**Crystal Structure of the Human Prostacyclin Synthase**

Prostacyclin synthase (PGIS) catalyzes an isomerization of prostaglandin H$_2$ to prostacyclin, a potent mediator of vasodilation and anti-platelet aggregation. Here we report the crystal structure of human PGIS at 2.15 Å resolution, which represents the first 3D structure of a Class III cytochrome P450. While notable sequence divergence has been recognized between PGIS and other P450s, PGIS exhibits the typical prism-shaped P450 fold with only moderate structural differences. The conserved acid-alcohol pair in the I helix of P450s is replaced by residues G286 and N287 in PGIS, but the distinctive disruption of the I helix and a nearby water channel remain conserved. Residue N287 appears to be situated for endoperoxide bond cleavage, suggesting a functional conservation of this residue in O-O bond cleavage. A combination of bent I helix and tilted B’ helix creates a channel extending from the heme distal pocket, which seemingly allows substrate binding; however, residue W282, placed at a distance of 8.4 Å from the iron, may serve as a threshold to exclude most heme ligands from binding. Additionally, a long “meander” region protruding from the protein surface may impede electron transfer. The PGIS structure presented here should provide an avenue to better understand the structure/function relationship of the atypical P450.

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**Cytochromes** P450 (P450s) are heme-containing enzymes involved in various vital processes including degradation of xenobiotics, biosynthesis of steroids and lipids as well as drug metabolism and carcinogenesis. P450s can be categorized according to their redox partners. Class I P450s have two electron-transfer partners: a reductase which is a NAD(P)H-dependent flavoprotein, and an iron-sulfur protein serving as the immediate electron donor to P450. Class II P450s use only one electron-transfer partner, P450 reductase. Class III P450s, such as prostacyclin synthase (PGIS), do not require any electron-transfer partner or molecular oxygen as endoperoxides or hydroperoxides are their sole substrates. There are also a few P450s which, for example, catalyze a peroxide-dependent hydroxylation or obtain electron directly from NAD(P)H, do not belong to the above categories and are grouped as Class IV P450s. While three-dimensional structures are available for a number of P450 enzymes, there are no X-ray structures of a Class III P450. Thus, the wide diversity of P450s will be helpful by the determination of tertiary structure of a Class III P450 for elucidating the differences in active site structure as well as providing information crucial for drug design.

PGIS catalyzes an isomerization of prostaglandin H$_2$ (PGH$_2$) to prostacyclin (PGI$_2$), which is a highly potent vasodilator and inhibits platelet aggregation. It is primarily expressed in endothelial and smooth muscle cells and is associated with endoplasmic reticulum and nuclear membrane. In tissues, the enzyme is found mainly in

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**Beamlines**

SP12B Protein X-ray Crystallography

13B1 Protein Crystallography

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Aorta. Sequence analysis and mutagenesis studies have identified two divergent regions in PGIS. One region is the Cys ligand loop, which consists of the consensus sequence of F(G/S)XGX(H/R)XCXG. In PGIS, the sequence of the Cys ligand loop is WGAGHNHCLG. Furthermore, a highly conserved region located in the I helix with an “acid-alcohol pair” in the consensus sequence of (A/G)GX(E/D)(T/S) is absent in PGIS. The protein per se also exhibits unusual features. For example, PGIS has only a few known heme ligands, in contrast to many P450s which bind various sizes and shapes of ligands. It was thus inferred that PGIS has a very limited space with a low “ceiling” at the heme distal side. In addition, the ferrous-CO complex of PGIS is very unstable. Collectively, analysis from sequence alignment and results from protein characterization suggest that PGIS is not a typical P450 with respect to the heme-binding and active site environments.

We previously reported a high-yield prokaryotic system for expression of human PGIS. The resulting recombinant PGIS was purified to electrophoretic homogeneity with enzymatic activity higher than the native PGIS isolated from bovine aorta. Using this recombinant expression system, we report here the determination of crystal structure of PGIS and elucidation of its exceptional features.

