Solution structure and topology of the N-terminal membrane anchor domain of a microsomal cytochrome P450: prostaglandin I₂ synthase

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The three-dimensional structure of a synthetic peptide corresponding to the N-terminal membrane anchor domain (residues 1–25) of prostaglandin I₂ synthase (also known as cytochrome P450 8A1), an eicosanoid-synthesizing microsomal cytochrome P450, has been determined by two-dimensional ¹H NMR spectroscopy in trifluoroethanol and dodecylphosphocholine which mimic the hydrophobic membrane environment. A combination of two-dimensional NMR experiments, including NOESY, TOCSY and double-quantum-filtered COSY, was used to obtain complete ¹H NMR assignments for the peptide. Using the NOE data obtained from the assignments and simulated annealing calculations, the N-terminal membrane domain reveals a bent-shaped structure comprised of an initial helix (residues 3–11), followed by a turn (residues 12–16) and a further atypical helix (residues 17–23). The hydrophobic side chains of the helix

and turn segments (residues 1–20) are proposed to interact with the hydrocarbon interior of the phospholipid bilayer of the endoplasmic reticulum (ER) membrane. The hydrophilic side chains of residues 21–25 (Arg-Arg-Arg-Thr-Arg) point away from the hydrophobic residues 1–20 and are expected to be exposed to the aqueous environment on the cytoplasmic side of the ER membrane. The distance between residues 1 and 20 is approx. 20 Å (1 Å = 0.1 nm), less than the thickness of a lipid bilayer. This indicates that the N-terminal membrane anchor domain of prostaglandin I₂ synthase does not penetrate the ER membrane.

Key words: cytochrome P450, membrane topology, NMR, synthetic peptide.

INTRODUCTION

Prostaglandin I₂ (PGI₂) is a potent inhibitor of platelet aggregation, vasoconstriction and leucocyte interaction with endothelium [1]. It plays an important role in a wide variety of physiological and pathological processes, such as haemostasis, atherogenesis, thrombosis and stroke. The biosynthesis of PGI₂ involves the efficient co-ordination of catalysis by prostaglandin H₂ (PGH₂) synthase (PGHS), which converts arachidonic acid into PGH₂ on the luminal side of the endoplasmic reticulum (ER), and PGI₂ synthase (PGIS), which converts PGH₂ into PGI₂ on the cytoplasmic side of the ER.

The eicosanoid-synthesizing cytochrome P450 (P450) PGIS has been purified from various tissues [2–4]. Human and bovine PGIS cDNAs have been cloned and sequenced and the amino acid sequences deduced [5]. Both PGIS proteins contain 500 amino acids with a calculated molecular mass of approx. 57 kDa. As a member of the P450 superfamily, PGIS shares considerable primary and secondary structural similarity with other microsomal P450s [5]. Structural and topological studies of the PGIS membrane anchor domain can directly provide valuable information in understanding how the co-ordination of the two ER-membrane proteins PGHS and PGIS is achieved in the biosynthesis of PGI₂.

Crystallographical structures are known for several soluble P450s, including P450cam [6], P450BM3 [7], P450ter [8] and engineered microsomal P450 2C5 with deletion of the N-terminal membrane anchor domain [9]. Several three-dimensional (3D) structural models of the catalytic domains of mammalian P450s have been constructed by homology modelling on the basis of the

crystal structures of the soluble P450s [10–13]. However, the structures of the N-terminal membrane anchors of mammalian P450s could not be modelled, because the soluble P450s used as templates lacked N-terminal membrane anchor domains. Many efforts have been made to elucidate aspects of the structures of N-terminal membrane anchor domains and to characterize their membrane-binding properties, including computer prediction [14], molecular modelling [13], protease susceptibility [15], chemical modification [16], genetic engineering [17] and immunocytochemistry [18] studies. As a result, a topology model for microsomal P450s has been proposed in which a large cytoplasmic domain of the protein is anchored to the membrane by one or two N-terminal transmembrane segments [14,19,20]. Recently, the more widely accepted structural model for the microsomal P450s has only one transmembrane segment in the N-terminal region, with the N-terminus itself exposed on the luminal side of the ER. However, this model does not fit the data for thromboxane A₂ synthase (TXAS) [21]. Hydropathy analysis for PGIS [22] indicates that the putative N-terminal membrane anchor domain of this protein is very similar to other membrane-bound microsomal P450s and different from that of TXAS. To obtain direct evidence for the characterization of the PGIS membrane anchor domain, two-dimensional (2D) ¹H NMR spectroscopy has been used to determine the 3D solution structure in a membrane-mimetic environment of a synthetic peptide corresponding to the N-terminal membrane anchor domain. The solution structure obtained suggests that the side chains of the first 20 residues of the peptide adopt a helix-turn structure and interact with the hydrocarbon interior of the ER membrane, and

