Lipocalin-type prostaglandin (PG) D synthase (L-PGDS) catalyzes the isomerization of PGH$_2$, a common precursor of various prostanooids, to produce PGD$_2$, an endogenous somnogen and nociceptive modulator, in the brain. L-PGDS is a member of the lipocalin superfamily and binds lipophilic substances, such as retinoids and bile pigments, suggesting that L-PGDS is a dual functional protein acting as a PGD$_2$-synthesizing enzyme and a transporter for lipophilic ligands. In this study we determined by NMR the three-dimensional structure of recombinant mouse L-PGDS with the catalytic residue Cys-65. The structure of L-PGDS exhibited the typical lipocalin fold, consisting of an eight-stranded, antiparallel β-barrel and a long α-helix associated with the outer surface of the barrel. The interior of the barrel formed a hydrophobic cavity opening to the upper end of the barrel, the size of which was larger than those of other lipocalins, and the cavity contained two pockets. Molecular docking studies, based on the result of NMR titration experiments with retinoic acid and PGH$_2$ analog, revealed that PGH$_2$ almost fully occupied the hydrophobic pocket 1, in which Cys-65 was located and all-trans-retinoic acid occupied the hydrophobic pocket 2, in which amino acid residues important for retinoid binding in other lipocalins were well conserved. Mutational and kinetic studies provide the direct evidence for the PGH$_2$ binding mode. These results indicated that the two binding sites for PGH$_2$ and retinoic acid in the large cavity of L-PGDS were responsible for the broad ligand specificity of L-PGDS and the non-competitive inhibition of L-PGDS activity by retinoic acid.

Lipocalin-type prostaglandin (PG)$_2$ D synthase (L-PGDS, prostaglandin H$_2$ D-isomerase, EC 5.3.99.2) (1–3) catalyzes the isomerization of the 9,11-endoperoxide group of PGH$_2$, a common precursor of various prostanooids, to produce PGD$_2$ with 9-hydroxy and 11-keto groups, an endogenous somnogen (4) and a modulator of pain responses (5), in the presence of sulf-hydryl compounds. L-PGDS is the only enzyme among members of the lipocalin gene family (6) that is composed of a group of lipid-transporter proteins, such as retinol-binding protein, β-lactoglobulin, major urinary protein, aphorodisin (6–8), epdidymal retinoic acid-binding protein (9), and tear lipocalin (10). L-PGDS has three Cys residues, Cys-65, Cys-89, and Cys-186, conserved among all species. Two of these Cys residues, Cys-89 and Cys-186, form a disulfide bridge, which is highly conserved among most, but not all lipocalins (6). This disulfide bridge can be removed without a significant loss of the enzymatic activity. On the other hand, Cys-65 is unique to L-PGDS, as it has never been found in other lipocalins. Moreover, the replacement of Cys-65 with Ser/Ala by site-directed mutagenesis led to complete loss of the catalytic activity of the recombinant rat (11), human, mouse, chicken (12), and bull and frog (13) enzymes, indicating that the Cys-65 residue is the key residue for the reaction catalyzed by L-PGDS.

L-PGDS is abundantly expressed in the central nervous system of various mammals, male genitals, human heart, and mouse adipocytes (14). L-PGDS is the same as β-trace (15, 16), a major protein in the human cerebrospinal fluid (17), and is secreted actively into various body fluids, such as the plasma and seminal plasma. L-PGDS binds a large variety of ligands, such as retinoids (18, 19), biliverdin, bilirubin, thyroid hormones (20), gangliosides (21), and amyloid β peptide (22), with high affinities ($K_d = 20$ nM to 2 μM). Thus, we thought that L-PGDS possesses dual functions as an extracellular lipophilic ligand-transporter protein as well as a PGD$_2$-synthesizing enzyme (3). In a recent study the function of non-mammalian L-PGDS homologue without the catalytic Cys-65 residue serves

**References**

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2. The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

3. The atomic coordinates and structure factors (code 2E4J) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

4. The chemical shift assignments for free form of L-PGDS have been deposited in the BioMagRes Data Bank (www.bmrbr.wisc.edu) under the accession number BMRB 10137.

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6. The abbreviations used are: PG, prostaglandin; L-PGDS, lipocalin-type PG synthase; HSQC, heteronuclear single quantum correlation; NOE, nuclear Overhauser effect; r.m.s.d., root mean square deviation.
as a carrier protein for lipophilic ligands but not as an enzyme (12).