PGIS exhibits the typical triangular prism-shaped P450 fold composed of 3 β-sheets and 12 α-helices with an edge length of approximately 65 Å and a thickness of approximately 40 Å (Fig. 1). Although the overall fold is similar to other P450s, PGIS differs in the location of several helices, heme environment and the meander region. The significant differences include the B’, F, G and I helices, which are important for substrate binding. The I helix (residues E270-L300) in PGIS is disrupted in the middle such that the N-terminal portion bends upward from the heme (Fig. 2). The bent helix pushes up the F/G loop while the B’ helix leans toward the I-helix, creating a substrate binding channel that connects the heme distal pocket to protein surface (Fig. 3). The I helix plays a critical role in P450 catalysis not only because it shapes the distal pocket, but it also hosts a conserved acid-alcohol pair. Together with several neighboring water molecules, the acid-alcohol region is involved in protonation of ferric peroxo intermediate in P450 catalyzed reaction. PGIS has no acid-alcohol pair, instead, G286-N287 are present in the corresponding positions. The carbonyl oxygen of G286 forms hydrogen bonds (H-bonds) to two water molecules, which in turn form H-bonds to D211 located at the F helix and L485 at the β3-2 strand. This arrangement is very similar to that in P450terp in which the carboxylate of the acidic residue (D270) forms an H-bond to Q185 (at the F helix) and a salt bridge with K419 (at the β3-2 strand). In addition, an H-bond between wat17 and Q285 of PGIS also has a structural counterpart in P450terp. Two PGIS water molecules form H-bonds to the carbonyl oxygen of A283 and side chain OH of T284. Residue A283 whose carbonyl oxygen is only 4 Å from the iron is homologous to A264 of P450BM3 and A267 of P450terp. Collectively, positions of well ordered water molecules in the vicinity of I helix are maintained in PGIS, suggesting that the enzyme is positioned for protonation of ferric peroxo intermediate, an earlier step of hydroxylation. It is worth noting that thromboxane synthase, a Class III P450, can catalyze hydroxylations of prostaglandin H2 analogs in the presence of iodosylbenzene. For residue N287, the side chain carbonyl oxygen forms H-bond to wat152 and the side chain amide group is positioned 4.7 Å from the iron. The role of N287 in PGIS appears to be homologous to N137 of coral allene oxide synthase, a catalase-type protein which converts the fatty acid hydroperoxide to an...
epoxide. In coral allene oxide synthase this side chain amide group binds the distal oxygen of the hydroperoxide, causing a homolysis of the peroxide bond that subsequently produces an alkoxyl radical. Previous studies have also shown that PGIS catalyzes homolysis of the peroxide bond of a hydroperoxide, accompanied by the formation of an alkoxyl radical and a heme intermediate with a Compound II-like optical spectrum. Therefore, the functional role of the threonine/serine residue in P450 mono-oxygenases involved in O-O bond cleavage is likely conserved as N287 in PGIS.

The PGIS structure reveals a well defined distal heme pocket (Fig. 3), which is surrounded by residues from the regions commonly used by other P450s for substrate binding: the I helix, the loop between the K helix and β1-4 strand, and the loop between two β3 strands at the C-terminus. Given that the size of PGIS substrate is large, two additional regions (the B’ and F’ helices) which are located at the substrate entrance channel but slightly farther from the heme may also contribute to substrate binding. Residues from these five substrate binding sites constitute a highly hydrophobic cavity with only two residues near the heme being hydrophilic; Q280 and N287. Notably, residue W282 sits 8.4 Å from the iron with its side chain nearly parallel to the heme. As a result, the active site volume of PGIS is only about 355 Å³, as compared to CYP3A4 of 520 Å³ and CYP2C9 of 670 Å³. The low active site volume may explain why PGIS has a limited number of ligands. Furthermore, the position of W282 also supports the "low ceiling" hypothesis proposed by Ullrich, and may also explain the instability of ferrous-CO complex that found in PGIS as mutation at this residue greatly stabilizes the ferrous-CO complex (H-C Yeh and L-H Wang, unpublished).

Substrate-free PGIS in solution has a six-coordinated iron with thiolate and water as the axial ligands. Surprisingly, the PGIS crystal structure with C441 as the axial ligand is five-coordinated, lacking a water ligand at the sixth position. The heme is embedded between the I and L helices but the interactions of the propionate groups with PGIS apoprotein are distinct. Most P450s have either the two propionates pointing toward the proximal side or one to the proximal and the other to distal side. Moreover, the two propionates each form two to three H-bonds with the protein directly or through water molecules. While the heme iron and the four pyrrole rings have unambiguous electron density, the positions of propionates in the PGIS crystal are ill-defined and show conformational heterogeneity in both monomers. Thus, the direct interactions between propionates and the protein are largely absent in PGIS, indicating a somewhat greater plasticity of the heme lodging. Weaker interactions between propionates and PGIS apoprotein may hint on the enzyme being deficient in mono-oxygenation reaction as computational analyses have shown that these interactions are crucial for hydrogen-atom abstraction from the substrate by oxyferryl intermediate, a final step of hydroxylation.

PGIS structure also reveals an unusual meander region (residues R393- P433) located on the proximal side of the heme. This region is markedly longer compared to other P450s and the coil structure protrudes to the protein surface. It is likely that this region interferes the electron transfer via a steric effect as it has long been thought that P450 redox partner delivers its electron from the proximal side. Unfortunately, knowing the PGIS structure at present gives no clear information as to why PGIS accepts no electrons from the reductase. Analysis of surface potentials of PGIS reveals no noticeable difference from other P450s on the proximal side; nor the dipole moment along the molecule axis which exerts a long-range electrostatic attraction with the redox partner. As P450 electron transfer system is complicated, enigma of PGIS in electron transfer remained unsolved.

In summary, we describe the first crystal structure of a Class III P450. PGIS structure closely resembles other P450s, although some notable differences are apparent. While the F/G loop, B’, F and G helices in PGIS appears to be responsible for binding different ligands, a wider substrate entrance opening is created thanks to the bent I helix and tilted B’ helix. These structural features, which should have favored binding of PGIS with ligands of various sizes and shapes, were restricted by the presence of the residue W282 which likely serves as a ceiling to
exclude most ligands. PGIS also reveals an unusual meander structure protruding from the protein surface which may impede electron transfer. Another important feature of the PGIS structure is the plasticity of the heme propionates that may have an important implication in understanding the enzymatic catalysis. The presence of N287 in the active site and its potential role in peroxide cleavage warrants further experimental investigation. The PGIS structure thus should provide an avenue to better understand the structure/function relationship of the atypical P450.