Abbreviations used: 2D, two-dimensional; 3D, three-dimensional; DPC, dodecylphosphocholine; DQF-COSY, double-quantum filtered COSY; ER, endoplasmic reticulum; NOE, nuclear Overhauser effect; P450, cytochrome P450; PGH₂, prostaglandin H₂; PGHS, PGH₂ synthase; PGI₂, prostaglandin I₂; PGIS, PGI₂ synthase; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TXAS thromboxane A₂ synthase.

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the membrane anchor segment does not traverse the bilayer completely.

MATERIALS AND METHODS

Materials

Dodecylphosphocholine (DPC) was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). DPC- $^2\text{H}_{38}$, trifluoroethanol (TFE)- $^2\text{H}_3$ and $^2\text{H}_2\text{O}$ were from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). TFE and trifluoroacetic acid (TFA) were obtained from Sigma (St. Louis, MO, U.S.A.) and Millipore (Bedford, MA, U.S.A.) respectively.

Peptide synthesis

A peptide corresponding to the N-terminal membrane anchor domain of human PGIS (MAWAALLGLLAALLLLLLLRRRTR, where single-letter amino-acid notation has been used) was synthesized manually using fluorenylmethoxycarbonyl-polyamide solid-phase methods [23]. After cleavage with TFA, the peptide was purified to homogeneity by HPLC on a Vydac C_4 reversed-phase column with a gradient of 0–80% acetonitrile in 0.1% TFA. Molecular mass of the purified peptide was determined by fast atom bombardment MS [24].

CD

The peptide corresponding to the N-terminal membrane anchor domain with residues 1–25 of human PGIS was dissolved in sodium phosphate buffer (pH 5.5) with different membrane-mimicking conditions. CD spectra were recorded on a JASCO-700C spectrometer (Easton, MD, U.S.A.) at ambient temperature in a 0.1 cm pathlength cell, and analysed as described by Chen et al. [25]. The experiments for the peptide were performed to obtain solution conditions and initial structural information for subsequent NMR spectroscopical studies.

NMR sample preparation

The HPLC-purified peptide was dissolved in water and lyophilized three times to completely remove TFA and acetonitrile, and then dissolved in 0.02 M sodium phosphate buffer (pH 5.5). The sample was freeze-dried and rehydrated at a final concentration of 5 mM in water containing 10% $^2\text{H}_2\text{O}$ and 30% TFE- $^2\text{H}_3$ or 550 mM DPC- $^2\text{H}_{38}$. For $^2\text{H}_2\text{O}$ experiments, the peptide was dissolved in buffer, lyophilized and then dissolved with 30% TFE- $^2\text{H}_3$ in $^2\text{H}_2\text{O}$.

NMR experiments

Proton NMR experiments were carried out on a Bruker AMX-600 spectrometer (Acton, MA, U.S.A.). All 2D experiments [double-quantum-filtered (DQF) COSY, TOCSY and NOESY] were performed under identical conditions (298 K and pH 5.5) in $^1\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$ solvents. NOESY spectra were recorded with mixing time of 150 and 250 ms. TOCSY spectrum was carried out with MLEV-16 (Malcolm Levitt's decoupling cycle) mixing sequence with total mixing time of 80 ms. All spectra were composed of 2048 complex points in the F_2 dimension and 512 real points in the F_1 dimension with 32–128 scans per t_1 increment. Quadrature detection was achieved in F_1 by the time proportional phase increment ('TPPI') method. The NMR data were processed using the Felix program (Molecular Simulation, Inc., San Diego, CA, U.S.A.). All free induction decays ('FIDs') were zero-filled to $2k \times 2k$ before Fourier transformation and 0–30° (for DQF-COSY and TOCSY) or 90° (for NOESY) shifted sine-bell window function was used in both dimensions. Complete ^1H

NMR spectrum assignments were obtained using standard methods [26] and Felix auto-assign strategy [27].

