The microsomal epoxide hydrolase has a single membrane signal anchor sequence which is dispensable for the catalytic activity of this protein

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The microsomal epoxide hydrolase (mEH) catalyses the hydrolysis of reactive epoxides which are formed by the action of cytochromes *P*-450 from xenobiotics. In addition it has been suggested that mEH might mediate the transport of bile acids. For the mEH it has been shown that it is co-translationally inserted into the endoplasmic reticulum. Here we demonstrate that the N-terminal 20 amino acid residues of this protein serve as its single membrane anchor signal sequence and that the function of this sequence can also be supplied by a cytochrome *P*-450 (CYP2B1) anchor signal sequence. The evidence supporting this conclusion is as follows: (i) the rat mEH and a CYP2B1–mEH fusion protein, in which the CYP2B1 membrane anchor signal sequence replaced the N-terminal 20 amino acid residues of mEH, was co-translationally inserted into dog pancreas microsomes in a cell-free translation system, whereas a truncated epoxide hydrolase with a deletion of the 20 N-terminal amino acid residues was not co-translationally inserted. (ii) The mEH and the CYP2B1–mEH fusion protein, but not the truncated epoxide hydrolase, were anchored in microsomes in a cell-free translation system and in membrane fractions derived from fibroblasts which expressed these proteins heterologously. These fibroblasts were also used to evaluate the significance of the mEH membrane anchor for the catalytic activity of mEH. The mEH, the truncated mEH and the CYP–EH fusion protein were found to be enzymically active. This result shows that the membrane anchor signal sequence of mEH is dispensable for the catalytic activity of this protein. However, truncated mEH was only expressed at low levels, which might indicate that this protein is unstable.

INTRODUCTION

The microsomal epoxide hydrolase (mEH; EC 3.3.2.3) plays a central role in the metabolism of several carcinogenic polycyclic aromatic hydrocarbons [1–4]. Cytochromes *P*-450 and the mEH catalyse, in this metabolism, a cascade of events culminating in the formation of dihydrodiol epoxides which are thought to be the ultimate carcinogenic metabolites of certain polycyclic aromatic hydrocarbons. It has been shown unequivocally for several microsomal cytochromes *P*-450, that they are co-translationally inserted into the endoplasmic reticulum (ER) [5] and are mainly exposed to its cytosolic face [6,7]. A co-translational insertion into the ER was also shown for mEH, and the N-terminus of this protein is not proteolytically processed during its insertion into membranes [8,9]. There is evidence that this enzyme has a similar orientation in the membrane to that of microsomal cytochromes *P*-450 [10,11]. However, a recent report [12] demonstrated that mEH was identical with a bile acid transport protein which could assume two opposite orientations within the membrane.

Theoretical calculations predict that the first 20 amino acid residues of mEH might serve as a membrane insertion signal sequence; however, other transmembrane domains have also been suggested for this enzyme [13]. In this report we have tried to find out whether or not the N-terminus of mEH serves as its single membrane anchor signal sequence in order to start to address the question of which structural features of mEH determine its orientation in the membrane of the ER.

For a cytochrome *P*-450, namely the bovine 17α-steroid hydroxylase, it has been shown, by its heterologous expression in COS 1 cells, that an anchor signal sequence is important for the catalytic activity of this protein [14]. However, for several cytochromes *P*-450 it has been demonstrated in a yeast system [15] and in bacterial expression systems [16,17], that their catalytic activities are retained in the absence of a membrane insertion signal. For cytochromes *P*-450 the structural constraints for catalytic activity might be rather strict, as this activity is not only dependent on the proper formation of the catalytic centres and on the incorporation of haem, but also on their interaction with cytochrome *P*-450 reductase and it appears that cytochromes *P*-450 do not fold correctly in a mammalian expression system upon deletion of their membrane anchor sequence. In the case of mEH one might expect less structural constraints as this enzyme requires neither a prosthetic group nor a second protein for catalytic activity. In this work we demonstrate that the natural membrane insertion signal of mEH is not necessary for the catalytic activity of this protein in mammalian cells.

