Prostacyclin synthase (PGIS), a cytochrome P450 enzyme, catalyzes the biosynthesis of a physiologically important molecule, prostacyclin. In this study we have used a molecular modeling-guided site-directed mutagenesis to predict the active sites in substrate binding pocket and heme environment of PGIS. A three-dimensional model of PGIS was constructed using P450bim-3 crystal structure as the template. Our results indicate that residues Ile67, Val76, Leu384, Pro355, Glu360, and Asp384, which were suggested to be located at one side of lining of the substrate binding pocket, are essential for catalytic activity. This region containing β1–3, β1–2, β1–3, and β1–4 strands is predicted well by the model. At the heme region, Cys441 was confirmed to be the proximal axial ligand of heme iron. The conserved Phe and Arg in P450BM-3 were substituted by Leu112 and Asp439, respectively in PGIS. Alteration of Leu112 to Phe retained the activity, indicating that Leu112 is a functional substitution for Phe. In contrast, mutant Asp439 → Ala exhibited a slight increase in activity. This result implies a difference in the heme region between P450bim-3 and PGIS. Our results also indicate that stability of PGIS expression is not affected by heme site or active site mutations.

Prostacyclin (PGI₂) is a potent inhibitor of platelet activation, vasoconstriction, and leukocyte interaction with endothelium (1, 2). It is considered to be an important vasoprotective molecule. Its biosynthesis in vascular endothelial cells is catalyzed by a series of enzymes of which PGI₂ synthase (PGIS) catalyzes the final conversion of prostaglandin H₂ (PGH₂) to PGI₂. PGIS was purified from bovine aortic tissues to homogeneous, exhibiting a slight increase in activity. This result implies that residues Ile67, Val76, Leu384, Pro355, Glu360, and Asp384, which were suggested to be located at one side of lining of the substrate binding pocket, are essential for catalytic activity. This region containing β1–3, β1–2, β1–3, and β1–4 strands is predicted well by the model. At the heme region, Cys441 was confirmed to be the proximal axial ligand of heme iron. The conserved Phe and Arg in P450BM-3 were substituted by Leu112 and Asp439, respectively in PGIS. Alteration of Leu112 to Phe retained the activity, indicating that Leu112 is a functional substitution for Phe. In contrast, mutant Asp439 → Ala exhibited a slight increase in activity. This result implies a difference in the heme region between P450bim-3 and PGIS. Our results also indicate that stability of PGIS expression is not affected by heme site or active site mutations.

Experimental Procedures

Materials—COS-1 cells (ATCC CRL-1650) were obtained from the American Type Culture Collection. pBluescript (SK+) plasmid DNA and eukaryotic expression vector pSH5 were from Stratagene. Human lung cDNA was from CloneTech. Cell culture media and antibiotics were from either Life Technologies, Inc. or HyClone Laboratories. DEAE-dextran and pGEX-2T plasmid DNA were from Pharmacia Biotech Inc. Isopropyl-β-D-thiogalactopyranoside, dimethyl sulfoxide, and chloroquine were from Sigma. [14C]Arachidonic acid ([14C]AA) was from Amersham Corp. PGH₂ was from Cayman Chemical (Ann Arbor, MI). Oligonucleotides were synthesized by Genosys (The Woodlands, TX). Linear-K preadsorbent TLC plates with silica gel (60 Å) and 250-μm thickness were from Whatman (LK6D).

Molecular Modeling—The strategy used for constructing the PGIS three-dimensional model followed that developed for TXAS (10) and other mammalian P450s (12). The strategy took into consideration six procedures: (i) sequence alignment, (ii) framework construction, (iii) loop structural determination, (iv) site-chain placement, (v) molecular docking, and (vi) energy minimization, which are briefly described below. A sequence similarity alignment was made for PGIS and the hemoprotein domain of the P450bim-3 (Fig. 1), and the main-chain conformation of PGIS was built with the Quanta-Charmm protein model-
cDNA, 2.5 units of DNA polymerase (Promega or Perkin-Elmer), and buffer for Pfu DNA polymerase were used. 1

GGGCC) and I36 (CGAGCACGTGGATCCATC), inner primer set). I35 (CATGGATCCGCGATGGCTT GAAGCTG), outer primer set; and I34 (CAGCCCCGCGATGGCTTG) and I37 (TGTGCACACAC GAAAGCTG), inner primer set). These primers were used in PCR to amplify a 400–500-basepair product using outer primers (pGEX-PGIS).

