 6: if the molecules involved pre-exist as stable dimers or larger oligomers, no change in the rotational mobility of the microsomal cytochromes P450 should be occasioned by cross-linking; if, on the other hand, cross-linking stabilizes transient dimers resulting from collisions, a change in rotational mobility should result because such mobility is strongly dependent on the diameter of the rotating species. Using this principle, a non-covalently-associated dimer of band 3 in erythrocyte membrane was shown to pre-exist prior to cross-linking by cupric phenan throline [7].

A method for estimating the rotational diffusion of cytochrome P450 molecules in microsomal membranes has been successfully developed [8]. Photolysis of bound CO from the haem groups of a population of cytochrome P450 molecules with a flash of linearly-polarized light gives rise to transient dichroism at 450 nm. The subsequent decay of absorption anisotropy is related to the rotational motion of cytochromes in this population by the following expression, if the reasonable assumption is made that rotation is about an axis normal to the membrane:

$$r(t) = A_1 \exp(-D_{\parallel} t) + A_2 \exp(-4D_{\parallel} t) + A_3 \qquad (1)$$

where $r(t)$ is absorption anisotropy at time t after the flash, $D_{\parallel}$ is the rotational diffusion coefficient and A_1, A_2, A_3 are constants which depend only on the angle between the haem plane and the normal to the plane of the membrane [9].

Employing both cross-linking and rotational diffu-

sion techniques in a series of parallel experiments, we find that cross-linking can be carried out efficiently at 20°C, at which there appears to be no measurable rotational movement of the cytochrome P450 molecules in the microsomal membrane for up to 400 μs. We infer from this that all the cytochrome P450 molecules in microsomes from β-naphthoflavone-induced rabbits may participate in molecular complexes of some kind at this temperature, and that it is likely that the close association of different molecules of cytochrome P450 is permitted within such complexes. At 35°C in such microsomes and at 20°C in microsomes from phenobarbitone-induced rabbits some decay of flash-induced dichroism is observed. This indicates that not all of the cytochrome P450 molecules in the membranes are included in large molecular complexes under these latter conditions.

2. Experimental

2.1. *Animals*

Male New Zealand White rabbits (1.7–2.0 kg body wt) obtained from Hop Rabbits Ltd., Chilham, Kent were used. Three animals were injected on 4 consecutive mornings with 50 mg/kg i.p. of a 50 mg/ml crude suspension of β-naphthoflavone (Aldrich, Milwaukee) in Mazola corn oil. Following the last dose, the rabbits were starved for 24 h prior to killing. Three further rabbits were allowed ad libitum consumption of drinking water containing 0.1% sodium phenobarbitone for 7 days. These animals were also starved for 24 h before killing.

2.2. *Preparation of microsomes*

The animals were killed by cervical dislocation and the livers from each group were pooled. Microsomes were prepared as in [6]. This method includes the washing of microsomal pellets twice with a pyrophosphate buffer of low ionic strength. The washed microsomal pellets were resuspended in TEG buffer (0.01 M Tris, 0.1 mM EDTA, 20% (v/v) glycerol; adjusted to pH 7.4 at 20°C with acetic acid) to final conc. 12.5 and 50 mg protein/ml for microsomes from β-naphthoflavone- and phenobarbitone-induced rabbits, respectively. The microsomes were frozen in aliquots and stored at −20°C. Transport from Canterbury to Zürich was carried out with the samples stored in solid CO_2.

2.3. *Estimation of the cytochrome P450 and protein content of the microsomes*

Protein was estimated by the Lowry method [10] using bovine serum albumin as standard. Cytochrome P450 was measured as in [11]. Estimates of 4.2 and 4.8 nmol/mg protein were obtained for the cytochrome P450 content of microsomes from the phenobarbitone- and β-naphthoflavone-induced animals, respectively.

2.4. *Cross-linking of cytochrome P450 with cupric phenanthroline*

Suspensions of microsomes (270 μl at 12.5 mg/ml) were diluted by thorough mixing with 550 μl of a solution of 70% (w/v) sucrose made up in TCEG buffer (0.05 M Tris, 0.01 mM EDTA, 20% (v/v) glycerol; adjusted to pH 8.0 at 20°C with HCl). The samples were then pre-incubated for 5 min at 20°C.

