Orientation of Cytochromes P450 in the Endoplasmic Reticulum†

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ABSTRACT: The orientation of eukaryotic cytochromes P450, with respect to the membrane of the endoplasmic reticulum, has been investigated. There is now good evidence that the tertiary structure of these proteins is essentially the same as that of the soluble bacterial isoenzyme cytochrome P450CI, with the exception of an extension at the N-terminus which is thought to form a membrane-anchoring sequence. The remainder of the molecule protrudes from the cytosolic face of the membrane so that it can interact with substrates and electron-donating proteins. Two models based on this structure have been considered, in which the plane of the heme of cytochrome P450 is oriented either parallel with or perpendicular to the plane of the membrane of the endoplasmic reticulum. The validity of these models has been assessed from the results of studies involving the binding of antipeptide antibodies directed toward known regions of cytochromes P450, modeling of the interaction of cytochrome P450 with cytochrome b_5, proposed intramolecular movements of cytochrome P450 during its catalytic cycle, and the partitioning of substrates for cytochrome P450 between the cytosol and membrane. It is concluded that cytochrome P450 is most likely oriented such that the heme is not fixed horizontal to the plane of the membrane of the endoplasmic reticulum and may well lie with the heme perpendicular to the membrane.

Cytochromes P450 are present in a wide variety of organisms, ranging from bacteria and yeast to plants and higher animals (Nebert et al., 1989). Through the application of recombinant DNA techniques, the amino acid sequences of over 100 apoproteins of cytochrome P450 have been deduced (Gonzalez et al., 1989). However, the only cytochrome P450 that has been crystallized and its structure solved is the soluble, bacterial isoenzyme, cytochrome P450CI (Poulos et al., 1987). Two of the major differences between this protein and the eukaryotic members of the cytochrome P450 superfamily are, first, the eukaryotic cytochromes P450 are all membrane-bound and, second, cytochrome P450CI is smaller than the eukaryotic forms by up to 100 residues. As eukaryotic cytochromes P450 are membrane-bound, this led some workers to suggest that these forms are intrinsic membrane proteins with structures that are strikingly different from that of cytochrome P450CI (Tarr et al., 1983; Ozols et al., 1985; Hudecek & Anzenbacher, 1988). However, it is becoming increasingly apparent that the structure of the eukaryotic forms is largely similar to that of cytochrome P450CI (Nelson & Strobel, 1988; Edwards et al., 1989a). The difference in length between cytochrome P450CI and the eukaryotic cytochromes P450 can be accounted for by an N-terminal membrane-anchoring sequence and two other regions of unknown function (Edwards et al., 1989a).

The relationship between eukaryotic cytochromes P450 and the membrane of the endoplasmic reticulum has yet to be determined. In order to investigate the overall topography, a model was devised in which an N-terminal membrane-anchoring sequence was attached to cytochrome P450CI in a number of different orientations with respect to the remainder of the protein. The validity of the resultant structures was assessed by examining the accessibility of antipeptide antibody binding sites on native eukaryotic cytochromes P450.

Experimental Procedures

Materials. N^{α}-9-Fluorenylmethoxycarbonyl amino acids were purchased from Millipore (U.K.) Ltd. (London, U.K.), and *tert*-butylthiocysteine was from Novabiochem AG (Läufelfingen, Switzerland). Keyhole limpet haemocyanin was from Cambridge Bioscience (Cambridge, U.K.). Polystyrene 96-well microtiter plates were from Dynatech Laboratories (Billinghurst, Sussex, U.K.), and conjugates of immunoglobulins with horseradish peroxidase were from ICN Biomedicals Ltd. (High Wycombe, Bucks, U.K.). All other chemicals were purchased from Sigma (Poole, Dorset, U.K.) or BDH Ltd. (Dagenham, Essex, U.K.) and were of analytical grade or the best equivalent.

Treatment of Animals and Preparation of Microsomal Fractions. Groups of male Wistar rats (200–250 g) from Harlan Olac Ltd. (Bicester, Oxon, U.K.) either were left untreated or were treated with a single intraperitoneal injection of 3-methylcholanthrene (80 mg/kg body weight) dissolved in corn oil, 48 h before killing. Male New Zealand White rabbits (2.5–3.0 kg) from Froxfield Farms Ltd. (Petersfield, Hampshire, U.K.) either were untreated or were administered acetone as a 1% (v/v) solution in their drinking water for 7 days. After the respective treatment period, animals were killed humanely in accordance with approved Home Office procedures, the livers were rapidly removed, and microsomal fractions were prepared as described previously (Boobis et al., 1980).