MATERIALS AND METHODS

Construction of expression plasmids coding for full-length mEH

The expression plasmid coding for full-length rat mEH in a cell-free translation system was constructed by insertion of the *Eco*RI/*Sal*I mEH cDNA insert of the plasmid pEH52 [13] into the *Eco*RI/*Sal*I site of vector pSP65 (Promega). The mammalian expression vector coding for mEH was constructed by filling in the *Eco*RI/*Sal*I overhangs of the mEH cDNA fragment, derived from pEH52 using T4 DNA polymerase, and inserting this blunt-ended fragment into vector pMPSV [18], which had been

Abbreviations used: ER, endoplasmic reticulum; mEH, microsomal epoxide hydrolase; δmEH, truncated epoxide hydrolase; CYP–EH; cytochrome *P*-450–mEH fusion protein; PMSF, phenylmethanesulphonyl fluoride; FCS, fetal-calf serum; DMEM, Dulbecco's modified Eagle's medium.

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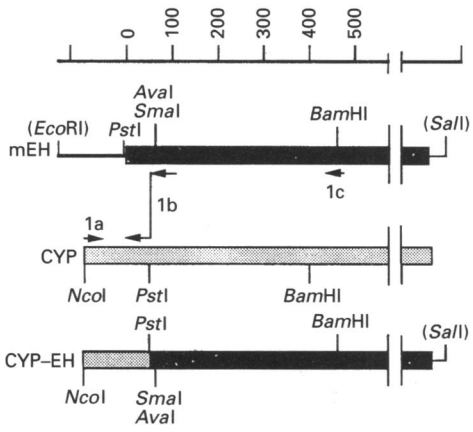
(a)



(b)

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--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
MET GLU PRO SER ILE LEU LEU LEU LEU ALA LEU LEU VAL GLY PHE
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--- --- --- --- --- --- --- --- --- --- --- --- MET TRP LEU
LEU LEU LEU LEU LEU VAL ARG GLY HIS PRO LYS SER ARG GLY ASN
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GLU LEU VAL LEU ALA SER LEU LEU GLY PHE VAL ILE TYR TRP PHE
PHE PRO PRO GLY PRO ARG PRO LEU PRO LEU LEU GLY ASN LEU LEU
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VAL SER ARG ASP LYS GLU....mEH
GLN ALA ARG ASP LYS GLU....CYP-EH
MET ALA ARG ASP LYS GLU....dEH
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Figure 1 Strategy for the generation of CYP–EH cDNA and amino acid sequence of mEH constructs

(**a**) The restriction maps of the various cDNAs are drawn to scale in the regions starting with the 5′ end of the cDNAs to the interrupted part of the scale. Restriction sites in parentheses are the cloning sites of mEH cDNA in the SKII vector (Stratagene). The numeration of the scale starts with the initiation codon of the mEH cDNA. mEH and CYP2B1 cDNA are aligned at the site which was used for the fusion of both cDNAs to generate the CYP–EH cDNA. The coding region of mEH cDNA is indicated by a black box and that of CYP2B1 cDNA by a shaded box. Untranslated regions are represented by a line. The designation of the PCR-primers is that used in the text. The fusion primer (1b) contains at its 5′ end sequences complementary to the mEH cDNA and at its 3′ end sequences complementary to the CYP2B1 cDNA. Note, that in the final complete CYP–EH cDNA, only the *NcoI*/*SmaI* fragment is derived from the PCR-amplified DNA. (**b**) The N-terminal sequences of mEH, δmEH (dEH) and CYP–EH fusion protein are displayed. The sequences are aligned at the site where all three sequences become identical. Dashes in the protein sequence indicate the absence of amino acid residues.

previously linearized with *Bam*HI and had been made blunt-ended using T4 DNA polymerase.