Cross-linking was achieved by adding 100 μl 10 mM $CuCl_2$ and 50 μl 2.5 mM *o*-phenanthroline. (150 μl of water was added to non-cross-linked control incubations.) Incubation was for 1 min at 20°C. Sample pH was 7.5 at this temperature. The reaction was quenched by adding 50 μl 2-mercaptoethanol.

Under the above conditions no deterioration of the cytochrome P450 spectrum occurs. Such spectra were monitored throughout the experiments to be described and no significant changes took place. There was also no evidence for the deterioration of the microsomal membrane by lipid peroxidation. Levels of malonaldehyde both prior to and subsequent to cross-linking were <1 nmol/mg protein (measurements by Dr C. Richter, ETH).

2.5. *Electrophoretic separation of microsomal proteins and estimates of extent of cross-linking*

Aliquots (135 μl) were removed from the above samples and analyzed by SDS–PAGE as in [6]. Staining was with Coomassie brilliant blue R (Gurr, stain 009120). In model experiments stained gels were scanned with a Gilford 250 spectrophotometer and the above cross-linking conditions were found to displace 17 ± 2% of the stained material from region X of the gels (see fig.3), where all the different forms of cytochrome P450 are believed to be located, to the dimer region of the gels. It is not known what proportion of the proteins migrating in region X are in fact cytochromes P450, nor to what extent each protein in this region binds stain molecules. If, however, we assume a uniform uptake of stain and that 2/3 of the stain in

region X is due to cytochromes P450, we arrive at the estimate that ~25% of all the cytochrome P450 molecules in the microsomes may be cross-linked. This is true only for microsomes derived from β-naphthoflavone-induced animals. Very little cross-linking occurs under these conditions in microsomes from phenobarbitone-induced animals [4].

2.6. *Measurements of absorption anisotropy*

The remaining portions of the microsomal samples were made 60% (w/w) with respect to sucrose and 4.9 μM with respect to cytochrome P450 content by the addition of 2.0 ml 70% (w/w) sucrose in TCEG buffer. Sucrose was added in order to inhibit vesicle tumbling and to reduce light scattering. Samples were then reduced with several grains of sodium dithionite and bubbled gently with CO for 1 min. Decay of absorption anisotropy was determined as in [12]. Briefly, the sample was excited at 540 nm by a vertically-polarized light pulse of 1–2 μs duration from a dye laser. Absorbance changes due to photolysis of the haem–CO complex were measured at 450 nm. About 500 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:

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

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

Following flash photolysis of suspensions of microsomes from β-naphthoflavone-induced rabbits in 60% sucrose, a decay in absorption anisotropy at 450 nm is observed over 0.4 ms at 35°C (fig.1). However, almost no decay is observed at 22°C. Thus, it appears probable that most or all of the cytochrome P450 molecules present in the microsomal membrane are immobile at the lower temperature. This contrasts with the situation in rat liver microsomes in which between 1/2 and 1/3 of the cytochrome P450 molecules present have measurable rotation in the ms time range at room

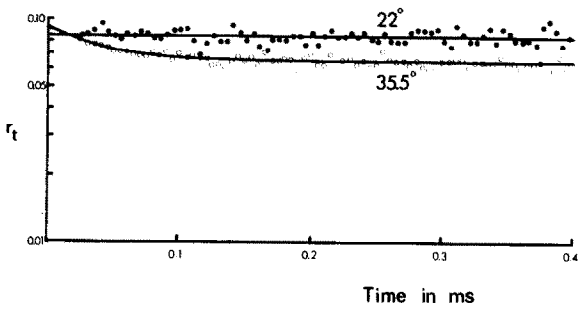

Fig.1. Time dependence of absorption anisotropy $r(t)$ after flash photolysis of cytochrome P450–CO in microsomes from β-naphthoflavone-induced rabbits suspended in 60% sucrose. The microsomes were not treated with cross-linking reagents. The curves are $r(t) = 0.083$ at 22°C (•) and $r(t) = 0.028 \exp(-t/40\ \mu s) + 0.064$ at 35°C (○).

temperatures ([8]; S. K. unpublished). A decay in absorption anisotropy is seen when microsomes from phenobarbitone-induced rabbits are used (fig.2). As rotational mobility is strongly dependent on the diameter of the rotating species, the simplest explanation for the lack of observable rotational movement of the cytochrome P450 molecules in microsomes from β-naphthoflavone-treated animals at 22°C is that the cytochromes are integrated into molecular aggregates or complexes of some kind.