Antipeptide Antibodies. The synthesis of peptides Tyr-Leu-Pro-Gly-Ser-His-Arg-Lys, corresponding to a region of rabbit cytochrome P450IIE1 (Khani et al., 1987) (region 6, Table I), and Gly-Arg-Asp-Arg-Gln-Pro-Arg-Leu, which occurs in both cytochromes P450IA1 (Sogawa et al., 1984) and P450IA2 (Sogawa et al., 1985) in the rat (region 8, Table I), their conjugation to a carrier protein, immunization of rabbits, and affinity purification of the antibodies were performed by using procedures described previously (Edwards et al., 1988, 1989b). Characterization of these antipeptide antibodies was by enzyme-linked immunosorbent assay (ELISA) as reported previously (Edwards et al., 1988). The binding titer was that amount of antibody that gave half-maximal binding to an antigen as determined by ELISA, expressed as the protein concentration of purified antibody preparations.

The characterization of the other antibodies described in this study has been published previously (Frey et al., 1985; Edwards et al., 1988, 1990).

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Identification of Antipeptide Antibody Binding Regions on Cytochrome P450. Ten regions of cytochrome P450 where antipeptide antibodies are known to bind to the native, membrane-bound isoenzymes have been identified (Table I). Information concerning the binding of antibodies to rat cytochrome P450IIB1 (regions 1, 2, 3, 5, 6, 9, and 10) has been drawn from the work of Frey et al. (1985) and De Lemos-Chiarandini et al. (1987). In these cases, binding was determined by electron microscopic analysis of the adsorption of gold-labeled antibodies to rat hepatic microsomal fraction. Only those antibodies which scored maximum binding (+++) have been considered in the present analysis. The binding of antipeptide antibodies to cytochromes P450IA1 and P450IA2 at regions 4 and 7 has been reported in detail elsewhere (Edwards et al., 1988, 1990). The binding of antipeptide antibodies to cytochrome P450IIE1 at region 6 and to cytochromes P450IA1 and P450IA2 at region 8 is described below.

Molecular Modeling. The models of membrane-bound cytochrome P450 were produced and manipulated by using the software package Desktop Molecular Modeller (Oxford Electronic Publishing) running on an IBM-PC compatible microcomputer. The coordinates for the C^{α} atoms for residues 10–414 of cytochrome P450CI were obtained from the Brookhaven Protein Data Bank.

The length of the N-terminal extension of the mammalian isoenzymes is thought to be approximately 23 residues (Edwards et al., 1989a). There is evidence that the majority of this forms a single membrane-anchor sequence (Sakaguchi et al., 1987; Monier et al., 1988; Szczesna-Skorupa et al., 1988; Edwards et al., 1989a; Vergères et al., 1989). The results of applying the algorithm of Garnier et al. (1978) suggest that this region forms an α-helix (Edwards et al., 1989a). However, it should be noted that the database from which this algorithm was derived did not contain any membrane-bound proteins. Nevertheless, in the present study, this region has been modeled as an idealised α-helix (3.6 residues/turn) (Chothia, 1984) where possible, of 32 residues in length and attached to residue 10 of cytochrome P450CI, the first N-terminal residue for which coordinates are available (Poulos et al., 1987). This represents 23 residues of extended structure plus the unassigned 9 N-terminal residues of cytochrome P450CI. Throughout this paper, the residues in this sequence are numbered from the N-terminus to the C-terminus as residues −23 to 9.

Results

The antibody raised against region 6 of cytochrome P450IIE1 was tested by ELISA against microsomal preparations obtained from rabbit livers. The animals were either untreated or treated with acetone to increase the specific content of cytochrome P450IIE1. The antipeptide antibody preparation had a binding titer of 7.0 μg/mL for microsomes from untreated rabbits and 1.5 μg/mL for those from acetone-treated rabbits. Thus, acetone treatment increased the specific binding of the anti-cytochrome P450IIE1 antibody by 5-fold, which is in accord with the known effect of this compound on the levels of cytochrome P450IIE1 (Koop et al., 1985).

The antibody raised against region 8 of rat cytochromes P450IA1 and P450IA2 was tested by ELISA against rat hepatic microsomal fractions from animals which were either untreated or treated with 3-methylcholanthrene to increase the specific contents of cytochromes P450IA1 and P450IA2. The antipeptide antibody preparation had a binding titer of 100 μg/mL for microsomes from untreated rats and 5 μg/mL for those from 3-methylcholanthrene-treated rats. Thus, there was a 20-fold increase in specific binding following 3-methylcholanthrene treatment, which is in accord with the known effect of this compound on the levels of cytochrome P450IA isoenzymes (Thomas et al., 1983).

