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MEMBRANE TOPOLOGY OF MICROSOMAL CYTOCHROME P-450: SATURATION TRANSFER EPR AND FREEZE-FRACTURE ELECTRON MICROSCOPY STUDIES

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SUMMARY: The rotation of cytochrome P-450 LM2 (CYPIIB4) incorporated into large microsomal-like lipid vesicles was investigated by saturation transfer EPR using ^{15}N- and ^{2}H-substituted spin labels. In combination with rotational diffusion, the distribution and size of protein particles in the bilayer were studied by freeze-fracture electron microscopy. The data from both methods suggest an oligomeric and membrane-spanning aggregate for the topology of microsomal cytochrome P-450.

The membrane topology of microsomal cytochrome P-450 (P-450) is at present a subject of much debate. Contrary to first predictions (1), recent biochemical data favor a localization with the main part of the protein outside the bilayer and only one or at most two membrane-spanning helical sequences (residues 3-21 of P-450 LM2) with the N-terminus on the luminal side (2-5 and citations therein). This view is supported by more recent secondary structure predictions (3,6). So far, this model does not take into account a possible oligomeric structure, although evidence for P-450 aggregates has been obtained from rotational diffusion experiments (7-10). However, the very slow rotation found by all investigators could also be interpreted in terms of a monomeric model with additional assumptions, e.g. electrostatic interactions between the cytoplasmic part of P-450 and the membrane surface (5,11).

The present paper reports, for the first time, rotational diffusion of P-450LM2 (CYPIIB4) in microsomal-like, large lipid vesicles, which were prepared according to a new method for the incorporation of P-450 into large unilamellar vesicles (12). In this way the problem of vesicle tumbling was circumvented from the very beginning, and any undue perturbation of the membrane system by the addition of chemicals to slow down the vesicle rotation could be avoided.

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Such large vesicles provide the additional advantage of controlling and analyzing the proper incorporation of the enzyme into the bilayer directly by observation of intramembrane protein particles (IMPs) using freeze-fracture electron microscopy. The use of large vesicles and study of the size and distribution of IMPs in combination with rotational diffusion remove much of the ambiguities in the interpretation of recent investigations. Rotational diffusion was measured by saturation transfer EPR (ST-EPR) using recent methodological developments that refine the method due to enhanced precision of the measurement (13,14) and improve sensitivity and resolution by use of ^{15}N- and ^{2}H-substituted spin labels (15).

MATERIALS AND METHODS

P-450 LM2 was prepared and spin labeled as described previously (8) except that the ^{15}N- and ^{2}H-substituted spin label perdeuterio N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinylmaleimide ([^{15}N,^{2}H] MSL) was used.

Subsequently the labeled enzyme was incorporated into large unilamellar vesicles (average diameter, 200-300 nm) by octylglucoside dialysis as described earlier (12), but with additional adsorption of the detergent by polystyrene beads. Non-incorporated protein was removed by gel filtration on Sepharose 4B followed by ultracentrifugation at 100,000 x *g* for 90 min to pellet the vesicles. Finally, the pellet was resuspended in buffer (0.1 M phosphate, pH 7.4, sometimes with glycerol) for the ST-EPR and freeze-fracture experiments. Vesicles were thoroughly characterized for structure, composition, residual detergent, size, homogeneity, and stability as described (12). Co-reconstitution of P-450 with NADPH-cytochrome P-450 reductase results in an enzymatically active monooxygenase system as shown by N-demethylation of benzphetamine (12).

EPR and ST-EPR spectra were recorded using a Varian E3 spectrometer modified to detect the second harmonic of absorption out-of-phase (v'_2) (16). Phase setting was performed according to the "self null method" (17); modulation amplitude was 2.8 G. The microwave power setting was adjusted to give an effective H_1 value of 0.2 G, depending on the dielectric properties of the sample. The recommendations given elsewhere (13,14) were closely followed. The sample was placed in a standard flat cell.

Rotational correlation times (τ_R) were determined by comparison of the spectra with reference spectra obtained from [^{15}N,^{2}H] MSL-labeled hemoglobin in aqueous glycerol and sucrose solutions. For quantitation the intensity ratio parameters introduced by Thomas et al. (17) and defined for ^{15}N-spin label spectra by Beth et al. (15) (L'/L, L"/L, and H"/H) were used.