Calculation of structures

The overall structure of the peptide was determined by the use of intra-residue, sequential and long-range nuclear Overhauser effects (NOEs). The Felix program was used for quantification of the NOE cross-peak volumes and for converting them into upper bounds of the interproton distances. NOE cross-peaks were segmented using a statistical segmentation function and characterized as strong, medium or weak, corresponding to upper-bound distance range constraints of 2.7, 3.5 and 5.0 Å respectively (1 Å = 0.1 nm). Lower bounds between non-bonded atoms were set to the sum of their van der Waals radii (approx. 1.8 Å). Pseudoatom corrections were added to interproton distance restraints where necessary [28]. Distance geometry calculation and the structural refinement were carried out on an SGI workstation using DGII, Simulated Annealing and NMR refinement programs within the Insight II package (Molecular Simulation, Inc.). A total of 190 (in TFE) and 155 (in DPC) NOE constraints and torsion angle restraints derived from coupling constants were used for the initial structures calculated from the DGII program. Energy refinement calculations, including restrained minimization/dynamics, were carried out in the best-distance geometry structures using the Discover program within the Insight II package.

RESULTS

Design and synthesis of peptide mimicking the putative N-terminal membrane domain of PGIS

Our previous peptidoliposome-reconstitution studies for the overlapping synthetic peptides corresponding to residues 1–28

Figure 1 CD spectra of the PGIS N-terminal peptide

Peptide (100 μM) was dissolved in 0.02 M sodium phosphate buffer (pH 5.5) alone (spectrum 1) or containing 10% (v/v) TFE (spectrum 2), 20% (v/v) TFE (spectrum 3), 30% (v/v) TFE (spectrum 4), 40% (v/v) TFE (spectrum 5) or 150 mM DPC (spectrum 6) in a 0.1 cm pathlength cell.

Figure 2 Expanded α H-NH region of the NOESY spectrum (150 ms mixing time) for the peptide in TFE/water

The amino acid residues (given in single-letter amino-acid notation) and numbering within the peptide are shown. The $d_{\alpha N}$ sequential connectivities are indicated.

(LP1) and 25–54 (LP2) of the putative N-terminal membrane anchor domain of PGIS and P450 2C1 have shown that both LP1 peptides of PGIS and P450 2C1 bound to the lipid bilayers, whereas neither LP2 peptides bound to the liposomes [22].

These results indicated that the membrane anchor segments in the N-terminal domain of PGIS and P450 2C1 could be localized within residues 1–25. In addition, both LP1 peptides in DPC micelles, 40 % (v/v) TFE or peptidoliposomes adopted similar helical structures as determined by CD spectroscopy [22]. Thus it is suggested that the helical structure of the LP1 peptides present in solution with TFE is comparable with the helical structure induced by peptidoliposomes or DPC micelles, which are membrane-mimetic reagents with a structure accepted to resemble that of membrane phospholipid [29]. On the basis of this information, a peptide corresponding to residues 1–25 of the N-terminal domain of PGIS was synthesized and used for the structural characterization. To find out whether the peptide with three residues shorter than the LP1 peptide of the PGIS N-terminal domain adopted a similar helical conformation, and to find the optimal condition of the peptide with the helical conformation in the membrane environment, a CD study was performed with increasing amounts of TFE or 150 mM DPC. The helical structure of the peptide was stabilized with 30 % (v/v) TFE in which the helical content was almost identical with the structure of the peptide in the presence of 150 mM DPC (Figure 1). Thus 30 % (v/v) TFE was used to prepare the peptide solution for initial NMR spectroscopy studies. The CD results also indicated that the 25-residue peptide had a similar membrane anchor function to the 28 residues of the PGIS LP1 peptide [22], and the membrane anchor segment was within the first 25 residues of the N-terminus of PGIS.