In order to gain some insight into the relative sizes of the freely-rotating and immobile populations of cytochrome P450 molecules under the various experimental conditions used, the data have been analysed by curve fitting procedures. Since the accuracy of the

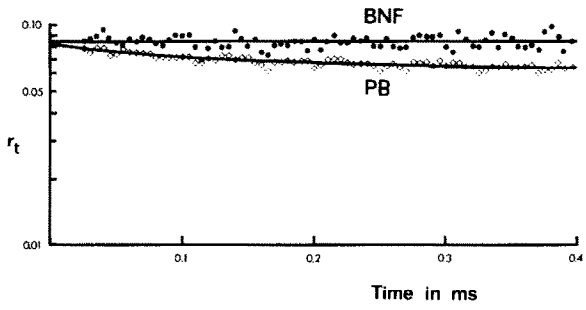

Fig.2. Time dependence of absorption anisotropy $r(t)$ after flash photolysis of cytochrome P450–CO in microsomes from β-naphthoflavone-treated rabbits at 22°C (•) and microsomes from phenobarbitone-treated rabbits at 20°C (-◇-). The microsomes were suspended in 60% sucrose. They were not treated with cross-linking reagents. The curves are $r(t) = 0.083$ (•) and $r(t) = 0.018 \exp(-t/128\ \mu s) + 0.062$ (-◇-).

data does not justify use of the full equation (1), we employ the following simplified expression:

$$r(t) = B_1 \exp(t/T_1) + B_2 \quad (3)$$

where T_1 is a time constant related to the rotational diffusion coefficient, and B_1, B_2 are constants.

When all the cytochrome P450 molecules present in the membrane are rotating, the expected time-independent residual anisotropy ratio, $r(\infty)/r(0)$, is as follows [9]:

$$r(\infty)/r(0) = A_3/r(0) = B_2/r(0) = \tfrac{1}{4}(3\cos^2\theta_N - 1)^2 \quad (4)$$

where we assume a 4-fold symmetry of the haem plane at 450 nm, and θ_N is the angle between the normal to the haem plane and the membrane normal. When all the cytochrome molecules are immobile, $r(\infty)/r(0) = 1$. Although the initial anisotropy, $r(0)$, is a function of the laser flash intensity, $r(t)/r(0)$ is independent of the flash intensity in the case of linear and circularly symmetric chromophores [13]. Model experiments with purified rat liver cytochrome P450 incorporated into lipid vesicles suggest that $r(\infty)/r(0)$ has a value of ~0.2 when almost all the cytochrome P450 molecules are rotating [14]. Here, measured values of $B_2/r(0)$ are 1 (in microsomes from β-naphthoflavone-treated rabbits at 22°C), 0.7 (in similar microsomes at 35°C) and 0.8 (in microsomes from phenobarbitone-treated rabbits at 20°C). These large $B_2/r(0)$ values suggest that there are considerable amounts of immobilised or very slowly rotating cytochrome P450 molecules ($T_1 > 5$ ms) in rabbit liver microsomes. If we assume that the value of the ratio, $r(\infty)/r(0)$, for freely mobile rabbit liver cytochrome P450 molecules is similar to that determined for rat liver cytochrome P450, it may be estimated that around 2/3 of the cytochrome P450 population are immobile in microsomal membranes from β-naphthoflavone-treated rabbits at 35°C and in microsomes from phenobarbitone-induced rabbits at 20°C, whereas virtually all the population is immobile at 22°C in microsomes from β-naphthoflavone-treated rabbits.

We have carried out cross-linking experiments in parallel with the above studies of decay of absorption anistropy. At 20°C a considerable extent of cross-linking of form 4 and form 6 cytochrome P450 to homodimers and heterodimers is permitted in microsomes from β-naphthoflavone-induced rabbits (fig.3).