Construction of expression plasmids coding for N-terminally truncated mEH

An *AvaI*/*SalI* mEH cDNA restriction fragment was isolated from plasmid pEH52 (for the location of these restriction sites: see Figure 1). This shortened mEH cDNA fragment lacks the region of the cDNA coding for the first 20 amino acid residues of mEH. In order to supply the initiation codon for protein biosynthesis to this shortened cDNA, we applied the procedure of Haymerle et al. [19] as follows: an unphosphorylated *NcoI* linker (GCCATGGC) was ligated to the previously blunt-ended *AvaI*/*SalI* ends of the truncated mEH (δmEH) cDNA restriction fragment. The recipient pMPSV expression vector for this fragment was linearized with *Bam*HI, blunt-ended using T4 DNA polymerase and also ligated to the unphosphorylated *NcoI* linker. For ligation of the cDNA fragment into the vector, the linker-modified shortened mEH cDNA fragment and the linearized linker-modified pMPSV vector were mixed, heated to 65 °C and passed through a Sephacryl S-1000 (Pharmacia) molecular-sieve column at 65 °C. Under these conditions the unligated strands of the *NcoI* linker are removed from both the insert and the vector to produce compatible sticky ends on both DNAs which were then ligated to each other using T4 DNA ligase. This construct (designated as pMPSV δmEH) was used for the heterologous expression of the truncated mEH in fibroblasts. The in frame ligation of the *NcoI* linkers to δmEH cDNA was verified by DNA sequencing.

In order to obtain a template for the cell-free translation of shortened mEH, the cDNA insert of pMPSV δmEH was removed by an *EcoRI*/*SalI* double restriction digest and inserted into the appropriately linearized transcription vector pSP65.

Construction of expression plasmids coding for the CYP2B1–mEH fusion protein

The fusion of CYP2B1 cDNA to mEH cDNA was achieved by using a procedure which enables two non-complementary cDNAs to ligate at any site [20]. A sample (1 ng) of plasmid pEH52 and 1 ng of plasmid pSV450, containing the entire coding region of the CYP2B1 cDNA [21], were co-amplified by PCR (30 cycles) in the presence of a 40000-fold excess of primers 1a (ACCATGGAGCCCAGTATC) and 1c (CTCATAGAAGGATCCAGG) and a 400-fold excess of the linking primer 1b (GGTTTCCTCCTTGTCCCGGGACTGCAGGAGGTTCCCC) (see Figure 1). The temperatures for denaturing, annealing and primer extension were 94 °C, 59 °C and 70 °C respectively. The resulting amplified CYP–EH cDNA fragment contained at its 5′ end an *NcoI* restriction site which includes the natural initiation codon of the CYP2B1 cDNA. At the 3′ end the PCR fragment includes the unique *Bam*HI site of mEH cDNA. The amplified fragment was inserted via its dA overhangs, which had been generated by the action of the *Taq* DNA polymerase into the complementary ends of the *XcmI*-linearized vector pDK101 [22]. From the resulting plasmid a cDNA fragment stretching from the *NcoI* site to the *SmaI* site of the fused cDNA (see Figure 1) was removed by an *NotI*/*SmaI* double digest (*NotI* is a restriction site in the polylinker of pDK101, 5′ to the cloning site of the PCR fragment) and inserted into the vector SKII(+) (Stratagene) containing the full-length mEH cDNA which had been previously linearized with *NotI* and *SmaI* in order to remove parts of the 5′ region of the mEH cDNA. In this way only a small part of the mEH cDNA, including its 5′ untranslated region and the first 61 bases of its coding region, were replaced by the PCR-fused CYP2B1–mEH cDNA fragment. From the resulting vector, SKII(+) CYP–EH, the complete CYP–EH cDNA insert was removed by an *NcoI*/*SalI* double digest, made blunt-ended by the action of T4 polymerase and inserted either into the mammalian expression vector pMPSV, which had been previously linearized by an *EcoRI*/*SalI* double digest and which had been blunt-ended by T4 DNA polymerase to yield the plasmid pMPSV CYP–mEH, or into the transcription plasmid pSP65, which had been linearized by *SmaI* to yield the plasmid SP65 CYP–mEH. The correct sequence of the template was verified by the DNA-sequence analysis of those parts of the template which were derived from the PCR reaction.

Cell-free transcription and translation

The SP6 promoter plasmids containing the various cDNA constructs were directly used for cell-free coupled transcription/

translation, which uses reticulocyte lysate (Promega) as the translation system. Conditions for the coupled transcription/translation reaction in the presence of [^{35}S]methionine were as described by the manufacturer (Promega). Protein biosynthesis was performed either in the presence or in the absence of dog pancreas microsomes (Promega) or alternatively these membranes were added post-translationally after EDTA (5 mM final) had been added to the translation assay. The translation products were analysed by SDS/PAGE analysis [23].