PGIS cDNA (wild-type) was subcloned into pGEX-2T vector (pGEX-PGIS). Transformed JM105 cells containing pGEX-PGIS DNA were cultured at 37 °C in 2 × YT medium to A600 = 0.8. Isopropyl-b-D-thiogalactopyranoside (0.2 mM) was then added to induce protein expression, and cells were further incubated at 37 °C for 3 h. Harvested cells were sonicated for 35 s and centrifuged at 1000 × g for 10 min. The pellet which contained the fused GST-PGIS protein was washed with lysis buffer (100 mM NaCl, 50 mM Tris-Cl, pH 8.0, 0.5% Triton X-100, 1 mM EDTA) and centrifuged before being applied on SDS-polyacrylamide gel electrophoresis gels. The ~68-kDa GST-PGIS protein was dissected out from the SDS-polyacrylamide gel electrophoresis gel and electroeluted. The antibody against purified GST-PGIS was prepared in rabbits by H.T.I. Bio-products (Ramona, CA).

COS-1 Transfection—COS-1 cells were grown to near-confluence in 100-mm tissue culture dishes in the presence of Dulbecco's modified Eagle's medium containing 2% fetal calf serum and 8% bovine calf serum at 37°C in a humidified 5% CO2 atmosphere. Transfection was performed by a procedure described previously (17) with some modifications. In brief, cells were activated in fresh medium for 2 h before transfection. After washing with phosphate-buffered saline (PBS) at 37°C, the cells were cultured at 37°C in 2 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum and chloroquine (60 μg/ml) for 30 min in a lamina hood. 5 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum and chlorine (60 μg/ml) was then added, and the cells were incubated for additional 3 h at 37°C in 5% CO2 atmosphere. After incubation, the cells were shocked with dimethyl sulfoxide (10%) for 2 min, and the medium was replaced with 12 ml of complete growth medium. The transfected cells were then incubated for 48–60 h. To harvest cells, COS-1 cells were scraped from the plate, pelleted by centrifugation, washed with PBS, and resuspended in 350 μl of PBS. The protein concentration of each sample was determined using BCA protein assay reagent kit (Pierce).

Antibody Development—To generate the expression vector for glutathione S-transferase (GST) and PGIS (GST-PGIS) fusion protein, PGIS cDNA (wild-type) was subcloned into pGEX-3T vector (pGEX-PGIS). Transformed JM105 cells containing pGEX-PGIS DNA were cultured at 37°C in 2 × YT medium to A600 = 0.8. Isopropyl-b-D-thiogalactopyranoside (0.2 mM) was then added to induce protein expression, and cells were further incubated at 37°C for 3 h. Harvested cells were sonicated for 35 s and centrifuged at 1000 × g for 10 min. The pellet which contained the fused GST-PGIS protein was washed with lysis buffer (100 mM NaCl, 50 mM Tris-Cl, pH 8.0, 0.5% Triton X-100, 1 mM EDTA) and centrifuged before being applied on SDS-polyacrylamide gel electrophoresis gels. The ~68-kDa GST-PGIS protein was dissected out from the SDS-polyacrylamide gel electrophoresis gel and electroeluted. The antibody against purified GST-PGIS was prepared in rabbits by H.T.I. Bio-products (Ramona, CA).

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Determination of PGIS Protein Level by Western Blot—Immunoblotting was performed by a procedure described previously (18). 15–20 μg of whole cells were boiled in an electrophoresis sample buffer (19) for 8–10 min before being applied to a 10% polyacrylamide minigel for electrophoresis. The resolved proteins were electrotransferred at 300–450 mA for 1 h to nitrocellulose membrane. Subsequently, the membrane was blocked with 3% non-fat milk in PBS and probed with a 1 to 1000 dilution of rabbit serum containing antibody against GST-PGIS fusion protein at 25°C for 1 h overnight. A secondary antibody of goat anti-rabbit IgG conjugated with horseradish peroxidase was used as recommended. The protein bands were visualized by incubation with either the peroxidase substrate 4-chloro-1-naphthol (Bio-Rad) or Supersignal CL-HRP substrate system (Pierce). The levels of wild-type and mutant proteins on the Western blots were scanned by densitometer as described below.