Our reference system is closely related to that described earlier (14) apart from the use of [^{15}N,^{2}H] MSL-labeled hemoglobin. This system is a reliable model for isotropic rotational diffusion, and consequently the τ_R determined this way for membrane systems can only be used as an effective correlation time. These effective values have to be interpreted carefully because the measured spectra depend not only on the rate of motion; problems in the analysis are discussed elsewhere (18). In the case of P-450 LM2 we could define the angle of the label relative to the membrane by cross-linking experiments and relating our data to time-resolved optical studies of the same system (9,11).

Freeze-fracture experiments were done as described (12). Freeze-fracture micrographs should, in principle, allow estimation of the size of the IMPs. However, many problems arise in practice; for a discussion, see a recent paper (19) in which an improved procedure was developed minimizing deformations and enlargement of particle size. This improved procedure provided evidence for an enlargement of 6 nm for 10 nm-IMPs due to shadowing caused by conventional freeze-fracture.

RESULTS

Freeze-fracture. The micrograph in Fig. 1A shows predominantly large, spherical proteoliposomes with a fairly homogeneous size distribution. The

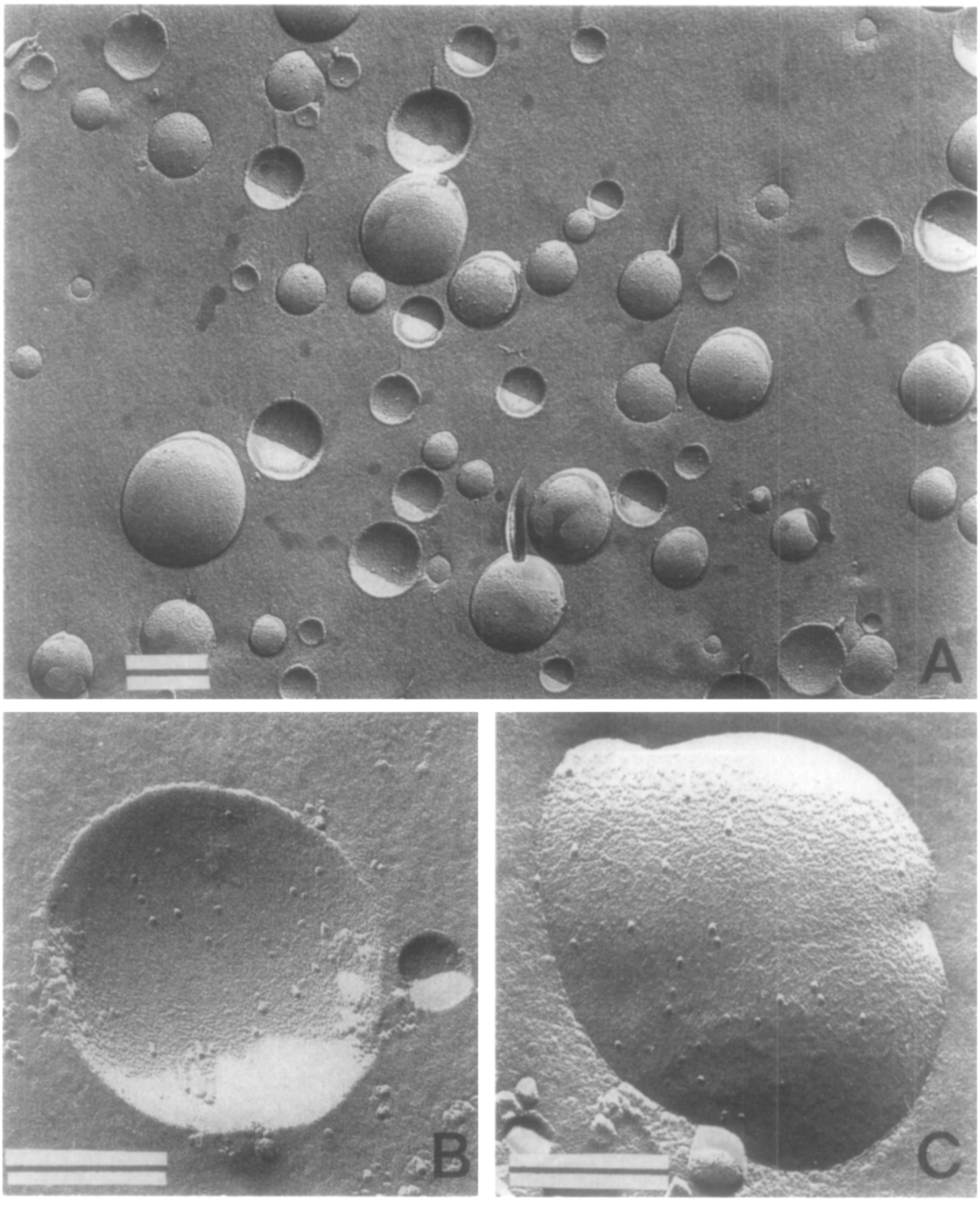


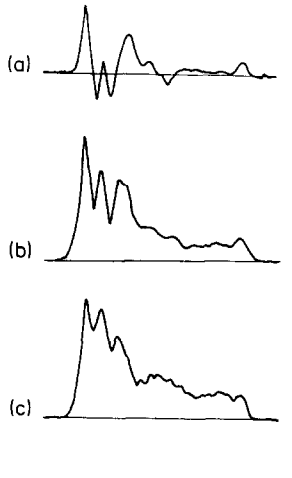
Fig. 1. Freeze-fracture electron micrographs of P-450 LM2-containing PC/PE/PA (2:1:0.06, w/w/w) vesicles prepared by octylglucoside dialysis/adsorption at 4 mg of protein/ml lipid suspension and lipid/protein ratio of 7.5 (w/w). The bars represent 200 nm.

absence of fracture steps on the fracture faces is in accord with a unilamellar nature of the vesicles, which was also indicated by single cross-fractured proteoliposomes (not shown). At higher magnification the presence of IMPs is clearly visible on concave (Fig. 1B) as well as convex (Fig. 1C) fracture faces. These findings provide evidence for a proper incorporation of P-450 into the membrane bilayer. The rarity of particles in the background ice indicates that almost all protein present in the vesicle fractions used for the experiments is incorporated into the vesicles.