Heterologous expression of mEH constructs in BHK21 fibroblasts

The BHK21 Syrian hamster kidney fibroblast cell line was used for the heterologous expression of the various mEH cDNA constructs, as it is known that these cells are almost devoid of an endogenous mEH enzyme activity [24]. In addition we were not able to detect the endogenous expression of mEH in these cells by immunoblotting. The cDNAs in expression vector pMPSV were co-transfected together with the plasmid LK444, which confers G418-resistance [25], into the fibroblasts as described in ref. [21]. G418-resistant clones were analysed by immunoblotting using a polyclonal anti-(rat mEH) serum. Three clones having the highest level of expression for either mEH or CYP2B1–mEH fusion protein or mEH with a deleted N-terminus were chosen for further analysis.

Membrane integration assay

In order to assay for the integration of mEH and modified mEH constructs into dog pancreas microsomes in a cell-free translation system, the translation mixtures (see above) were alkaline-extracted with sodium carbonate, pH 11.5, and pelleted by centrifugation as described [7], except that the translation mixtures were not adjusted with NaOH to pH 11.5 but were diluted 1:10 with 0.1 M sodium carbonate, pH 11.5. The pellets and the supernatants were analysed by SDS/PAGE followed by fluorography.

In order to assay for the integration of mEH and modified mEH constructs in a cellular system BHK21 fibroblasts, which express these proteins heterologously, were harvested by a short trypsin treatment, washed in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal-calf serum (FCS) and homogenized in a glass–Teflon homogenizer with a tight-fitting pestle in buffer A [0.1 M sucrose, 10 mM Hepes, pH 7.4, 1 mM EDTA and 0.5 mM phenylmethanesulphonyl fluoride (PMSF)]. The homogenate was first centrifuged at 4000 ***g*** for 10 min at 4 °C and the resulting supernatant was centrifuged at 150000 ***g*** in an airfuge for 15 min. The pellet was either resuspended in buffer A or in 0.1 M sodium carbonate buffer and layered either on buffer A containing 0.25 M sucrose or on an alkaline sucrose cushion as described [26]. The membrane fraction was pelleted by centrifugation as performed for the analysis of the cell-free translation products. Analysis of the resulting membrane fractions and supernatants was carried out by immunoblotting [27] using a polyclonal anti-(rat mEH) antibody. The immunoblot was developed by a fluorographic exposure using the ECL system (Amersham).

Determination of cellular mEH enzyme activity

The mEH enzyme activity of the various BHK21 fibroblast cell lines was determined as follows: the fibroblasts were grown in cell culture, isolated by mild trypsinolysis and washed with DMEM containing 10% FCS followed by phosphate-buffered saline. The cells were resuspended in KBS (10 mM sodium phosphate buffer, pH 7.4, 110 mM KCl, 1 mM EDTA, 60 units/ml Trasylol, 0.5 mM PMSF) and were lysed by sonication on ice. The enzyme activity of the lysed cells was determined with 0.6–1 mg of cellular protein using benzo[a]pyrene 4,5-oxide as substrate [28]. Under these conditions the enzyme activity was linear with time and protein concentration.

RESULTS

Construction of templates coding for modified mEH proteins

The N-terminal sequences of mEH, CYP2B1–mEH fusion protein (in which the 20 N-terminal amino acids of mEH had been replaced by the membrane anchor sequence of CYP2B1) and mEH with an N-terminal deletion of 20 amino acids is given in Figure 1. The N-terminal sequence of mEH contains a negatively charged amino acid residue at position 4 followed by a hydrophobic core of 16 amino acid residues. This core precedes a stretch of 15 amino acid residues containing positively and negatively charged amino acids followed by the remaining peptide chain. In mEH with a shortened N-terminus, the negatively charged amino acid residue and the hydrophobic core is removed. In CYP2B1–mEH fusion protein a negatively charged amino acid residue at position 2 is followed by a hydrophobic core of 19 residues. This core is followed by a stretch of 15 amino acid residues containing five positive charges followed by the remaining peptide chain.