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Fig. 1. Alignment of the amino acid sequence of human PGIS with partial sequence of P450 BM-3. The secondary structural elements of P450 BM-3 are indicated by * (a helix) and double asterisks (b strand) underlines. A-L denote the a helices and b1–b4 denote the b strands. Amino acid residues selected for mutation in this work are marked by asterisks. Numbers of amino acid residue are indicated on the right.
Assay of PGIS Activity—The assay was performed by mixing whole cell homogenates (80–80 μg in PBS) with 5.5 μg of purified sheep PGH₂ synthase (20) provided by Dr. A.-L. Tsai, and [14C]AA (10 μM) was added to the mixture in a total volume of 100 μl. After addition of [14C]AA, the tube was vortexed for the initial 30 s and incubated for 3 min. The reaction was terminated with 40 μl of methanol and 1 x citric acid (4:1, v/v). Organic products generated were extracted with 300 μl of diethyl ether twice. The organic extract which contained 92% ± 2% (n = 10) of the radioactivity was concentrated under nitrogen to less than 60 μl and applied to a TLC plate. The TLC plate was chilled on ice before placing in a developing tank (on ice) in the organic phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100, v/v; upper phase) (21). After development, the radioactive signals on the TLC plate were detected by autoradiography. PGH₂ was hydrolyzed to 6-keto-PGF₁α during extraction, therefore the amount of PGI₂ converted from PGH₂. The fractions containing 6-keto-PGF₁α were collected, dried, and resuspended in methanol. The organic extract was dried under nitrogen and resuspended in methanol. The extract was applied to reverse-phase HPLC as described above. The organic products were extracted by the procedure described previously (22). After development, the radioactive signals on the TLC plate were detected by autoradiography. PGI₂ was hydrolyzed to PGF₁α, which was visualized by iodine vapor. The radioactive signal of 6-keto-PGF₁α on TLC plate was determined by co-migration with [14C]6-keto-PGF₁α, which was visualized by iodine vapor. The radioactive signal of [14C]6-keto-PGF₁α on the autoradiography was detected by using a densitometer (UMAX Vista-T630 or Epson ES-800C scanners with the Adobe Photoshop program), and the intensity of each signal was analyzed on a Macintosh computer using the public domain NIH Image program.

Radioimmunoassay was also used to determine the catalytic activity of PGIS. Whole cell homogenate (60 μg of protein) was incubated with PGH₂ (5 μM) at room temperature for 3 min, the reaction was terminated, and the organic products were extracted by the procedure described above. The organic extract was dried under nitrogen and resuspended in methanol. The extract was applied to reverse-phase HPLC as described previously (22). The fractions containing 6-keto-PGF₁α were collected, dried, and resuspended in methanol. 6-Keto-PGF₁α contents in these extracts were measured by radioimmunoassay as described previously (23).