NMR assignment

2D NMR spectra of the peptide were recorded in the presence of 30 % (v/v) TFE- $^2\text{H}_3$ or DPC- $^2\text{H}_{38}$. ^1H NMR assignments were

Table 1 Proton chemical shifts for the peptide corresponding to residues 1–25 of PGIS

The conditions used for the spectrum collection and analysis are described in the Materials and methods section. Others relate to H_γ , H_δ and other side-chain protons. n.d., not determined.

Residue	Chemical shift (p.p.m.) in solvent									
	TFE					DPC				
	HN	H α	H β 1	H β 2	Others	HN	H α	H β 1	H β 2	Others
Met ¹	8.172	3.971	1.853	1.757	n.d.	n.d.	4.059	2.381	n.d.	2.494
Ala ²	8.288	4.428	1.303	n.d.	n.d.	8.672	4.444	1.616	n.d.	n.d.
Trp ³	7.820	4.407	3.219	n.d.	7.041, 7.459, 7.415	8.425	4.435	3.318	3.403	7.447, 10.567
Ala ⁴	7.734	3.896	7.734	3.896	1.159	8.672	3.979	1.468	n.d.	n.d.
Ala ⁵	7.305	4.092	1.347	n.d.	n.d.	7.286	4.066	1.597	n.d.	n.d.
Leu ⁶	7.507	4.117	1.717	1.568	1.431, 0.817	7.602	4.073	1.901	n.d.	1.013
Leu ⁷	7.950	4.005	1.879	1.802	1.618, 0.756	8.236	4.065	n.d.	n.d.	n.d.
Gly ⁸	7.819	3.837	n.d.	n.d.	n.d.	7.885	3.790	n.d.	n.d.	n.d.
Leu ⁹	7.654	4.186	1.792	1.657	0.817	8.632	4.059	1.697	2.010	1.011
Leu ¹⁰	8.202	4.018	1.741	1.520	1.300, 0.802	8.361	4.021	1.688	1.965	0.979
Ala ¹¹	7.720	4.281	1.197	n.d.	n.d.	8.398	3.912	1.543	n.d.	n.d.
Ala ¹²	7.582	4.049	1.494	n.d.	n.d.	8.142	3.919	1.550	n.d.	n.d.
Leu ¹³	8.325	4.020	1.745	1.607	0.817	8.182	4.010	1.679	1.966	0.962
Leu ¹⁴	7.832	4.036	1.790	1.644	1.212, 0.802	8.271	4.021	1.688	1.965	0.979
Leu ¹⁵	8.042	3.962	1.851	1.763	1.390, 0.725	8.427	4.025	1.778	1.954	1.020
Leu ¹⁶	8.460	3.978	1.927	1.864	1.738, 1.643	8.051	4.072	1.893	n.d.	n.d.
Leu ¹⁷	8.512	3.998	1.955	1.509	0.756	8.262	4.047	1.689	n.d.	1.014
Leu ¹⁸	8.895	4.033	1.914	1.854	1.448	8.134	4.070	1.882	n.d.	0.963
Leu ¹⁹	8.238	4.152	1.899	1.654	1.304	8.118	4.072	1.883	n.d.	0.962
Ser ²⁰	7.766	4.170	3.214	n.d.	n.d.	8.182	4.063	3.593	3.717	n.d.
Arg ²¹	7.900	4.345	1.815	1.693	1.631, 3.109, 7.167	8.235	4.149	1.848	n.d.	3.250
Arg ²²	8.143	4.345	1.815	1.681	1.570, 3.146, 7.117	7.732	4.269	1.916	n.d.	2.050, 2.161, 3.201
Arg ²³	8.006	4.280	1.888	1.851	1.658, 1.608, 7.130	7.815	4.271	1.853	2.122	3.258
Thr ²⁴	7.748	4.295	4.206	n.d.	1.159	7.848	4.317	4.280	n.d.	1.365
Arg ²⁵	7.928	4.296	1.755	1.594	3.129, 7.131	7.816	4.355	1.833	2.086	3.261

Figure 3 Plot of the difference between α H chemical shifts observed and the random coil chemical shifts described in the text

The peptide was dissolved in TFE/water (A) and DPC (B).

achieved using the standard sequential assignment technique [26]. The assignment procedure involves identification of spin systems and sequential assignment [27,30] using a combination of TOCSY [31,32], DQF-COSY [33] and NOESY [34] spectra recorded in TFE and DPC respectively. The expanded NOESY spectrum of the peptide in TFE is shown in Figure 2. In addition, each step of the assignment was double-checked using an auto-assignment program according to the manufacturer's instructions for the Felix package. The complete proton resonance assignments for the peptide in TFE and DPC are summarized in Table 1.