The genetic and biochemical properties of L-PGDS have been extensively studied as described above, whereas the structural information on L-PGDS and its complexes with various ligands remain to be elucidated. To understand the molecular basis of the reaction mechanism and of the ligand recognition, we determined the solution structure of recombinant mouse L-PGDS mutant, which contains the catalytic Cys-65 residue, by NMR spectroscopy and clarified the ligand binding mode by molecular docking.

EXPERIMENTAL PROCEDURES

NMR Spectroscopy—In this study we used the recombinant Δ1–24-C89A,C186A mouse L-PGDS mutant, in which the N-terminal signal sequence and the intermolecular disulfide bridge between Cys-89 and Cys-186 were removed, but the catalytically active Cys-65 residue was retained. This mutant shows the same catalytic activity and retinoid-binding activity as those of the wild type enzyme (19). Uniformly 15N- and 13C-labeled Δ1–24-C89A,C186A mouse L-PGDS mutant was prepared as reported previously (19) except that Escherichia coli BL21(DE3) cells were cultivated in M9 minimal medium containing [15N]ammonium chloride (1 g/liter) and/or 13C glucose as described previously (19). The all-atom 15N, 13C chemical shifts were indirectly calibrated from each gyromagnetic ratio (24). Backbone and side-chain assignments were obtained from two-dimensional 1H,15N HSQC, 13C,1H HSQC, three-dimensional HNCO, HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HBHA(CBCACO)NH, and HCCCH-two-dimensional total correlation spectroscopy (25, 26). NOE EIs were collected from three-dimensional 15N-edited NOE (100-ms mixing time) and 13C-edited NOE (100-ms mixing time) spectra (25, 26). Backbone amide groups slowly exchanging with the solvent were identified from a series of two-dimensional 1H,15N HSQC spectra after a rapid buffer exchange to D2O. The retinoic acid binding site was investigated by two-dimensional 1H,13N HSQC spectra. The protein and the ligands were combined at a molar ratio of 1:1. All the NMR data were processed with NMRPipe (27) and analyzed with the NMRView (Merck). A table containing the chemical shift assignments of L-PGDS has been deposited in the BioMagResBank data base under the accession number 10137.

The binding of L-PGDS to ligands was monitored by an NMR titration of 15N-labeled L-PGDS with unlabeled ligands using 1H,15N HSQC experiments. The combined 1H and 15N chemical shift changes over the range of the titration from 0 to 2 eq of ligands are plotted. Because of the instability of PGH2 in solution, the stable PGH2 analog, U-46619, was used to replace PGH2 for the interaction. The overall chemical structure of U-46619 is identical to PGH2 except for one endoperoxide oxygen that was replaced with carbon at C-9 (28).

Structure Calculation—NOE restraints were classified into four categories, strong, medium, weak, and very weak, corresponding to the distance restraints of 1.8–2.8, 1.8–3.4, 1.8–4.2, and 1.8–5.0 Å, respectively. The ψ and φ torsion angle restraints were evaluated from the 15N, Hα, 13Cα, and 13Cβ chemical shifts using the TALOS program (29). The restraints deduced from intramolecular hydrogen bonds of protein backbone, which were identified by H-D exchange experiments, were classified into two groups; between the amide proton and the carbonyl oxygen of 1.5–2.5 Å and between the amide nitrogen and the carbonyl oxygen of 2.5–3.5 Å (30). The initial solution structures were calculated using the distance geometry algorithm in the CNS programs (31). The structure optimization and energy minimization were achieved by a simulated annealing algorithm. The final 15 lowest energy structures were analyzed by using the MOLMOL (32) and PROCHECK programs (33). Structural statistics for the 15 structures are included in Table 1. Graphical representations were prepared using RASMOL and PyMOL. All of these structures have been deposited in the Protein Data Bank under accession code 2E4J.

Enzyme Assay—The L-PGDS activity was measured by incubating the enzyme at 25 °C for 1 min with [1-14C]PGH2 (final concentration of 1.25–40 μM) in 50 μl of 0.1 M Tris/HCl (pH 8.0) containing 1 mg/ml IgG and 1 mM dithiothreitol (11). [1-14C]PGH2 was prepared from [1-14C]arachidonic acid (2.20 GBq/mmol, PerkinElmer Life Sciences) as described previously (1). The all-trans-retinoic acid was dissolved in Me2SO to give a stock solution of 2 mM. The concentrations of all-trans-retinoic acid were determined spectroscopically based on its molar absorption coefficient, ε336, for all-trans-retinoic acid = 45,000 M−1 cm−1 (34). Various concentrations of all-trans-retinoic acid were added to the reaction mixture. Aggregates were removed by centrifugation, and the retinoic acid concentration was calibrated by spectrophotometry at 336 nm after the assay experiments. The U-46619 was dissolved in 0.1 M Tris/HCl (pH 8.0) to give a stock solution of 2 mM.