Cell-free translation of mEH and modified mEH proteins

In order to study whether the N-terminal sequence of mEH is necessary for co-translational insertion and anchoring of this protein into microsomal membranes, we translated mEH, truncated mEH and CYP2B1–mEH fusion protein in the absence and presence of dog pancreas microsomes. The translation mixtures were subsequently subjected to alkaline treatment and membrane fractions were recovered by centrifugation. To assay for non-specific binding of the translated proteins to membranes, membranes were also added post-translationally before centrifugation. The results of this experiment are displayed in Figure 2. mEH and CYP2B1–mEH fusion protein which were translated in the presence of the microsomes were found almost exclusively in the microsomal pellet, whereas shortened mEH, translated under identical conditions, was alkaline-extracted into the supernatant after centrifugation. Alkaline extraction of translational mixtures which did not contain membranes, or which had been supplied with membranes post-translationally, followed by SDS/PAGE analysis showed that approximately equal amounts of the *in vitro*-translated mEH and the CYP2B1–mEH fusion protein were found in the supernatant and in the pellet after centrifugation. Under these conditions shortened mEH was mainly found in the supernatant. In another experiment (results not shown) mEH and modified mEH proteins were translated in the presence of dog pancreas microsomes and the membranes were treated either with an alkaline solution as above, or with 20 mM Tris buffer, pH 8.0. Again as for the experiment described above mEH and CYP2B1–mEH fusion protein were not alkaline-extracted from the membranes, whereas truncated mEH was. The treatment of the membranes at pH 8.0, however, extracted much less shortened mEH from the membranes than did the alkaline treatment, indicating that under near neutral conditions the shortened mEH remained associated with the membranes.

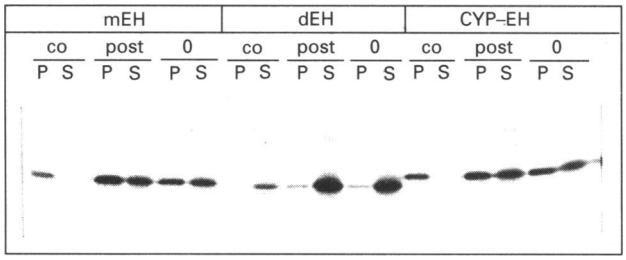

Figure 2 Cell-free membrane integration assay

In vitro translation of mEH, δmEH (dEH) and CYP–EH fusion protein was performed in the presence (co) or absence (0) of dog pancreas microsomes. In the experiments designated as post, the microsomes were added post-translationally. After the translation was completed, the translational assays were treated with an alkaline solution and centrifuged. The resulting pellets (P) and supernatants (S), each a third of the original translation assay, were analysed by SDS/PAGE followed by fluorography.

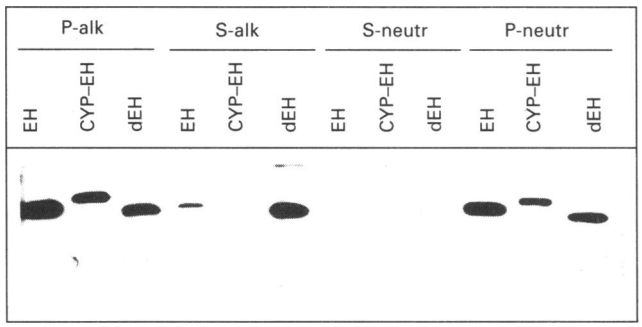

Figure 3 Cellular membrane integration assay

mEH, δmEH (dEH) and CYP–EH cDNA were expressed heterologously in fibroblasts. The cells were isolated, disrupted by homogenization, and membrane fractions were recovered as described in the Materials and methods section. The resulting membranes were treated either with an alkaline (alk) or with a near neutral (neutr.) pH solution and were recovered by centrifugation. The resulting pellets (P) or supernatants (S) were analysed by SDS/PAGE followed by immunoblotting, using as the first antibody a polyclonal anti-mEH serum. Only a portion of the membrane and the supernatant fractions resulting from the last centrifugation step were analysed. For the membranes these were 2.5, 5, or 25 mg of protein derived from fibroblasts expressing mEH, CYP–EH or δmEH respectively. The corresponding portion of the supernatants was analysed in each case.