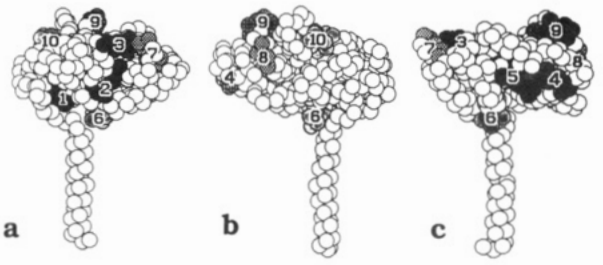

FIGURE 1: Model for membrane-bound cytochrome P450 with its heme parallel to the plane of the membrane. Only the C^{α} atoms have been represented in the model. The coordinates for residues 10–414 were obtained from the crystal structure of cytochrome P450CI, and to this a membrane-anchoring sequence has been modeled onto the N-terminus. This membrane-anchoring region, consisting of residues −23 to 9, has been depicted as an α-helix and is considered to span the membrane of the endoplasmic reticulum. Only the first 20–30 residues of the N-terminus, i.e., the "tail" region, are considered to be in contact with the membrane; the location of the globular portion of the protein is considered to be largely cytosolic with minimal contact with the membrane. The modeled protein (a) has been rotated around an axis running though the center of the protein and parallel to the anchoring helix by 120° (b) and 240° (c). The 10 predicted binding sites of the antipeptide antibodies have been shaded.

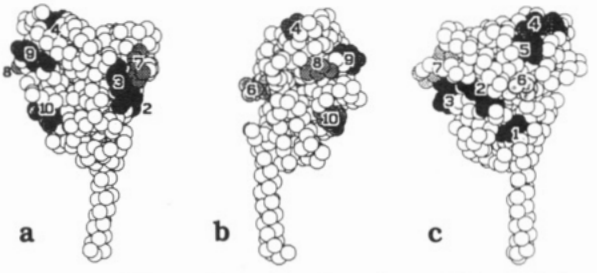

FIGURE 2: Model for membrane-bound cytochrome P450 with its heme perpendicular to the plane of the membrane. This model was constructed in a similar way to the structures shown in Figure 1, except that the heme is perpendicular to the membrane (a). The model has been rotated around an axis running though the center of the protein and parallel to the anchoring helix by 120° (b) and 240° (c) as in Figure 1. The 10 predicted binding sites of the antipeptide antibodies have been shaded.

The positions of the 10 antipeptide antibody binding sites were located on a model of cytochrome P450, based on the predicted positions of the α-helices, which has been described previously (Edwards et al., 1989a). Region 1 was placed between helices A and B. Region 2 was sited in the loop region prior to the C-helix. Region 3 was positioned at the beginning of the C-helix, region 4 at the C-terminal end of the D-helix and region 5 at the beginning of the E-helix. Region 6 covered the loop region between helices F and G. Region 7 spanned the C-terminal end of the G-helix to within 4 residues of the I-helix. Region 8 comprised a region including the C-terminus of the I-helix and the N-terminus of the J-helix. Regions 9 and 10 both occurred at parts of the protein predicted to be extended in the eukaryotic cytochromes P450 compared with cytochrome P450CI (Edwards et al., 1989a); these regions were midway between the J- and K-helices, and the K- and L-helices, respectively. These locations are all depicted in Table I.

All 10 of the binding sites for the anti-peptide antibodies were predicted to occur on the surface of cytochrome P450 (Figures 1 and 2). This is consistent with the results of studies showing that all of the antibodies bound to native, membrane-bound, microsomal cytochromes P450.

Table I: Location of the 10 Antipeptide Antibody Binding Sites on a Generalized Model of Eukaryotic Cytochrome P450 Based on the Structure of Cytochrome P450CI[a]

region	positions of synthetic peptides in various cytochromes P450	predicted position of antibody binding sites in model of cytochrome P450
1	IIB1 61–72	56–67
2	IIB1 108–116	92–99
3	IIB1 122–131	105–114
4	IA1 174–182 = IA2 171–179	138–146
5	IIB1 186–193	150–161
6	IIB1 225–232 and IIE1 227–234	183–192
7	IA2 290–296	224–230
8	IA1 356–363 = IA2 350–357	265–269
9	IIB1 315–323	274–282
10	IIB1 398–408	326–335

[a] The table details the positions of the synthetic peptides as they occur in rat cytochromes P450 (except for cytochrome P450IIE1 which was from the rabbit). The predicted binding sites of the antibodies raised against these peptides on the model of cytochrome P450 are shown. In regions 4 and 8, the respective peptide sequences were identical in both cytochromes P450IA1 and P450IA2. Two antipeptide antibodies were directed against region 6, one toward cytochrome P450IIB1 and the other toward cytochrome P450IIE1.