RESULTS

Substrate Binding Pocket—A three-dimensional model based on the crystal structure of the hemoprotein domain of P450BM-3 was constructed. Details of the PGIS model will be published elsewhere. The backbone structure of the PGIS model matched well with that of P450BM-3, especially the helical regions (data not shown). The crystal structure of P450BM-3 reveals a long binding pocket lined with mostly nonaromatic hydrophobic residues (9). We compared the substrate access channel of the PGIS model with that of P450BM-3 by superimposing the substrate binding pocket structure of PGIS model on that of P450BM-3 crystal structure. Residues which were predicted from the three-dimensional model to be important in substrate binding pocket and heme site are shown in Fig. 2. Along the substrate access channel of PGIS were hydrophobic residues, Ile⁶⁷, Val⁷⁶, Thr²⁰⁶, Leu²¹⁰, Leu²¹³, and Phe⁴⁷⁶, which correspond to Phe⁴², Tyr⁴³, Leu¹⁸¹, Met¹⁸⁵, Leu¹⁸⁸, and Leu⁴³⁷ of P450BM-3 Channel, respectively. To test the hypothesis that these hydrophobic residues were important in PGIS catalytic activity, the cDNA sequences at these six positions were altered individually to code for recombinant PGIS mutants: Ile⁶⁷Val, Thr²⁰⁶Asp, Leu²¹⁰Asp, Thr²⁰⁶Asp, Leu²¹⁰Asp, and Leu²¹⁰Asp were 5.1, 8.3, and 6.8% of the wild-type enzyme, respectively. By contrast, catalytic activity of mutants Thr²⁰⁶Lys, Val⁷⁶Glu, or Phe⁴⁷⁶Val was not significantly different from that of the wild-type (100, 97, and 83%, respectively; Table I and Fig. 4). These results indicate that, as predicted by the three-dimensional model, Ile⁶⁷Val, Val⁷⁶Glu, and Leu²¹⁰Asp are markedly reduced to the level of the mock-transfected control as analyzed by TLC (Fig. 4). The TLC results were confirmed by radioimmunoassay of 6-keto-PGF₁α fractions separated by reverse-phase HPLC (data not shown). The catalytic activities of PGIS mutants from multiple experiments expressed as percent of the wild-type PGIS activity are summarized in Table I. The activities of Ile⁶⁷ → Lys, Val⁷⁶ → Asp, and Leu²¹⁰ → Asp were 5.1, 8.3, and 6.8% of the wild-type enzyme, respectively. By contrast, catalytic activity of mutants Thr²⁰⁶ → Val, Leu²¹³ → Glu, or Phe⁴⁷⁶ → Val was not significantly different from that of the wild-type (100, 97, and 83%, respectively; Table I and Fig. 4). These results indicate that, as predicted by the three-dimensional model, Ile⁶⁷Val, Val⁷⁶Glu, and Leu²¹⁰Asp are important in substrate access to the active site, while contrary to prediction from the model, Thr²⁰⁶Val, Leu²¹³Glu, and Phe⁴⁷⁶Val are not critically involved in substrate access.
Val, Glu360, A and B. PGIS activity was significantly diminished in Asp364 COS-1 cells was comparable with that of the wild-type (Fig. 3, Glu360, Leu384, and Asp364 are important in substrate binding were suggested to be involved in substrate binding (Fig. 2). A and B, the position of PGIS is indicated on the right side, and the protein size in kilodaltons derived from molecular mass markers is indicated on the left side. Vector denotes pSG5 without PGIS insert.

Arg47, corresponding to Arg72 in PGIS model, is considered to be a “gate-keeper” for P450BM-3 enzyme. To determine whether this charged residue plays a similar role in PGIS, we altered it to Gru or Gin by site-directed mutagenesis. These two mutants expressed a similar quantity of proteins on Western blots as the wild-type (Fig. 3A) and had comparable catalytic activities as the wild-type enzyme (Table I and Fig. 4).

Ravichandran et al. (9) predicted from the crystal structure of P450BM-3 that β1–4 strand was part of the substrate binding sites and residues Ala328, Ala330, and Met354 (on β1–3) were involved in the substrate binding. In this region, according to sequence alignment and PGIS modeling, three corresponding amino acid residues, Pro355, Glu360, and Leu384, respectively, were suggested to be involved in substrate binding (Fig. 2). A nearby residue in TXAS, Arg413 (on β1–4) corresponding to Asp364 in PGIS, was suggested to have interaction with substrate (11). Each of these four amino acid residues in the full-length PGIS cDNA was individually altered to Pro355 → Val, Glu360 → Gru, Asp364 → Val, and Leu384 → Asp. The level of each mutant protein expressed in transient transfected COS-1 cells was comparable with that of the wild-type (Fig. 3, A and B). PGIS activity was significantly diminished in Asp364 → Val and Leu384 → Asp mutants (6.8 and 6.9%, respectively; Table I and Fig. 4), whereas Pro355 → Val and Glu360 → Gru mutants obtained a fraction of the wild-type enzymatic activity (34 and 44%, respectively). These results indicate that Pro355, Glu360, Leu384, and Asp364 are important in substrate binding as predicted by molecular modeling.

Heme Environment—A cysteine residue which serves as the proximal axial ligand for the heme iron through a thiolate bond is conserved among all P450s. There are only two Cys residues in PGIS, Cys231 (on aG) and Cys441, of which Cys441 corresponds to the consensus P450 cysteine (Figs. 1 and 2). Mutation of Cys441 to Ser by site-directed mutagenesis resulted in a diminished enzyme activity (13%; Table I and Fig. 4), without alteration in the expressed protein level (Fig. 3B). Mutation of Cys231 to Ser did not alter the catalytic activity (98%) (Table I and Fig. 4).