Membrane integration of mEH and modified mEH proteins in fibroblasts

In order to verify whether the mode of membrane integration found for mEH and modified mEH proteins in a cell-free translation system faithfully reflected this process in an intact cell, we expressed these proteins in BHK21 hamster kidney fibroblasts and analysed for the integration of mEH, CYP2B1–mEH fusion protein and shortened mEH into cellular membrane fractions. The membranes were either treated with an alkaline solution or with a near neutral solution (see the Materials and methods section) and were isolated by centrifugation. The resulting supernatants and pellets were analysed by SDS/PAGE, followed by immunoblotting using an anti-mEH serum as the first antibody. The results of this experiment are displayed in Figure 3. After alkaline and neutral treatment of the membrane fraction, mEH and CYP2B1–mEH fusion protein remained mainly integrated in the membrane fractions. The shortened mEH was, however, extracted from membrane fractions mainly at alkaline pH and not so much under near neutral pH conditions. Even the inclusion of 1 M KCl in the neutral extraction buffer did not result in the membrane extraction of δmEH (results not shown). Thus it can be said that in the intact mammalian cell, as well as in a cell-free system, mEH as well as CYP2B1–mEH fusion protein are integrated into the membranes, whereas truncated mEH is only peripherally associated with membranes.

Table 1 mEH enzyme activity and concentration of mEH-related proteins in the parental BHK21 cell line and in BHK21 cells which express mEH and modified mEH proteins heterologously

The cellular mEH enzyme activity towards benzo[a]pyrene 4,5-oxide was measured. The enzyme activity of each cell line was determined in three separate pools of cells. The enzyme assays were done in triplicate for each pool. The cellular concentration of the heterologously expressed proteins was determined by immunoblotting of the cellular homogenates from a single cell-harvest of each cell line. Serial dilutions (four concentrations) of the cellular protein of each cell line were analysed in two separate immunoblots. As standard 10.0; 5.0; 2.5 and 1.25 ng of a homogeneous mEH preparation were mixed with 50 μg of cellular protein of BHK21 cells and analysed by immunoblotting. The absorbance of the immunosignals was integrated. The intensity of the immunoblot signals was linear with the amount of protein analysed. The first antibody was an anti-(rat mEH) serum raised in goat. Abbreviation: n.d., non detectable.

Cell line	Specific mEH activity (pmol/min per mg)	[mEH-related protein] (mg/mg of cellular protein)	Relative catalytic turnover number*
BHK21	1.2 ± 0.4	n.d.	n.d.
BHK-mEH	266.0 ± 5.6	1.0	1.0
BHK-CYP–mEH	65.4 ± 3.4	0.6	0.4
BHK-δmEH	9.3 ± 0.9	0.05	0.7

* Defined as the ratio of the catalytic turnover number of mEH in BHK-mEH cells to the catalytic turnover number of modified cellular mEH proteins and calculated from the specific mEH activities found in the cellular protein extracts and the cellular concentration of the heterologously expressed proteins.

Catalytic function of mEH and of modified mEH proteins

The catalytic function of mEH, CYP2B1–mEH fusion protein and of truncated mEH was evaluated in cells which stably express these proteins heterologously. As a control this enzyme activity was determined in the parental cell line. The mEH enzyme activity was determined using benzo[a]pyrene 4,5-oxide. This enzyme assay was shown to be highly sensitive [28]. Enzyme activity was determined after the cells had been broken by means of a short ultrasonification at 4 °C. The mEH enzyme activity of the cellular homogenates is given in Table 1. Cells expressing mEH or modified mEH proteins had an mEH enzyme activity which was clearly higher than that of BHK21 cells. Cells expressing mEH had 4-fold and 28-fold higher mEH enzyme activity compared with CYP–EH- or δmEH-expressing cells respectively.

In order to correlate mEH enzyme activity of the various cell lines with the level of heterologously expressed proteins, we determined their level of expression by immunoblotting using a serial dilution of the cellular homogenates and of a homogeneous epoxide hydrolase preparation. The first antibody in this assay was an antiserum directed against mEH. For quantification, the fluorography of the immunoblot was scanned and the signal intensities obtained for mEH and modified mEH proteins were integrated. The signal intensities were linear with protein concentration. By comparison of the signal intensities obtained for purified mEH with those obtained for heterologously expressed proteins, we estimated the content of these proteins in cellular homogenates. These values are given in Table 1. In this ex-

periment we found that the cellular expression levels of CYP–EH fusion protein and of δmEH were lower than the heterologous expression of mEH by a factor of two and 20 respectively. Thus the catalytic turnover number of δmEH is only about 30% less than that of mEH, whereas CYP–EH displayed 40% of the catalytic activity of mEH (see Table 1).