Molecular Modeling—Docking of retinoic acid and PGH2 to solution structure of L-PGDS was performed with AutoDock, Version 3.0 (35). This program allows ligand structures to dock in a conformationally flexible manner to a protein and adopts a rigid-body protein approximation to speed up the calculation of binding free energy. The initial structures of the ligands were constructed by the Maestro molecular modeling package (Schrödinger Inc., Portland, OR). The AMBER (36) and Gasteiger-Marsili (37, 38) atomic charges were loaded on the protein and ligand atoms as partial charges, respectively. A docking space of the ligands was defined with a rectangular box (33.8 × 45.0 × 33.8 Å3) that covers the interior space of β-barrel of the protein. Grids of probe-atom interaction energies were computed on the boxes. The number of grid points in the
The ligand probes were then docked by Lamarckian genetic algorithm. Flexibility was allowed for all exocyclic torsion angles of the ligands by freely rotating the torsional bonds. One Lamarckian genetic algorithm calculation generated 270,000 conformers by varying the molecular position in the docking space and torsion angles of the ligand and ranked the conformers by their binding free energy. Each docking experiment consisted of a series of 200 Lamarckian genetic algorithm simulations.

RESULTS

Structure Determination—We determined the solution structure of the Δ1–24-C89A,C186A mouse L-PGDS, which showed the same catalytic activity and retinoid binding activity as those of the wild type enzyme (19) by using NMR-derived distance constraints. Nearly complete assignments for backbone and side-chain 1H, 13C, and 15N resonances of L-PGDS were accomplished. The detailed restraint data for the structure calculation of L-PGDS are summarized in Table 1. A total of 1653 NOE restraints were employed for this structure calculation, including 706 intraresidue, 428 sequential, and 147 medium-range (i-i+2, i-i+3, i-i+4) and 372 long-range restraints. In addition, the 348 dihedral angle restraints were obtained from the TALOS program, and the 80 hydrogen bond restraints were observed from H-D exchange experiments. Using the CNS program, 4000 structures were calculated. The 15 structures of the lowest total energy structures with no distance restraint violations greater than 0.5 Å, and no torsion angle restraint violations above 5° were selected for further analysis as shown in Fig. 1A. Ramachandran ϕ-ψ plots for the ensemble of 15 structures indicate that 72.8% of the non-glycine and non-proline residues are found in the most favored region, and 25.4% were found in the additional allowed regions. Several residues in the N terminus are found around the border of the disallowed region probably due to the lack of experimental information on the ϕ-ψ angle values (39). A ribbon diagram of a representative lowest energy NMR structure of L-PGDS is shown in Fig. 1B. The superposition of backbone atoms clearly shows
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that the calculated structures were well converged (Fig. 1A). The overall average r.m.s.d. values to the mean structure for backbone heavy atoms and all heavy atoms including side chains of L-PGDS (residue 25–189) were 1.24 ± 0.25 and 1.73 ± 0.23 Å, respectively. The average r.m.s.d. values for backbone heavy atoms and all heavy atoms in the regular secondary structure elements were 0.41 ± 0.07 and 0.85 ± 0.07 Å, respectively.

Structure Overview of L-PGDS—L-PGDS exhibited the typical lipocalin fold, consisting of an eight-stranded, antiparallel β-barrel with a repeated +1 topology. Secondary structural elements of L-PGDS are comprised of nine β-strands (A, amino acid residues 41–50; B, 63–71; C, 76–84; D, 89–98; E, 104–107; F, 115–121; G, 128–135; H, 144–149; I, 177–179) plus two short 3₁₀ helices (H1, 36–39; H2, 56–60) and one long α-helix (H3, 157–171) (Fig. 1B). In NMR ensemble structures of L-PGDS, the first eight residues at the N terminus, the EF loop, and GH loop are less defined due to the HN signal broadening of these loop regions. Therefore, these regions are indicated by the dashed line in Fig. 1B. The hydrophobic cavity exists in the barrel, which consists of eight strictly antiparallel β-sheets and is largely open at one end (Fig. 1C). Four relatively flexible loops connect neighboring β-strands in a pairwise manner at the end of the barrel. The cavity has an unusually bifurcated shape and a larger entry than those in other lipocalins. The size of the cavity is about 17 Å deep, 16 Å wide, and 8 Å thick. Analysis of the inner accessible surface of the cavity shows that there are two distinct pockets adjacent to each other, pocket 1 and pocket 2. Pocket 1 is located at the middle of the cavity, and pocket 2 is located at a relatively lower part of the cavity. For comparison, the cavity of representative lipocalins, retinoic acid-binding protein is illustrated in Fig. 1D, and the size of the cavity is about 17 Å deep, 8 Å wide, and 8 Å thick.