In the heme binding region of cytochrome P450, an Arg or a His residue is conserved which forms a hydrogen bond with the β-ring propionate group of the heme moiety. Alignment of amino acid sequence of the three-dimensional model of PGIS with that of P450BM-3 did not reveal a corresponding Arg or His. Instead, it suggested that Asn439 of PGIS was the corresponding residue (Figs. 1 and 2). This raised the possibility that the heme binding environment of PGIS may differ from that of other P450s and Asn439 may be functionally important in heme binding. To test this hypothesis, Asn439 was altered to Ala. The protein level of mutant Asn439 → Ala in transient transfected COS-1 cells is similar to that of the wild-type. Contrary to the hypothesis, mutation of Asn439 to Ala resulted in a slight increase in the PGIS activity.

Crystallographic structure of P450BM-3 suggests that Phe87 of P450BM-3 is involved in heme interaction (9). However, neither sequence alignment nor molecular modeling disclosed a corresponding Phe residue around this region in PGIS. The corresponding residue derived from molecular modeling was Leu112 (Fig. 2). To test if this Leu residue can provide hydrophobic interactions with heme and substrate as the Phe residue in other P450s does, Leu112 was mutated to Asp, Phe, or Gru by site-directed mutagenesis. The catalytic activity of mutant Leu112 → Phe was only slightly lower (89%) than that of the wild-type, while the activity of mutants Leu112 → Asp and Leu112 → Gru was reduced to 11 and 15% of the wild-type enzyme, respectively (Table I).

Pro113 was mutated as a control. Although situated next to Leu112, this residue was not located at the vicinity of heme or substrate pocket on the PGIS model. Pro113 → Ala mutant as predicted from the model retained most of the catalytic activity (Table I).

**DISCUSSION**

Molecular modeling coupled with site-directed mutagenesis is a powerful tool in studying the structure-activity relationship of cytochrome P450 enzymes. PGIS is a new family of P450 which shares several enzymatic and spectral characteristics with TXAS. Based on our previous observations that the crystallographic structure of P450BM-3 serves as a suitable template for constructing the three-dimensional model of TXAS (10), we have taken a similar approach in constructing the three-dimensional model of PGIS, and the results indicate that the generated model is valuable for identifying the amino acid residues involved in substrate binding and heme environment.

**Substrate Binding Pocket**—The PGIS model suggests that the substrate access channel of PGIS is similar to that of P450BM-3 in that it is very long and lined with hydrophobic residues. This result is not surprising since the substrate (PGH2) for PGIS is a 20-carbon metabolite of arachidonate, a substrate for P450BM-3. Our site-directed mutagenesis results indicate that Ile67, Val76, and Leu384, which are situated along the lower portion of the channel according to the PGIS model (Fig. 2), are critically important in PGIS catalytic activity. Mutations of these hydrophobic residues to charged residues reduced the catalytic activity to the background value. Hence, this portion of the binding pocket in PGIS is comparable with that of P450BM-3.
Two residues (Pro\textsuperscript{355} and Glu\textsuperscript{360}) are located inside the lower portion of the binding pocket adjacent to heme (Fig. 2). Mutations of these two residues, Glu\textsuperscript{360} → Gly and Pro\textsuperscript{355} → Val retained 44 and 34\% activity, respectively, suggesting that these two charged residues are involved in catalytic activity. We have shown previously that TXAS Arg\textsuperscript{471}, which is not predicted to be located at the binding pocket based on P450\textsubscript{BM-3} structure, is important in TXAS catalytic activity (11). This residue corresponds to PGIS Asp\textsuperscript{364}. Mutation of Asp\textsuperscript{364} to Val reduced the catalytic activity to the background value, indicating that these two corresponding residues are similarly important in PGIS and TXAS activities. According to the PGIS model, this charged residue was located near the lining of the substrate binding pocket but not in the vicinity of PGH\textsubscript{2} or heme (Fig. 2). Taken together, these results indicate that, with minor exceptions, the lower part of the substrate binding pocket containing β1–1, β1–2, β1–3, and β1–4 strands in PGIS model (Fig. 2) is comparable with that of P450\textsubscript{BM-3}.