It is convenient to consider the orientation of the globular portion of the protein in terms of the position of its heme. All possible orientations have been considered, but the main features of these orientations can be summarized in two basic models in which the protein lies such that the heme is either parallel with or perpendicular to the surface of the membrane.

The model with the heme parallel to the membrane is depicted in Figure 1. This model has been drawn with the side containing the F- and G-helices lying against, or close to, the membrane. The N-terminal anchor runs directly from Asn-10 into the membrane. In this orientation, access of antibodies to regions 1 and 6, and possibly to regions 2, 4, and 5, is likely to be blocked by the membrane. It is also possible to model the protein with the opposite side of cytochrome P450 nearest to the membrane, but then access of the antibodies to regions 3, 7, 9, and 10 would be impaired.

The second model is shown in Figure 2. The protein is oriented with an edge adjacent to the membrane such that it is in an upright position with the heme held perpendicular to the plane of the membrane. To model this orientation, it was necessary to introduce a turn in the polypeptide backbone at residues 7–10 in order to join the N-terminal anchor to the remainder of the protein. In this orientation, all 10 of the antipeptide antibody binding sites are fully exposed and accessible in the cytosol.

Discussion

The importance of the N-terminal region of cytochrome P450 as a membrane insertion and anchoring sequence has been demonstrated by site-directed mutagenesis experiments. When rat cytochrome P450IIB1 is truncated by 43 residues at the N-terminus, it is still expressed but is not incorporated into membranes (Monier et al., 1988). Similarly, rabbit cytochrome P450IIC2 shortened by 25 residues at the N-terminus is not incorporated into the endoplasmic reticulum (Kemper & Szczesna-Skorupa, 1989). In contrast to these findings, Yabusaki et al. (1988) have reported that when rat cytochrome P450IA1 lacking 30 residues from the N-terminus was expressed in *Saccharomyces cerevisiae*, the shortened protein was found associated with the microsomal fraction. However, these authors did not determine whether attachment was by insertion or adsorption. Further evidence for the importance of the N-terminus of cytochrome P450 in membrane insertion has been obtained from studies of hybrid proteins composed of secretory proteins to which was attached the N-teminus of cytochrome P450. Such secretory proteins are normally translocated across the endoplasmic reticulum. However, the hybrid forms were incorporated into the endoplasmic reticular membrane (Sakaguchi et al., 1987; Monier et al., 1988; Szczesna-Skorupa et al., 1988).

The N-terminal region of cytochrome P450 also appears to contain a stop-transfer sequence. Monier et al. (1988) have shown that substitution of the 39 N-terminal residues of the secretory protein, rat growth hormone, for the 43 N-terminal residues of rat cytochrome P450IIB1 results in complete translocation of the cytochrome P450 across the membrane. Site-directed mutation of Asp-2 to Lys and of Leu-3 to Arg of rabbit cytochrome P450IIC2 converted the N-terminal sequence from a stop-transfer signal to a translocation signal.

De Lemos-Chiarandini et al. (1987) have shown that an antipeptide antibody directed to the N-terminal 31 residues of cytochrome P450IIB1 binds strongly to purified cytochrome P450, but not to intact microsomes. However, another antibody directed to residues 24–38 of the same cytochrome P450 bound equally to both microsomal and purified cytochrome P450IIB1. This shows that only the extreme N-terminal region is embedded in the microsomal membrane.

These studies clearly demonstrate the role of the N-terminal region of cytochrome P450 in anchoring the protein to the endoplasmic reticulum and also indicate that the length of this region is 20–30 residues. An N-terminal region of this length is sufficient to allow only a single transmembrane anchor, possibly in the form of a helix (Eisenberg, 1984). However, it has been suggested previously by several groups that this N-terminal region may comprise two transmembrane segments in the form of a hairpin, such that the N-terminus is at the cytosolic side of the membrane and a small region is exposed on the lumenal side of the membrane (Nelson & Strobel, 1988; Bernhardt et al., 1989; Brown & Black, 1989). This model was suggested to explain the observation that the N-terminus of rabbit cytochrome P450IIB4 can be labeled with fluorescein isothiocyanate (Bernhardt et al., 1983), a compound which was not expected to cross membranes (Nilsson et al., 1973). However, these labeling studies were performed using a purified preparation of the protein in the absence of the microsomal membrane. In such a preparation, those regions of the protein normally inaccessible to fluorescein isothiocyanate, due to their location in the membrane or on its lumenal side, will be fully exposed. This has been demonstrated by Vergères et al. (1989), who found that the N-terminus of purified rat cytochrome P450IIB1 was readily labeled when the protein was in solution but not when incorporated in liposomes.