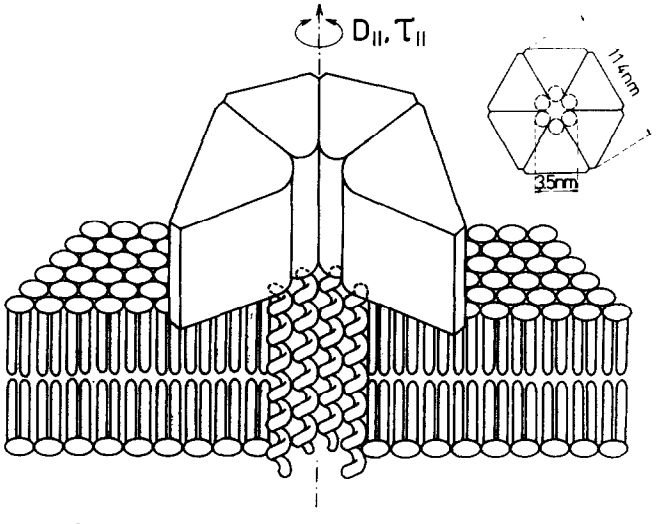
The surface density of the IMPs has not been analyzed quantitatively. However, in each preparation studied the particle density increased with increasing protein content. A rough estimate of the IMP density from the almost flat central surfaces of very large vesicles showed that the calculated particle density in every case was much below that expected for the corresponding lipid/protein ratio, which is an indication that P-450 aggregation had taken place.

Most IMPs are visualized as particles with roughly the same diameter, about 9-11 nm. With correction of this value as discussed under Methods, the IMP size in our P-450-containing proteoliposomes can be estimated as roughly 3-5 nm.

Saturation Transfer EPR. It was important for the measurement to prove that the label is rigidly attached to the enzyme molecule; the EPR spectra



(2)



(3)

Fig. 2. ST-EPR (v'2) spectra of [15N, 2H] MSL-labeled P-450 LM2: (a) in aqueous buffer solution; (b) in large PC/PE/PA vesicles (data as in Fig. 1); (c) as in (b), but after cross-linking of the proteins (suspensions were incubated in a 4% glutaraldehyde solution for 10 h at 4° C).

Fig. 3. Model for membrane topology of microsomal P-450. An oligomeric (possibly hexameric), membrane-spanning aggregate is shown as rotating around an axis parallel to the membrane normal.

observed in solution as well as in the membrane are characteristic of strongly immobilized labels (not shown). Fig. 2a shows the ST-EPR spectrum of P-450 in aqueous solution, from which an average τ_R of about 185 ns was estimated based on the parameters L'/L and L"/L. For a monomeric P-450 molecule assumed to be a sphere with a radius of 2.4 nm, one would expect a τ_R of about 21 ns (7). Consequently, the measured value can be understood taking into account the well-known fact that P-450 in solution exists as a hexameric aggregate, thus indicating that the v'_2 spectrum indeed reflects the overall rotation of P-450. A possible contribution to the measured τ_R from vesicle rotation is negligible because of the large size of the vesicles used (diameter, 200-300 nm).