DISCUSSION

Based on theoretical calculations, the N-terminal 20 amino acid residues of mEH are the most hydrophobic domains of human, rat and rabbit mEH [10]. Hydropathy analysis using two different methods indicated that mEH was either anchored only via its N-terminal region into the membrane [10] or contained several transmembrane loops [13]. Some experimental evidence supports the former model. Craft et al. [10] employed a membrane impermeant fluorescent probe which binds covalently to proteins to evaluate whether mEH is mainly exposed to the lumen of the ER or to its cytosolic side. They found that this probe labelled mEH but not UDP-glucuronosyltransferases, which are thought to be entirely exposed to the luminal face of the ER. Thus these authors concluded, on the basis of this experiment and the hydropathy profile of the mEH, that this protein is exclusively exposed to the cytosolic face of the ER and is anchored into the membrane most likely via its N-terminus. However, very recently [12] a bile acid transport protein, which was identified as being identical with mEH, was found to assume two topological orientations in the membrane as shown by the accessability of an epitope to its cognate antibody and by protease protection experiments in the presence of an intact membrane.

In the present work, we were able to show that the 20 N-terminal amino acid residues of mEH function as its sole membrane anchor signal sequence. In addition we demonstrated that the function of this signal can also be mediated by a cytochrome *P*-450 (CYP2B1) membrane anchor sequence. This conclusion is clearly supported by the results obtained in a cell-free system (Figure 2) and in a cellular system (Figure 3). The mEH and CYP2B1–mEH fusion protein which had been translated in a cell-free system in the presence of dog pancreas microsomes remained in these microsomes after their alkaline extraction followed by centrifugation (Figure 2). Under these conditions shortened mEH was almost exclusively found in the supernatant. mEH and CYP2B1–mEH fusion protein, which had been synthesized in the absence of microsomes, were found in equal amounts in the pellet and in the supernatant, independent of whether microsomes were added post-translationally or not. The presence of some mEH and CYP2B1–mEH fusion protein in the pellet, in the absence of microsomes, could be explained by the aggregation of these newly synthesized proteins in the absence of microsomal membranes during the translation reaction. Similar observations were made by others studying the membrane integration of cytochromes *P*-450 [7]. In these experiments it was found that cytochromes *P*-450, when translated in the absence of the signal recognition particle but in the presence of microsomes, were also partially recovered in the pellet after alkaline extraction and centrifugation of the translation mixture. This is most likely due to an aggregation of the newly synthesized cytochrome *P*-450 in the absence of its co-translational insertion into membranes.

Because it is known that the cell-free translation system might not mimic the cellular integration of some proteins faithfully [29], we had to verify whether mEH and CYP2B1–mEH fusion protein, but not truncated mEH, would also be anchored in the membrane in intact cells. For this purpose we stably expressed these proteins in BHK21 hamster kidney fibroblasts. We chose this cell line because it is known to be almost devoid of an endogenous mEH enzyme activity as determined with benzo[a]pyrene 4,5-oxide [24]. In addition we could not detect the expression of an endogenous hamster mEH by immunoblotting using a polyclonal anti-(rat mEH) serum.

The results displayed in Figure 3 show that, as in the cell-free system, heterologously expressed mEH and CYP2B1–mEH fusion protein, but not truncated mEH, were anchored in membrane fractions as the former two proteins, but not the latter protein, were resistant to alkaline extraction of cellular membrane fractions. However, after extraction at near neutral pH, all three proteins remained associated with the membrane fractions. The latter result indicates that shortened mEH may be peripherally associated with cellular membranes.

Our results do not establish whether mEH and CYP2B1–mEH fusion protein were localized in the ER of the BHK21 hamster kidney fibroblasts, or in other subcellular membrane compartments, as we did not attempt to perform a subcellular fractionation of the fibroblast membranes. However, studies employing immunofluorescence microscopy of BHK21 fibroblasts which express mEH heterologously, indicate at least that this protein was largely associated with membrane structures resembling ER and/or Golgi membranes (results not shown).

In order to study whether the 20 N-terminal amino acid residues of mEH are not only necessary for its co-translational insertion into cellular membranes but also for the catalytic activity of mEH, we determined the enzyme activity of rat mEH and modified mEH proteins in the above-mentioned fibroblasts, which stably express these proteins heterologously. We used benzo[α]pyrene 4,5-oxide as the substrate for this assay. The stable expression system was preferred to a transient expression system because it is known that in the latter system the heterologous expression level of a protein might vary from one transfection to the next [30], whereas in the stable expression system these difficulties are not encountered, once a stable expressing cell clone has been selected.