From the foregoing, it is apparent that the overwhelming balance of evidence favors a model for cytochrome P450 in which the protein is attached to the membrane by a single N-terminal anchor of 20–30 residues. The rest of the protein, including the heme, would be located on the cytosolic side of the endoplasmic reticulum. This has been confirmed by studies using specific antibodies (Thomas et al., 1977; De Lemos-Chiarandini et al., 1987) and proteases (Vlasuk et al., 1982; Brown & Black, 1989) on whole microsomes. However, the orientation of the extramembranous globular portion of the protein with respect to the plane of the endoplasmic reticulum still has to be determined.

It has been shown previously that there is a large degree of similarity between the primary structures and the predicted secondary structures of cytochrome P450CI and eukaryotic

cytochromes P450 (Nelson & Strobel, 1988, 1989; Edwards et al., 1989a). Although there are some notable differences between these forms (Edwards et al., 1989a), there would appear to be sufficient similarity to use the structure of cytochrome P450CI as a basis for modeling the structure of the eukaryotic cytochromes P450 (Nelson & Strobel, 1988, 1989; Edwards et al., 1989a). There is good reason to believe that this approach is valid, as there is much evidence that the structure of proteins is conserved throughout evolution, often at the expense of amino acid sequence homology (Bajaj & Blundell, 1984; Blundell et al., 1987).

It has been suggested previously that eukaryotic cytochrome P450 is oriented such that its heme is parallel to the plane of the membrane (Nelson & Strobel, 1988). Therefore, such a model was considered first. Cytochrome P450CI can be regarded as a flattened triangular prism with two of the sides parallel to the plane of the heme (Poulos et al., 1987). When the model of eukaryotic cytochrome P450, based upon the structure of cytochrome P450CI, is viewed such that the protein is oriented with the heme parallel to the plane of the membrane, one of the sides must face the membrane with the other completely exposed to the cytosol. The position of residues 1–9 in cytochrome P450CI could not be determined by X-ray crystallography as they are too flexible (Poulos et al., 1987). Thus, Asn-10 was the first residue in the sequence that could be resolved. It is located on the side of cytochrome P450CI containing the F- and G-helices. Consequently, modeling of an N-terminal anchor is more readily accomplished if this side of the protein faces the membrane. If the N-terminal anchor were attached so that the other side of the enzyme faced the membrane, it would require an extension rather longer than appears to be available (an additional 30 Å), unless there is a large difference in the eukaryotic isoenzymes in the position of the residues equivalent to 10–35 of cytochrome P450CI.

Both of these orientations, with the heme parallel to the plane of the membrane, are consistent with the results of electron paramagnetic resonance studies performed by Rich et al. (1979) from which it was concluded that the heme of cytochrome P450 is held rigidly in the plane of the membrane surface. However, when these experiments were performed, it was assumed that cytochrome P450 is an integral membrane protein and it is not clear what effect the drying process involved in the preparation of samples would have on the structure of the heme-containing cytosolic portion of cytochrome P450. Further, the results of studies of the binding of antipeptide antibodies to native cytochromes P450 in the microsomal membrane are not compatible with a parallel orientation. In either of the possible parallel orientations of cytochrome P450, access of several of the antipeptide antibodies considered here, to their binding sites, would be prevented by the microsomal membrane. Yet all of the antibodies readily bind to the native, membrane-bound cytochromes P450, indicating unimpeded access to their respective binding sites. In addition, there is evidence that the electron-donating protein cytochrome b_5 interacts with residues Arg-72, Arg-112, Gln-343, Lys-344, Phe-350, and Arg-364 of cytochrome P450CI (Stayton et al., 1989). Cytochrome b_5 does not normally reduce cytochrome P450CI. However, if one assumes that its site of interaction with this isoenzyme is analogous to that of eukaryotic cytochromes P450, this would lie between regions 3 and 9. In either of the parallel orientations, such a binding site would not be accessible to cytochrome b_5, as this is also membrane-bound. With the side containing the L-helix nearer the membrane, this binding site would be occluded by the membrane, and with the side containing the F- and G-helices nearer the membrane, the binding site would be >30 Å above the membrane so that cytochrome b_5 would require an unfeasibly long anchoring sequence for such an interaction to occur. In addition, based upon the observations of Poulos et al. (1987), the hinged movement of the F- and G-helices, thought to occur during substrate binding and product release, is likely to be restricted if the side of the protein containing this region faces the membrane.