Recently, the crystal structure of Δ1–24-C65 mouse L-PGDS without enzyme activity has been deposited to the Protein Data Bank (codes 2CZT and 2CZU). Comparison of the crystal and solution structures of mouse L-PGDS revealed that both structures were similar to each other and are characterized by their overall profiles with a large cavity that consisted of two pockets as described above.

Mapping of the Binding Sites by Chemical Shift Perturbation—The ligand interaction sites of L-PGDS were determined using the NMR chemical shift perturbation method (40) in which backbone resonances of L-PGDS in the complexes with retinoic acid and U-46619 were recorded with successive addition of all trans-retinoic acid or PGH₂ analog, U-46619 (Fig. 2, A and B). To allow quantification of the observed chemical shift changes, backbone resonances of L-PGDS in the complexes with retinoic acid and U-46619 were assigned (supplemental Table S1). Fig. 2A presents the magni-
Therefore, the ribbon representation of L-PGDS backbone, the carboxyl group of retinoic acid is located at the bottom of pocket 2 and surrounded by several aromatic residues. Upon retinoic acid binding, large chemical shift changes (>0.08) were observed at the region containing residues His-116, Ser-117 (β-strand F) and residues Phe-132, Ser-133, Gly-135 (β-strand G) (Fig. 2B), similar to those observed for the retinoic acid binding. In addition, residues around Cys-65 catalytic center, Ala-60, Leu-62, Met-64, Cys-65, Thr-82, Asn-87, and Tyr-149 also show large chemical shift changes (>0.08). These residues are located around pocket 1 in the solution structure of L-PGDS (Fig. 2D). β-Strands F and G and pocket 1 should contain the major contact sites for U-46619. Therefore, U-46619 binds in a different position from retinoic acid.

Kinetic Studies of L-PGDS Mutants—To clarify the difference between U-46619 binding and PGH₂ binding, the inhibition of L-PGDS reaction by U-46619 was investigated. Lineweaver-Burk plots of L-PGDS activity in the presence of various concentrations of U-46619 (see Fig. 4B) revealed an unchanged y axis intercept and an altered x axis, indicating that U-46619 inhibited L-PGDS in a competitive manner. This result clearly shows that U-46619 binds to the catalytic site of L-PGDS in the same manner as PGH₂.

To identify the residues important for the PGH₂ binding, the catalytic activities of several mutants, C89A,C186A/S45A, C89A,C186A/T67A, and C89A,C186A/S81A, were measured. As shown in Table 2, the mutants C89A,C186A/S45A and C89A,C186A/S81A showed a comparable Kₘ value to C89A,C186A, whereas the mutant C89A,C186A/T67A showed a 5–6-fold higher Kₘ value. This indicates that Thr-67 is important for the recognition of PGH₂. In addition, Ser-45, Thr-67, and Ser-81 are not important for retinoic acid binding because these mutants have nearly the same Kₘ values for retinoic acid to C89A,C186A. These results suggest that the region around Cys-65 is related to PGH₂ binding but not to retinoic acid binding. The catalytic activities and Kₘ values for retinoic acid of mutants C89A,C186A/H116E and C89A,C186A/H116A were measured (Table 2). Both Kₘ and Kₖ values of mutant C89A,C186A/H116E were higher than that of C89A,C186A. The mutant C89A,C186A/H116A showed a 3-fold higher Kₘ value but almost the same Kₖ value for retinoic acid. These results may indicate that the positive charge around His-116 contributes to the PGH₂ and retinoic acid binding and that His-116 is involved in PGH₂ binding but not in retinoic acid binding.

DISCUSSION

Unusually Bifurcated Cavity of L-PGDS—Fig. 1C clearly shows that the cavity of L-PGDS has an unusually bifurcated shape and a larger entry than those in other lipocalins.
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Analysis of the inner accessible surface shows that there are two distinct pockets adjacent to each other. Pocket 1 is located at the middle of the cavity, and pocket 2 is located at a relatively lower part of the cavity. Most of the residues located around the pocket 2 are aromatic and hydrophobic. In contrast, the pocket 1 is surrounded by several polar residues such as Ser or Thr and is more hydrophilic than the pocket 2. The two pockets are separated by residues Tyr-149 and Thr-67. These data indicate that the two binding pockets in the large cavity of L-PGDS should be responsible for the broad ligand specificity of L-PGDS.