We found that the expression level of δmEH was rather low. Screening of further clones which had been transfected with the template coding for δmEH did not yield clones expressing higher levels of this protein. Also others have found that the expression of a truncated membrane protein (cytochrome *P*-450) which was devoid of a membrane anchor signal sequence led to a 5-fold reduction in the expression level of this protein as compared with the full-length membrane protein [14]. This might be due to toxic effects of the truncated protein for the cell expressing it or due to degradation of the truncated protein.

The δmEH-expressing cells had a 28-fold lower mEH enzyme activity compared with mEH-expressing cells. However, the cellular expression level of δmEH was also found to be 20-fold lower than that of mEH (Table 1). We conclude from these results that the catalytic activity of δmEH is approximately as high as that of mEH. Therefore the membrane anchor signal sequence of mEH, unlike the analogous sequences in cytochromes *P*-450, appears to be dispensable for the catalytic activity of mEH in a mammalian system.

In contrast with the low expression of δmEH in BHK21 cells, a rather high level of expression was achieved for the CYP–EH fusion protein. However, comparison of mEH enzyme activity found for the CYP–EH-expressing cells with the expression level of this protein (Table 1) indicates that the catalytic activity of CYP–EH is a factor of two lower than that of mEH. However, we found some variability of mEH enzyme activity of the CYP–EH-expressing cells. Therefore we can not conclude whether the CYP–EH fusion protein has exactly the same catalytic activity as mEH. Nevertheless our data indicate that the

membrane anchor of mEH can be functionally replaced by another membrane anchor. This result opens up the possibility of altering the membrane orientation of mEH by site-directed mutagenesis of charged amino acid residues flanking the mEH membrane anchor signal sequence and of determining the consequences of this alteration for the catalytic activity of this protein. More importantly the impact of the cellular orientation of mEH on its toxicological function can thus be studied.

The membrane orientation of single membrane-spanning ER proteins was shown to be dependent on the charge difference [Δ (C-N)] of charged amino acid residues preceding the hydrophobic core of the membrane anchor and charged amino acid residues following this hydrophobic core [31,32]. For most cytochromes *P*-450 this charge difference is positive and these proteins have an N_{exo}/C_{cyt} orientation in the membrane of the ER. This orientation is also expected for the CYP2B1–mEH fusion protein described in the present work. However, for mEH of the human, the rat and the rabbit this charge difference has a value of -2, -1 and -3 respectively. This value would indicate that these proteins have an N_{cyt}/C_{exo} orientation in the membrane of the ER. Experimental data indicate that the rat mEH has an N_{exo}/C_{cyt} orientation in the membrane [10,11]; however, these findings are at variance with a recent report on the membrane topology of mEH [12].

For a cytochrome *P*-450, namely CYP2C2, it has been shown that this protein has an N_{exo}/C_{cyt} orientation in the ER and that replacing the negatively charged amino acid residue preceding the hydrophobic core of the membrane anchor of this protein by a positively charged amino acid residue converted the mutated CYP2C2 into a protein which was exposed to the lumen of the ER [7]. However, to our knowledge the catalytic activity of this mutated cytochrome *P*-450 has not as yet been reported. We are trying at present to invert the orientation of mEH in the membrane of the ER by replacing the negatively charged amino acid residue preceding the hydrophobic core of the mEH membrane anchor by positively charged amino acid residues. As the catalytic activity of mEH, unlike that of cytochromes *P*-450, is not dependent on ancillary proteins (i.e. cytochrome *P*-450 reductase) and the incorporation of a prosthetic group, it might be possible that by altering the orientation of mEH in the ER the catalytic activity of this protein would not be impaired. This experiment would clarify whether the functional folding of integral membrane proteins proceeds in a similar manner on both sides of the membrane of the ER in a mammalian cell. It has recently been shown that an mEH which was secreted into the periplasmic space of bacteria was catalytically active [33]. However, it has been argued that the functional folding of integral membrane proteins like cytochromes *P*-450 proceeds differently in bacteria and in mammalian cells [14,16,17].

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