As the evidence was against the model described above, the other extreme was considered, in which cytochrome P450 is oriented with its heme perpendicular to the plane of the membrane. This model required the introduction of a turn in the polypeptide chain before the N-terminal membrane-anchoring sequence could be attached to Asn-10. The existence of such a turn is strongly supported by the presence of a number of conserved proline residues in this region of all eukaryotic cytochromes P450 sequenced to date. Proline residues are frequently associated with β-turns and loop regions (Chou & Fasman, 1978; Leszczynski & Rose, 1986).

The orientation of the heme group of cytochrome P450 with respect to the membrane has been investigated by flash photolysis using a preparation of purified cytochrome P450 reconstituted in a phospholipid membrane and suspended in a solution of 60% sucrose. Although it is possible that a cytochrome P450 may have a different orientation when reconsituted in a phospholipid membrane compared with a microsomal preparation, the conditions employed did conserve the integrity of the enzyme, as was determined by spectrophotometric measurements. Angles of 41°, 55°, and 71° between the plane of the heme of cytochrome P450 and the lipid membrane were calculated from the decay in absorption anisotropy (Kawato et al., 1982; Gut et al., 1983). It was not possible to determine which of these calculated angles was the most accurate (Kawato et al., 1982) because of the inadequacy of the mathematical model used to calculate the tilt angle. However, these results do provide evidence that the orientation of the heme with respect to the membrane is not parallel and probably exceeds 45°.

In the second model postulated, the active site of the protein is positioned away from the membrane. This conflicts with the view that substrates for cytochrome P450 gain access to the active site directly from the membrane, where they are thought to accumulate as a result of their hydrophobic nature (Parry et al., 1976; Ebel et al., 1978; Kühn-Velten et al., 1989). Evidence that the active site of cytochrome P450 is located in the endoplasmic reticulum comes from studies of the partitioning of compounds that induce spectral changes in cytochrome P450 when bound to its active site. Under conditions which caused such compounds to be extruded from the membrane, there was a simultaneous decrease in the magnitude of their cytochrome P450 binding spectra (Ebel et al., 1978; Taniguchi et al., 1984). Hence, the addition of substances that cause cytochrome P450 substrates to partition out of the membrane phase would be expected to result in a decrease in enzyme activity. However, the opposite effect has been observed. The addition of organic solvents has been shown to cause up to a 7-fold increase in aldrin epoxidase activity (Wolff et al., 1989), and the stimulatory effect of albumin on the activity of aryl hydrocarbon hydroxylase activity has long been recognized (Alvares et al., 1970; Nebert & Gielen, 1972).

The importance of protein in the partitioning of bilirubin has been investigated by Leonard et al. (1989). They observed that bilirubin partitions into lipid vesicles or microsomes in the absence of albumin but in its presence bilirubin partitions

out of the membrane phase. However, this does not occur on the addition of phospholipids or microsomal lipids to the aqueous phase, indicating that, like albumin, microsomal proteins may have a role in binding hydrophobic compounds. The effect of albumin on aryl hydrocarbon hydroxylase activity described by Alvares et al. (1970) was dependent on the concentration of microsomal protein present in the incubation mixture. At low microsomal protein concentrations, there was a large stimulation of activity by albumin, but this effect progressively decreased as the concentration of microsomal proteins present was increased. Only in the presence of albumin was the reaction rate proportional to the enzyme concentration. It would appear that albumin (or microsomal protein at sufficiently high concentrations) provides a means for the substrate to access the cytochrome P450 active site, possibly directly. Those experiments in which partitioning of cytochrome P450 substrates in the microsomal membrane has been studied were all performed in the absence of protein in the aqueous phase. Such systems are somewhat artificial because the fact that the cytosol normally contains a high concentration of protein (in the order of 100 mg/mL) is ignored. The results of such studies should therefore be interpreted with caution. Thus, although the active site of cytochrome P450 appears to be located some distance from the endoplasmic reticular membrane, cytosolic proteins, microsomal proteins, and lipids appear to form a contiguous hydrophobic phase from which substrate can partition to the active site of cytochrome P450, itself hydrophobic, without entering the aqueous phase of the system. Hydrophilic substrates may enter the active site directly from the cytosol. This provides a simple explanation of substrate binding and does not require a complex model of multiple substrate access channels for compounds of different hydrophobicities such as that postulated by Brown and Black (1989), for which there is no experimental evidence.

In the proposed model for cytochrome P450, in which its heme is perpendicular to the membrane, the protein has been positioned so that there is minimum contact with the membrane. However, it is also possible to model the protein while still perpendicular, so that one edge is in contact with the membrane, by tilting the protein by approximately 45° in either direction. However, the position of the antipeptide antibody binding sites does not support such contact, as binding sites for antibodies occur on both edges.