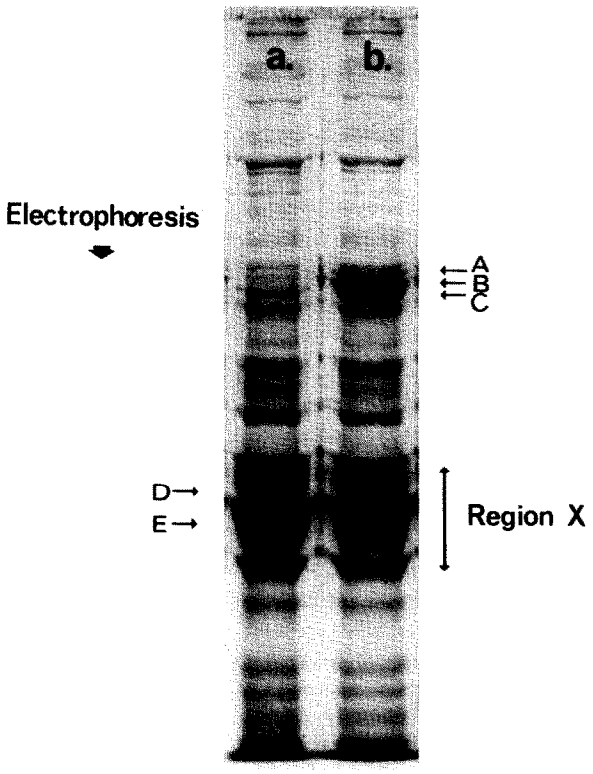

Fig.3. SDS gel electrophoresis of microsomes from β-naphthoflavone-induced rabbits (a) prior to and (b) subsequent to cross-linking with cupric phenanthroline as in section 2. Bands D and E represent form 6 and form 4 cytochrome P450, respectively [5]. Bands A–C represent the following dimeric species: form 6 + form 6 (A); form 4 + form 6 (B); form 4 + form 4 (C).

Model experiments (see section 2) indicate that around 1/4 of the total cytochromes P450 in the microsomes are involved in dimer formation under these conditions. The lack of decay of flash-induced anisotropy at 22°C indicates that there cannot be a significant subpopulation of cytochrome molecules which are freely rotating and thus independent of molecular aggregates at this temperature. This suggests that cross-linking is unlikely to be occuring between freely-diffusing molecules, but rather between cytochromes that are integral to the molecular aggregates. It would therefore appear probable that molecular complexes involving cytochrome P450 in microsomal membranes permit the close mutual association of a substantial proportion of the participating cytochrome molecules.

Since there is no decay in flash-induced absorption anisotropy at 22°C in microsomes from β-naphthofla-

vone-induced animals, no further information regarding the mechanism of cross-linking can be inferred from comparisons of absorption anisotropy at this temperature prior to and subsequent to cross-linking treatment. However, when decay of flash-induced dichroism is studied at 35°C in microsomes cross-linked at 20°C, almost no significant change in the decay is seen relative to that in untreated microsomes observed at 35°C (not shown). This finding further supports the view that cytochromes P450 molecules in pre-existing aggregates are cross-linked.

We conclude that cytochrome P450 molecules in rabbit microsomal membranes demonstrate a propensity to become involved in molecular aggregates in which contact between different cytochrome molecules seems to be permitted. This propensity is particularly marked at lower temperatures in microsomes derived from β-naphthoflavone-induced rabbits, but remains substantial at temperatures which approach the physiological value. A higher proportion of cytochromes P450 is mobile in microsomes from phenobarbitone-induced rabbits than in microsomes from β-naphthoflavone-induced rabbits. This may result from the differing nature of the cytochromes present, or from differences in the membrane lipid phase. Studies of the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene do not reveal any abrupt changes in the fluidity of the lipid phase of microsomes from β-naphthoflavone-induced rabbits over 0–40°C that could account for the complete loss of observable mobility of cytochrome P450 at 22°C (P.R.M., R.B.F., unpublished).

We have no information regarding the possible involvement of other proteins, such as cytochrome P450 reductase, in molecular aggregates involving cytochrome P450. Our data are consistent with the 'cluster' model of [3] in which a number of cytochrome P450 molecules are envisaged as being grouped around a centrally-located cytochrome P450 reductase molecule. This model was put forward to explain the biphasic kinetics of reduction of cytochrome P450, rapid phase reduction being thought to reflect reduction of the cytochrome molecules within the cluster and slow phase reduction resulting from the reduction of satellite cytochrome P450 molecules which were postulated to occur in the unaggregated form free in the membrane. However, recent studies have indicated that rapid-phase reduction is limited by diffusion in the membrane, at least in a model system [15], and this is not consistent with the 'cluster' model as proposed.

Clearly, further investigation is required to clarify the organisation of the components of this electron-transfer chain in endoplasmic reticulum membrane and the role of freely-rotating and immobile forms of cytochrome P450 in the monooxygenation of substrates.

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