By contrast, the upper portion of the substrate binding pocket of PGIS was not as well predicted from the P450\textsubscript{BM-3} structure. Only one of the four predicted residues is important in substrate binding and catalytic activity: Leu\textsuperscript{210} → Asp lost the activity, whereas Thr\textsuperscript{206} → Val, Leu\textsuperscript{213} → Glu, and Phe\textsuperscript{476} → Val retained the activity completely. It has been indicated that Met\textsuperscript{185} and Leu\textsuperscript{437} in P450\textsubscript{BM-3} form strong van der Waals interactions (9). These two residues correspond to Leu\textsuperscript{210} and Phe\textsuperscript{476} in PGIS by modeling. As mentioned above, the mutant Leu\textsuperscript{210} → Asp completely lost the catalytic activity as predicted from the P450\textsubscript{BM-3} structure, whereas mutation of Phe\textsuperscript{476} to Val surprisingly had no effect on the activity. The discrepancy between the predicted and experimental data by site-directed mutagenesis indicates that the molecular model around the upper portion of the channel of PGIS (Fig. 2) containing most of αF helix (Thr\textsuperscript{206}, Leu\textsuperscript{210}, and Leu\textsuperscript{213}) is different from that of P450\textsubscript{BM-3}. Since the polarity and size of AA are different form PGH\textsubscript{2}, it is expected to find disagreement between the structure of P450\textsubscript{BM-3} (using AA as substrate) and PGIS (using PGH\textsubscript{2} as substrate) around the substrate access channel.

Arg\textsuperscript{72} in PGIS model is corresponding to Arg\textsuperscript{47} in P450\textsubscript{BM-3} structure. This P450\textsubscript{BM-3} Arg residue is located at the mouth of its access channel in strand β1–2 close to the molecular surface and is not well defined in the crystal structure (9). Mutation of Arg to Glu in P450\textsubscript{BM-3} blocks the enzymatic reaction, and this residue was suggested to be important in substrate recognition and binding (9, 24). Surprisingly, mutation of the conserved Arg in PGIS to charged residue, Glu or Gln, of comparable size did not significantly change the catalytic activity (101 and 80\%, respectively). These results imply that the charge group of this Arg is not important in substrate access and binding in PGIS. Unlike the P450\textsubscript{BM-3}, which is a soluble enzyme, the PGIS has a trans-membrane domain at its N-terminal, which is close to the entrance of the substrate binding pocket. We speculated that the entrance of substrate channel of PGIS differs from that of P450\textsubscript{BM-3} because of the influence of membrane topology of PGIS on substrate channel orientation.

Heme Environment—The three-dimensional model of PGIS predicts Cys\textsuperscript{441} to be the proximal axial ligand for heme iron. This prediction was confirmed by site-directed mutagenesis. Hatae et al. (25) have recently obtained a similar result. This cysteine provides the thiol moiety to coordinate the heme. It has been shown that P450s exhibit a conserved sequence motif (F-GS-X-G-X-R/H-X-C-hy-G, where by denotes any hydrophobic residue) at the cysteine region near the C terminus of P450.

| TABLE I |
|---|
| Activity of PGIS expressed in COS-1 cells transiently transfected with wild-type and mutant PGIS cDNAs |

The activity was determined by TLC as described under "Experimental Procedures." Densitometric estimates of the 6-keto-PGF\textsubscript{1α} bands on TLC of the wild-type enzyme were set as 100\%.