Secondary structure identification from NMR data

As a standard approach, upfield shifts of the α H chemical shifts relative to the random coil values have been proposed as one of the markers for characterization of peptide helical structures in solution [35]. In particular, upfield shifts in four adjacent residues are characteristic of the presence of local helical structures. Alternatively, β -sheets are recognized as downfield shifts of three adjacent residues. For the PGIS N-terminal peptide in TFE and DPC, the upfield shifts of the α H chemical shifts, as compared with random coils [26], were observed in most of the residues of the peptide (Figure 3). These are indicative of considerable

helical content within the peptide, but the chemical-shift index method is not completely foolproof or always correct [35]. The further identification of the residues with helical structure was based on the NOE method. Using the sensitive indication of $d_{\alpha N}(i, i+2)$, $d_{\alpha N}(i, i+3)$, $d_{\alpha\beta}(i, i+3)$ and $d_{\alpha N}(i, i+4)$ connectivities of the NOESY spectra [35], the helical structure was defined further in the regions with residues 3–11 for TFE and 3–10 for DPC, and an atypical helical structure in the regions with residues 17–23 for TFE and 18–24 for DPC (Figure 4), which were also supported by the qualitative comparison of $^3J_{NH\alpha}$ coupling constants from the NH- α H cross-peaks in the DQF-COSY spectrum (Figure 4). The $^3J_{NH\alpha}$ coupling constants were obtained by direct measurement of $^3J_{NH\alpha}$ values and by comparing the intensities of NH- α H cross-peaks, which are grouped as strong ($J > 6$ Hz), medium ($J = 4$ –6 Hz) and weak ($J < 4$ Hz). Weak and strong $^3J_{NH\alpha}$ coupling constants have been used to identify helical and β -sheet structures respectively [36,37]. Out of the 25 residues of the peptide, 17 had weak $^3J_{NH\alpha}$ coupling constants, which is consistent with the helical structure assignment in general. The presence of several $d_{\alpha N}(i, i+2)$ NOEs suggests that at least part of the peptide conformational population is 3_{10} -helix, i.e. in equilibrium with α -helix [38]. The next step was to define the 3D structural nature of the peptide.

3D structural description of the peptide

The exact nature of the 3D structure of the folded peptide was deduced on the basis of the quantitative NOE data by structural calculations using DGII, simulated annealing and NMR refinement programs in the Insight II software package. After quantification of the NOE data using Felix auto-assignment software, 190 (in TFE) and 155 (in DPC) NOEs that were well distributed over all the residues of the peptide were used as distance restraints to calculate the structures in TFE and DPC that agreed with the data. Satisfactory results were obtained, as no large violations in the bond lengths and angles occurred upon the introduction of these NOEs into the calculation. To avoid the problem of local energy minima for the 3D structure, individual simulated annealing and energy minimization were run several times and the structures and energies were compared. The structure with minimal energies that agreed with the different experiments was used to display the conformation. Out of the 50 structures calculated, ten structures with the lowest energies were chosen for final analysis. Figures 5(A) and 5(C) show the overlay of the ten structures in TFE and DPC respectively, with small variations. The overall shape of the peptide in TFE or DPC can be described as a bent-shape with helix-turn conformation comprised of a typical helix (residues 3–11), followed by a turn structure (residues 12–16) and a further atypical helix on the C-terminal side of the peptide (residues 17–23). The turn structure was composed of the side-chain hydrophobic contacts of the inter-residues within residues 12–16 (Ala-Leu-Leu-Leu-Leu). Furthermore, the essential similarity of the 3D structures of the peptide in TFE and DPC from residue 1–20, predicted as the membrane anchor domain, is apparent from a comparison of Figures 5(A) and 5(C). The determined 3D structure of the membrane anchor domain of the peptide in DPC was clearly very similar to that obtained in TFE. It is apparent that the peptide structure induced in TFE is comparable with that induced by DPC micelles.