Molecular Docking Studies—To explore the ligand binding mode, we constructed two molecular models bound to retinoic acid or PGH₂. In the retinoic acid-docking model (Fig. 3A), the retinoic acid lies on the β-strand F and G, and the cyclohexene ring of retinoic acid can be placed into the hydrophobic pocket 2. Structural comparison with other retinoid binding lipocalins showed that aromatic residues important for retinoid binding are also conserved in L-PGDS. These aromatic residues, Phe-34, Phe-39, Tyr-105, and Tyr-149, are well defined (the side chain r.m.s.d. values = 0.4–0.7 Å) for L-PGDS and make up the aromatic cluster around the pocket 2 at the bottom of the β-barrel. The distances between these aromatic residues of L-PGDS and the α-ionone ring are within 4 or 5 Å, indicating the relatively strong van der Waals contacts (Fig. 3B). Corresponding distances in the crystal structure of retinoic acid-binding protein-retinoic acid complex (Protein Data Bank code 1EPA) also range from 3.0 to 4.4 Å. Therefore, the aromatic cluster of L-PGDS, located at the bottom of the pocket 2, is considered to be important for retinoid binding. Both L-PGDS (Kᵣ = 80–150 nM) and retinoic acid-binding protein (Kᵣ = 900 nM) exhibit relatively strong affinity to retinoid acid. In the docking model, an aliphatic side chain of retinoic acid is pointed toward the open entry of the β-barrel. The terminal carboxylate group is located in the vicinity of the positively charged region (β-strand F). This retinoid acid binding model is consistent with the results of NMR titration experiments and mutant analysis.

In the PGH₂-docking model (Fig. 3C), the PGH₂ was in the middle of the cavity, and the cyclopentane ring of PGH₂ almost completely occupied pocket 1. In this model the thiol group of Cys-65 is located within a 5-Å distance from the oxygen atoms of the 9,11-endoperoxide group of PGH₂ (Fig. 3D), indicating that these atoms are in contact distance with each other. This is consistent with the previously proposed PGH₂ reaction mechanism by Urade et al. (11). In the proposed mechanism, conversion of PGH₂ is initiated by a nucleophilic attack of the active intrinsic thiol of Cys-65 to the oxygen at C-11 of PGH₂. The extrinsic sulphydryl group withdraws the hydrogen at C-11 to produce PGD₂ in the following reaction step. Ser-45, Thr-67, and Ser-81 residues were close to oxygen atoms of the endoperoxide group of PGH₂ with distances of about 4 Å (Fig. 3B). Biochemical studies of the cane toad homolog of L-PGDS suggested that these residues may be involved in the recognition of the oxygen atoms of the endoperoxide group of PGH₂ (13). Present mutant analyses indicated that Thr-67 is the key residue among these residues. The cyclopentane ring and α and ω chains of PGH₂ were not fixed in their spatial relationship in solution. However, the α chain, with a negative charge at the carboxyl group, turned on the positive-charged region arising from His-116 and Lys-137 located at the edge of the cavity of L-PGDS. This PGH₂ binding model supports that PGH₂ binds to the region around the Cys-65 in the catalytic pocket 1, which is suggested by the U-46619 binding experiments. Interestingly, the overall structure of PGH₂ in our complex model is similar to the previously reported free form structure of U-46619 in a membrane-mimicking environment (28).

Non-competitive Inhibition of Retinoic Acid—An overlay of the retinoic acid-docking model and the PGH₂-docking model is shown in Fig. 4A. The terminal portions of the aliphatic chain of retinoic acid and the α chain of PGH₂ are slightly overlapped...
In both binding models of retinoic acid and PGH$_2$, there is a possibility that each of the carboxyl groups in the presence of various concentrations of all-trans-retinoic acid (Fig. 4C) revealed the unchanged an $x$ axis intercept and an altered y axis, indicating that all-trans-retinoic acid inhibited L-PGDS activity with the estimated $K_m$ value of $5–6 \mu M$ in a non-competitive manner against PGH$_2$; this is consistent with the current docking model (Fig. 3C). These results provide the direct evidence for two binding sites for PGH$_2$ and retinoic acid in the large cavity of L-PGDS.

The solution structure described here is in good agreement with the previously reported structural information from the fluorescence quenching assay and mutational studies (19). These data revealed that the Trp-43 residue contributed to the large cavity of L-PGDS.

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