Although cytochrome P450 has been modeled with the heme oriented at 90° to the membrane, it has not been possible to determine the precise angle of the heme with respect to the membrane of the endoplasmic reticulum. However, conformations at or near the perpendicular are favored, as these allow the greatest access of the antibodies to their respective binding sites on the surface of the protein.

Both of the models of cytochrome P450 discussed have been considered as rigid (Figures 1 and 2). This is a reasonable view since there would be little flexibility within the N-terminal helix and this structure is likely to be attached through hydrophobic interactions to the body of the protein, which itself forms a compact globular structure and again this does not allow for much flexibility. Brown and Black (1989) have suggested that cytochrome P450 may adopt a rigid orientation. They postulated that, in addition to the N-terminal region, there may also be an interaction of the region equivalent to the J-helix of cytochrome P450CI with the membrane. Cytochrome P450 has a high hydrophobic moment at this position, and a peptide fragment from this region was obtained only after prolonged exposure of microsomes to trypsin followed by stringent washing. However, a high hydrophobic moment is indicative of a surface helix (Eisenberg, 1984); there is no information to suggest an interaction of such a structure with membranes; indeed, the high negative charge in this region may actually repel it from the membrane. Although the peptide fragment from this region was recovered after stringent washing, this does not mean that it was tightly bound because more than 85% of the protein was unaccounted for and may remain adhered to the microsomes. The possibility that the orientation adopted may be flexible, for example, oscillating between the two models illustrated (Figures 1 and 2), cannot be entirely rejected, but as there is no experimental evidence to support a flexible structure, we consider it more likely that cytochrome P450 is rigidly oriented in the endoplasmic reticulum. If both cytochrome P450 and the reductive proteins are held rigidly, then the proteins will be restricted to lateral and rotational movements only, and this should allow regions of electron transfer on these proteins to come into contact more efficiently than would occur if the proteins were highly flexible.

In conclusion, most of the available evidence supports a model in which cytochrome P450 is anchored to the membrane by a single transmembrane N-terminal helix of 20–30 residues in length with the globular part of the protein in the cytosol. The orientation of the cytosolic domain of the protein with respect to the membrane has also been considered. It is concluded that models of cytochrome P450 in which the heme lies parallel to the plane of the membrane are not supported by the results of a number of studies including those with antipeptide antibodies. Therefore, an alternative model has been considered in which the heme lies at or near perpendicular to the membrane. Such a model is supported by evidence from the binding of antipeptide antibodies, the proposed position of cytochrome b_5 binding, and the partitioning of cytochrome P450 substrates and allows free intramolecular movement of the protein during catalysis.

Registry No. Cytochrome P450, 9035-51-2; heme, 14875-96-8.

References

Alvares, A. P., Schilling, G., Garbut, A., & Kuntzman, R. (1970) *Biochem. Pharmacol. 19*, 1449–1455.

Bajaj, M., & Blundell, T. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 453–492.

Bernhardt, R., Ngoc Dao, N. T., Stiel, H., Schwarze, W., Friedrich, J., Jänig, G.-R., & Ruckpaul, K. (1983) *Biochim. Biophys. Acta 745*, 140–148.

Bernhardt, R., Kraft, R., & Ruckpaul, K. (1989) in *Cytochrome P-450: Biochemistry and Biophysics* (Schuster, I., Ed.) pp 320–323, Taylor & Francis, London.

Blundell, T. L., Sibanda, B. L., Sternberg, M. J. E., & Thornton, J. M. (1987) *Nature (London) 326*, 347–352.

Boobis, A. R., Brodie, M. J., Kahn, G. C., Fletcher, D. R., Saunders, J. H., & Davies, D. S. (1980) *Br. J. Clin. Pharmacol. 9*, 11–19.

Brown, C. A., & Black, S. D. (1989) *J. Biol. Chem. 264*, 4442–4449.

Chothia, C. (1984) *Annu. Rev. Biochem. 53*, 537–572.

Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251–276.

De Lemos-Chiarandini, C., Frey, A. B., Sabatini, D. D., & Kreibich, G. (1987) *J. Cell Biol. 104*, 209–219.

Ebel, R. E., O'Keeffe, D. H., & Peterson, J. A. (1978) *J. Biol. Chem. 253*, 3888–3897.

Edwards, R. J., Singleton, A. M., Sesardic, D., Boobis, A. R., & Davies, D. S. (1988) *Biochem. Pharmacol. 37*, 3735–3741.

Edwards, R. J., Murray, B. P., Boobis, A. R., & Davies, D. S. (1989a) *Biochemistry 28*, 3762–3770.

Edwards, R. J., Singleton, A. M., Boobis, A. R., & Davies, D. S. (1989b) *J. Immunol. Methods 117*, 215–220.