Membrane topology of the N-terminal domain of PGIS

By analysing the 3D structure of the peptide, the hydrophobic side chains of the N-terminal helix and turn (residues 1–20) were

Figure 4 Amino acid sequence of the PGIS N-terminal peptide and a survey of sequential and medium-range NOEs, spin-spin coupling constants of the peptide in TFE/water (A) and DPC (B)

The residues (1–25) within the peptide are indicated at the top of each panel in single-letter amino-acid notation. Medium-range NOEs $d_{\alpha N}(i, i+1)$, $d_{\alpha N}(i, i+2)$, $d_{\alpha N}(i, i+3)$, $d_{\alpha N}(i, i+4)$ and $d_{\alpha \beta}(i, i+3)$ are indicated by lines starting and ending at the position of the interacting residues. The values of the $^3J_{\text{NH}\alpha}$ coupling constant are reported as strong ($J > 6$ Hz; S), medium ($J = 4\text{--}6$ Hz; M) or weak ($J < 4$ Hz; W) respectively. The locations of the helical structures are shown at the bottom of the Figure.

found to be exposed on the surface of the molecule. These results indicated that the side chains of the residues interacted with the hydrocarbon interior of the phospholipid bilayer of the ER membrane (Figures 5B and 5D). The hydrophilic side chains of residues 21–25 (Arg-Arg-Arg-Thr-Arg) point away from the hydrophobic residues 1–20 and are expected to be exposed to the aqueous environment on the cytoplasmic side of the ER membrane (Figures 5B and 5D). Thus the N-terminal membrane anchor segment of PGIS can be localized further within residues 1–20.

Additional information was observed from the 3D structure in which the distance between residues 1 and 20 is approx. 20 Å, less than the thickness of ER lipid bilayer [39]. This indicates that the N-terminal membrane anchor domain in PGIS does not penetrate the ER membrane. To obtain an overall topological arrangement model for the membrane-bound PGIS, the 3D solution structure

of the N-terminal membrane anchor segment was grafted on to a 3D working model of the PGIS catalytic domain constructed by homology modelling using P450BM3 as a template (Figure 6) [13,40].

DISCUSSION

The aim of the present study was to analyse the 3D structure of the N-terminal domain of human PGIS, whose sequence is believed to anchor the protein to the ER membrane as determined by our hydrophobic plot, CD spectroscopy and peptidoliposome-binding studies [22]. It is particularly interesting to identify the residues in the N-terminal domain responsible for the ER membrane anchor function, because PGIS shares high sequence similarity with other microsomal P450s [22] and can be seen as

Figure 5 Overlay of ten 3D structural models in TFE (A, B) and DPC (C, D) in the absence (A, C) or presence (B, D) of side chains of the PGIS peptide

Hydrophobic (grey lines) and hydrophilic (black lines) residues are plotted. The 3D structures of the PGIS peptide were obtained by using the NMR refinement programs within the Insight II package after dynamic studies and energy minimization. The structures of the side chains were added by using a molecular modelling approach.

one of the general models for the P450 superfamily. Understanding the details of the structure and membrane anchor function of PGIS will provide valuable information in understanding the structures and functions of this crucial domain in other microsomal P450s.

Synthetic peptides have been widely used to mimic parts of proteins for biochemical and structural biological studies. This provides a way of examining the structure and function of selected portions of native proteins, particularly for membrane-bound proteins that are difficult to crystallize for X-ray studies [41–44].

The NMR data in the present study for the synthetic peptide mimicking the N-terminal membrane anchor domain of PGIS gave convincing evidence for the presence of a well-defined tertiary structure comprised of a helix-turn-helix conformation in a hydrophobic environment. These NMR data provide the first experimental details of the secondary and tertiary structures of the crucial N-terminal membrane anchor domain of the

Figure 6 Overall topology of the N-terminal membrane anchor domain of PGIS

The NMR 3D structure of residues 1–25 (dark line) of human PGIS was grafted on to a 3D structural working model (backbone) constructed by homology modelling with QUANTA-CHARMM software package using P450BM3 as a template [13,40], and then inserted into the ER membrane with the first 20 hydrophobic residues. The proposed substrate access channel is indicated with an arrow.

eicosanoid-synthesizing P450. It confirms further our previous conclusion by CD and peptidoliposome studies [22] in which the N-terminal domain of PGIS appears to have a single membrane anchor segment.