| Constructs | PGIS activity relative to wild-typea | % |
|---|---|---|
| Wild-type | 100 | 100 |
| pSG5 (vector) (n = 9) | 4.1 ± 2.6 | 4.1 ± 2.6 |
| Substrate channel region | | |
| Ile\textsuperscript{87} → Lys (n = 6)b | 5.1 ± 3.8 | 5.1 ± 3.8 |
| Val\textsuperscript{176} → Asp (n = 3) | 8.3 ± 1.3 | 8.3 ± 1.3 |
| Thr\textsuperscript{206} → Val (n = 5) | 100 ± 13.9 | 100 ± 13.9 |
| Leu\textsuperscript{210} → Asp (n = 6) | 6.8 ± 4.0 | 6.8 ± 4.0 |
| Leu\textsuperscript{213} → Glu (n = 3) | 97 ± 10.9 | 97 ± 10.9 |
| Phe\textsuperscript{476} → Val (n = 5) | 83 ± 11.2 | 83 ± 11.2 |
| Arg\textsuperscript{72} → Glu (n = 3) | 101 ± 4.1 | 101 ± 4.1 |
| Arg\textsuperscript{72} → Gln (n = 4) | 80 ± 19.4 | 80 ± 19.4 |
| Pro\textsuperscript{355} → Val (n = 3) | 34 ± 6.3 | 34 ± 6.3 |
| Glu\textsuperscript{360} → Gly (n = 4) | 44 ± 5.5 | 44 ± 5.5 |
| Asp\textsuperscript{364} → Val (n = 3) | 6.8 ± 3.4 | 6.8 ± 3.4 |
| Leu\textsuperscript{364} → Asp (n = 7) | 6.9 ± 5.2 | 6.9 ± 5.2 |
| Heme environment | | |
| Cys\textsuperscript{441} → Ser (n = 2) | 13 | 13 |
| Cys\textsuperscript{441} → Ser (n = 2) | 98 | 98 |
| Asp\textsuperscript{439} → Ala (n = 4) | 124 ± 11.0 | 124 ± 11.0 |
| Leu\textsuperscript{112} → Phe (n = 4) | 89 ± 15.3 | 89 ± 15.3 |
| Leu\textsuperscript{112} → Asp (n = 4) | 11 ± 5.5 | 11 ± 5.5 |
| Leu\textsuperscript{112} → Gly (n = 6) | 15 ± 4.3 | 15 ± 4.3 |
| Pro\textsuperscript{113} → Ala (n = 6) | 83 ± 6.3 | 83 ± 6.3 |

a Mean ± standard deviation of three to nine experiments. The values for Cys\textsuperscript{441} → Ser and Cys\textsuperscript{441} → Ser are mean of two experiments.

The number of separate transfection experiments is given in parentheses.
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proteins (26). This motif in PGIS, WGAGHNHCLG, differs from the conserved motif by two residues: substitutions of Trp (W) for Phe (F) and Asn (N) for Arg/His (R/H). Since Phe to Trp substitution also occurs in nitric oxide synthase, we altered the second substitution from Asn$^{439}$ to Ala. This mutant surprisingly exhibited a slight increase in the catalytic activity. Asn$^{439}$ is unlikely to be the corresponding conserved Arg or His. This result implies that structure around the heme environment of PGIS is different from that of other P450s.

The crystal structure of P450$_{BM-3}$ predicted that Phe$^{87}$ forms close van der Waals interactions with the heme on the distal side and the corresponding residue in P450$_{cam}$ provides hydrophobic interactions with the substrate camphor (27). The corresponding residue in PGIS is Leu$^{112}$. It is interesting to note that alteration of Leu$^{112}$ to Phe retained the catalytic activity whereas change of it to a charged residue (Asp) or a smaller residue (Gly) reduced the activity markedly. This result indicates that Leu can substitute Phe in this region to provide hydrophobic interaction with heme and probably also with PGH$_2$.

Despite a marked loss of catalytic activity, mutants, Cys$^{441}$ → Ser and Leu$^{112}$ → Asp or Leu$^{112}$ → Gly, in which the heme environment is severely perturbed still expressed intact proteins as detected by Western blots. By contrast, TXAS in which the heme ligation or environment is perturbed by site-directed mutation expresses a very low level of proteins in cells (11). The stability of TXAS protein expression requires heme in a correct orientation. On the other hand, stability of PGIS protein appears to be less dependent on heme. These results epitomize major structural differences between PGIS and TXAS despite a similar substrate binding pocket.

Conclusion—Guided by the three-dimensional model, we have identified nine amino acid residues which are near or line the substrate binding pocket and are important in catalytic activity, especially the residues in β1–1, β1–2, β1–3, and β1–4 strands region. We have also confirmed Cys$^{441}$ as the proximal axial ligand of heme iron. However, the model is imperfect and fails to predict amino acid residues in other regions of the active site pocket especially in the αF helix region, heme environment and the substrate entrance gate-keeper. These inconsistencies between the model and the experimental data offer an interesting opportunity for further experiments to elucidate the different and potential new structural characteristics of PGIS. One approach is to refine the model based on the site-directed mutagenesis data from which additional functionally important residues can be identified. It is hoped that, through these experiments, the PGIS active site pocket and heme site can be more precisely mapped. Eventually, these structures will have to be confirmed by three-dimensional structure derived from x-ray crystallography and/or NMR spectroscopy.

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