Edwards, R. J., Singleton, A. M., Murray, B. P., Sesardic, D., Rich, K. J., Davies, D. S., & Boobis, A. R. (1990) *Biochem. J. 266*, 497–504.

Eisenberg, D. (1984) *Annu. Rev. Biochem. 53*, 595–623.

Frey, A. B., Waxman, D. J., & Kreibich, G. (1985) *J. Biol. Chem. 260*, 15253–15265.

Garnier, J., Osguthorpe, D. J., & Robson, B. (1978) *J. Mol. Biol. 120*, 97–120.

Gonzalez, F. J. (1989) *Pharmacol. Rev. 40*, 243–288.

Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., & Kawato, S. (1983) *J. Biol. Chem. 258*, 8588–8594.

Hudecek, J., & Anzenbacher, P. (1988) *Biochim. Biophys. Acta 955*, 361–370.

Kawato, S., Gut, J., Cherry, R. J., Winterhalter, K. H., & Richter, C. (1982) *J. Biol. Chem. 257*, 7023–7029.

Kemper, B., & Szczesna-Skorupa, E. (1989) *Drug Metab. Rev. 20*, 811–820.

Khani, S. C., Zaphiropoulos, P. G., Fujita, V. S., Porter, T. D., Koop, D. R., & Coon, M. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 638–642.

Koop, D. R., Crump, B. L., Nordblom, G. D., & Coon, M. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4065–4069.

Kühn-Velten, N., Meyer, I., & Staib, W. (1989) *J. Steroid Biochem. 33*, 33–39.

Leonard, M., Noy, N., & Zakim, D. (1989) *J. Biol. Chem. 264*, 5648–5652.

Leszczynski, J. F., & Rose, G. D. (1986) *Science 234*, 849–855.

Monier, S., Van Luc, P., Kreibich, G., Sabatini, D. D., & Adesnik, M. (1988) *J. Cell Biol. 107*, 457–470.

Nebert, D. W., & Gielen, J. E. (1972) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 31*, 1315–1325.

Nebert, D. W., Nelson, D. R., & Feyereisen, R. (1989) *Xenobiotica 19*, 1149–1160.

Nelson, D. R., & Strobel, H. W. (1988) *J. Biol. Chem. 263*, 6038–6050.

Nelson, D. R., & Strobel, H. W. (1989) *Biochemistry 28*, 656–660.

Nilsson, R., Peterson, E., & Dallner, G. (1973) *J. Cell Biol. 56*, 762–776.

Ozols, J., Heinemann, F. S., & Johnson, E. F. (1985) *J. Biol. Chem. 260*, 5427–5434.

Parry, G., Palmer, D. N., & Williams, D. J. (1976) *FEBS Lett. 67*, 123–129.

Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687–700.

Rich, P. R., Tiede, D. M., & Bonner, W. D. (1979) *Biochim. Biophys. Acta 546*, 307–315.

Sakaguchi, M., Milhara, K., & Sato, R. (1987) *EMBO J. 6*, 2425–2431.

Sogawa, K., Gotoh, O., Kawajiri, K., & Fujii-Kuriyama, Y. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5066–5070.

Sogawa, K., Gotoh, O., Kawajiri, K., Harada, T., & Fujii-Kuriyama, Y. (1985) *J. Biol. Chem. 260*, 5026–5032.

Stayton, P. S., Poulos, T. L., & Sligar, S. G. (1989) *Biochemistry 28*, 8201–8205.

Szczesna-Skorupa, E., Browne, N., Mead, D., & Kemper, B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 738–742.

Taniguchi, H., Imai, Y., & Sato, R. (1984) *Biochem. Biophys. Res. Commun. 118*, 916–922.

Tarr, G. E., Black, S. D., Fujita, V. S., & Coon, M. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6552–6556.

Thomas, P. E., Lu, A. Y. H., West, S. B., Ryan, D., Miwa, G. T., & Levin, W. (1977) *Mol. Pharmacol. 13*, 819–831.

Thomas, P. E., Reik, L. M., Ryan, D. E., & Levin, W. (1983) *J. Biol. Chem. 258*, 4590–4598.

Vergères, G., Winterhalter, K. H., & Richter, C. (1989) *Biochemistry 28*, 3650–3655.

Vlasuk, G. P., Ghrayeb, J., Ryan, D. E., Reik, L., Thomas, P. E., & Levin, W. (1982) *Biochemistry 21*, 789–798.

Wolff, T., Wanders, H., & Guengerich, F. P. (1989) *Biochem. Pharmacol. 38*, 4217–4223.

Yabusaki, Y., Murakami, H., Sakaki, T., Shibata, M., & Ohkawa, H. (1988) *DNA 7*, 701–711.