To date, the N-terminal membrane anchor domains of membrane-bound P450s are described as ‘transmembrane’ segments, because it was believed that the segments could penetrate the ER membrane with the N-terminus exposed on the ER luminal side. This description is not supported by the 3D solution structure of the PGIS N-terminal domain. Figure 6 shows the overall membrane topology of the N-terminal domain of PGIS after the structure of the segment was grafted on to the backbone of a 3D working model of PGIS. It is clear that residues 1–20 of PGIS, which is believed to be in contact with the ER membrane, is only approx. 20 Å long and this length is not enough to penetrate the ER lipid bilayer [39]. This suggests that the N-terminal membrane anchor domain of the eicosanoid-synthesizing P450 PGIS is not ‘transmembranous’. This conclusion is also in agreement with other studies [22,45] in which the results indicated that the N-terminus was not exposed on the luminal side of the ER.

Current X-ray structural studies have indicated that the catalytic portion of PGHS is on the luminal side of the ER [46]. PGH₂ produced by PGHS in the lumen of ER thus needs to cross the lipid bilayer and access the PGIS substrate channel on the cytoplasmic side through the substrate entrance facing the ER membrane. On the basis of the topological model of PGIS described in Figure 6, the N-terminal membrane anchor domain is near the substrate channel opening. This observation is also supported by our recent immunofluorescent studies [45] on membrane-bound human PGIS using site-specific antibodies. Peptides corresponding to the surface immediately near or

approx. 10–20 Å away from the opening were used to prepare site-specific antibodies. The site-specific antibodies were used to probe the accessibility of the substrate access channel opening in transiently transfected COS-1 cells expressing recombinant human PGIS and ECV cells expressing endogenous human PGIS. Immunofluorescence staining was performed for cells selectively permeabilized with streptolysin O and cells whose membranes were permeabilized with detergent. Antibodies to peptides in the immediate vicinity of the substrate channel bound to their targets only after general permeabilization with Triton X-100. In contrast, the two antibodies to peptides further from the channel opening bound to their targets even in cells with intact ER membranes. These observations allowed us to propose a topology model in which the PGIS substrate access channel opening is positioned close to the ER membrane and the N-terminal membrane anchor domain is close to the channel opening [22,45]. These findings suggest that the highly hydrophobic N-terminal membrane anchor segment may also play an important role in modulating channel conformation and substrate orientation. It should be mentioned that the enzyme reaction mechanism of PGIS and TXAS was done by excellent spectroscopic studies using the soluble protein [47]. However, little information is available for the delivery of the hydrophobic substrate PGH₂ (produced in the ER lumen) into the active site of PGIS, which is located on the cytoplasmic side of the ER for the membrane-bound synthase. Our studies described in the present report are particularly interested in how PGH₂ is delivered differently to the active sites of the membrane-bound TXAS and PGIS. In particular, we have demonstrated that the features of the N-terminal membrane anchor of TXAS and PGIS are quite different [22].

Despite PGIS sharing considerable primary and secondary structure similarities with other microsomal P450s [48], it is a unique P450, because PGIS lacks mono-oxygenase activity, which is one of the common features of other microsomal P450s. This makes it unclear as to the degree of structural similarity between the N-terminal membrane anchor of PGIS and the other microsomal P450s. In comparison, PGIS shares 50% sequence identity within the first 20 residues of the N-terminal membrane anchor domain with P450 2C1, a mono-oxygenase, which is much greater than in other regions in the two proteins; however, the hydrophilic residues 21–25 are not conserved between the two P450s. This suggests that other microsomal P450s may have similar secondary and tertiary structures in the first 20 residues and have the same membrane anchor format in the helix-turn segment. Characterization of the secondary and tertiary structures of the N-terminal region of P450 2C1 and human P450 will provide more information to enable a general N-terminal membrane anchor topology for other microsomal P450s to be constructed.

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