The v'_2 spectrum in Fig. 2b exhibits the typical shape of μs rotational motion. From the parameters L'/L and L"/L an average effective τ_R of about 36 μs for the rotation of P-450 in the membrane was estimated, which is different from the τ_R value derived from H"/H of about 50 μs. That difference provides direct evidence for the anisotropic character of the rotation as is expected for a protein rotating in a bilayer membrane. The spectra were unaffected by pellet formation (not shown), while glutaraldehyde-induced cross-linking inhibited the rotation nearly completely (Fig. 2c). The corresponding τ_R values of cross-linked P-450 calculated from L'/L, L"/L, and H"/H are about 370, 190, and 400 μs, respectively.

The measured τ_R values can only be used as effective correlation times. However, rotational diffusion studied by optical methods proved that there is strict uniaxial rotation around an axis parallel to the membrane plane in which P-450 LM2 is embedded (9,11). In this case the correlation time characterizing this rotation in EPR is usually related to the diffusion coefficient $D_{||}$ by $\tau_{||} = 1/(6 \cdot D_{||})$ (see Fig. 3). For $\tau_\perp >> \tau_{||}$ a relation was derived (20) which relates the effective τ_R to the true $\tau_{||}$ characterizing the uniaxial rotation. According to this relationship, τ_R amounts to $2 \cdot \tau_{||}$ for $\theta = 90°$ and increases with decreasing θ. For $\theta = 0°$, τ_R becomes totally insensitive to rotation about the parallel axis. The experimental finding that the τ_R values for the rotation of P-450 deduced from all spectral regions are strongly influenced by inhibition of the protein motion caused by glutaraldehyde cross-linking (Fig. 2C) definitely suggests that the nitroxide z axis has an orientation near to $\theta = 90°$. When this is taken into account, an upper limit for $\tau_{||}$ can be estimated as about 18 μs.

DISCUSSION

The interpretation of the measured correlation times requires theoretical predictions for the diffusion coefficient, $D_{||}$. Greinert et al. (9) adapted the general solution of the problem for cylindrically shaped membrane proteins or

aggregates (of radius a) in a membrane of viscosity η to the case of proteins not entirely spanning the bilayer but immersed only up to a depth of h:

$$D_{||} = \frac{kT}{4\ \pi\ \eta\ a^3}\left[\frac{h}{a} + \frac{8}{3\pi}\right]^{-1}$$

Assuming realistic values for η between 1 and 2.5 P (21), we have calculated $D_{||}$, and hence $\tau_{||}$, for different models of the protein size, shape, aggregational state, and membrane topology. Only an oligomeric and membrane-spanning protein aggregate consisting of most probably 6 P-450 molecules of shape and size schematically shown in Fig. 3 would rotate with a $\tau_{||}$ between 8 and 22 μs. This range is in good agreement with the experimentally determined value of 18 μs as the upper limit. It is suggested that the aggregate is arranged like a cake on the membrane surface and anchored by its 6 helices, which may form a channel- or bundle-like structure with a diameter of about 3.5 nm estimated from the diameter of the N-terminal helix of P-450 LM2, which is roughly 1.2 nm. Therefore the very slow rotation is primarily due to interaction of the aggregate with the membrane surface or, assuming partial immersion of the head part, with the hydrophobic core of the bilayer. By comparison, the frictional contribution of the anchor is nearly negligible.

The data from freeze-fracture experiments suggest such a structure. The occurrence of distinct IMPs demonstrates that the anchor is membrane-spanning, and estimation of the real diameter for the IMPs gives a value of at least 3 to 5 nm, excluding an anchor of only 1 or 2 helices. The striking similarity between the size and shape of the IMPs observed and the 7-helical bundle of the bacteriorhodopsin monomer (22) is of interest.

Although other models could be considered, the present model is certainly one of the simplest consistent with the rotational diffusion and freeze-fracture data. It also takes into account recent results from biochemical studies and the known 3-dimensional structure of a bacterial P-450 concerning size and shape of the monomeric subunits in the membrane (2-6,23). Because of a lack of knowledge of the membrane viscosity, the number of subunits as well as a possible small immersion of the main part of the molecule into the bilayer cannot be determined unambiguously. Therefore, Fig. 3 should be considered only as a rough schematic representation. Such a structure may have important consequences for various aspects of the monooxygenase cycle, such as electron transfer from the reductase, cooperativity, allosteric effects, substrate access, and product channeling, some of which have been discussed recently by Stier et al. (10), who have also proposed an oligomeric model for P-450, but with a different membrane topology.

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