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Characterization of a Major Secretory Protein in the Cane Toad (*Bufo marinus*) Choroid Plexus as an Amphibian Lipocalin-type Prostaglandin D Synthase

Daisuke Irikura[1,2], Takashi Inui[1], Carsten T. Beuckmann[1], Kousuke Aritake[1], Gerhard Schreiber[3], Masashi Miyano[2], Tsuyoshi Inoue[4] and Yoshihiro Urade[1,*]

[1]Department of Molecular Behavioral Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan; [2]Structural Biophysics Laboratory, RIKEN Harima Institute, 1-1-1 Kouto, Sayo, Hyougo, 679-5148, Japan; [3]Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia; and the [4]Department of Engineering Osaka University, Yamadaoka, Suita, Osaka 565-0871, Japan

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Here we report the enzymatic and ligand-binding properties of a major secretory protein in the choroid plexus of cane toad, *Bufo marinus*, whose protein is homologous with lipocalin-type prostaglandin (PG) D synthase (L-PGDS) and is recombinantly expressed in *Xenopus* A6 cells and *Escherichia coli*. The toad protein bound all-*trans* retinal, bile pigment, and thyroid hormones with high affinities ($K_d = 0.17$ to 2.00 μM). The toad protein also catalysed the L-PGDS activity, which was accelerated in the presence of GSH or DTT, similar to the mammalian enzyme. The K_m value for PGH_2 (17 μM) of the toad protein was almost the same as that of rat L-PGDS (14 μM), whereas the turnover number (6 min^{-1}) was approximately 28 fold lower than that of rat L-PGDS. Site-directed mutagenesis based on a modeled structure of the toad protein revealed that Cys^{59} and Thr^{61} residues were crucial for the PGDS activity. The quadruple $Gly^{39}Ser/Ala^{75}Ser/Ser^{140}Thr/Phe^{142}Tyr$ mutant of the toad protein, resembling mouse L-PGDS, showed a 1.6 fold increase in the turnover number and a shift in the optimum pH for the PGDS activity from 9.0 to 8.5. Our results suggest that the toad protein is a prototype of L-PGDS with a highly functional ligand-binding pocket and yet with a primitive catalytic pocket.

Key words: amphibian, lipocalin, lipocalin-type prostaglandin D synthase, prostaglandin D_2, site-directed mutagenesis.

Abbreviations: L-PGDS, lipocalin-type prostaglandin D synthase; PG, prostaglandin; DMSO, dimethyl sulfoxide; reverse T3, 3,3′,5′-triiodo-L-thyroxine; T3, 3,3′,5-triiodo-L-thyroxine; T4, L-thyroxine.

Epithelial cells of the choroid plexus are highly specialized in the synthesis of a variety of secretory proteins that enter the cerebrospinal fluid (*1*). The mammalian choroid plexus actively produces transthyretin, a thyroxine-binding protein, and β-trace, a 26-kDa glycoprotein identified as lipocalin-type prostaglandin (PG) D synthase [L-PGDS; EC 5.3.99.2; (*2, 3*)]. On the contrary, transthyretin was not produced in the cane toad choroid plexus, but a 20-kDa polypeptide product was synthesized and secreted (*4*). The cDNA for this amphibian choroid plexus protein was isolated from toad, *Bufo marinus* (*4*) and *Xenopus laevis* (*5, 6*). The bullfrog (*Rana catesbeiana*) choroid plexus also contained a very large amount of mRNA for a 20-kDa lipocalin (*7*). Sequence analysis and a homology search in databases of primary structure indicated that this amphibian choroid plexus protein was a member of the lipocalin superfamily and was the most homologous (~40% identity) one to mammalian L-PGDSs (*8, 9*). The lipocalin superfamily is composed of secretory transporter proteins for various lipophilic molecules, such as β-lactoglobulin, plasma retinol-binding protein, major urinary protein, and epididymal retinoic acid-binding protein. All these proteins share a common feature of binding and transporting small lipophilic molecules such as retinoids and thyroids (*10, 11*). In the presence of sulfhydryl compounds, L-PGDS catalyses the isomerization of the 9,11-endoperoxide group of PGH_2, a common precursor of various prostanoids, to produce PGD_2, having 9 hydroxy and 11 keto groups (*12*). L-PGDS is the first member of the lipocalin superfamily to be recognized as an enzyme (*9, 13*). Therefore, the amphibian choroid plexus protein may also be associated with the L-PGDS activity.

In this study, we expressed the recombinant major secretory protein of the cane toad choroid plexus in *Xenopus* A6 cells and *Escherichia coli*, purified them to apparent homogeneity, and determined its PGDS activity. We then identified the catalytic centre and activation residue of the cane toad protein by site-directed mutagenesis. Furthermore, we analysed the binding activity of this protein toward lipophilic substances such as biliverdin, bilirubin, 3,3′,5′-triiodo-L-thyroxine (reverse T3), 3, 3′,5-triiodo-L-thyroxine (T3), thyroxine (T4), and all-*trans* retinal by fluorescence

*To whom correspondence should be addressed. Tel: +81 66 872 4851, Fax: +81 66 872 2841, E-mail: uradey@obi.or.jp

quenching of intrinsic tryptophan residues. Our results suggest that the cane toad protein is a prototype of L-PGDS, having a highly functional ligand-binding pocket and yet a primitive catalytic pocket.

EXPERIMENTAL PROCEDURES

Materials—Dimethyl sulfoxide (DMSO), dithiothreitol (DTT), and glutathione (GSH) were purchased from WAKO Pure Chemical (Osaka, Japan). Thrombin, biliverdin, bilirubin, all-*trans* retinal, reverse T3, T3, and T4 were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Purification of Cane Toad Choroid Plexus Protein Recombinantly Expressed in Xenopus A6 Cells—We used the full-length cDNA for cane toad L-PGDS homologue (EMBL/GenBank/DDBJ^{TM} Accession Number X67952) (*4*) for protein expression. We generated cDNA for C-terminus FLAG-tagged cane toad L-PGDS homologue by PCR using the sense and anti-sense primers; 5′-ATG AAG GGA CTC GTG CTC AGC TTC GCC CTC-3′ and 5′-TTA CTT GTC ATC GTC GTC CTT GTA GTC CAC TTC TGA CAT GCA GCT ATC AGT-3′, respectively. The cDNA was ligated into pcDNA 3.1/V5-His-TOPO (Invitrogen, SanDiego, CA, USA). This plasmid was sequenced to confirm the correct sequence. Used for transfection, cells of the *Xenopus laevis* cell line A6 (derived from kidney epithelial cells, ATCC# CCL102) were cultured at 28°C in NCTC 109 medium (Invitrogen), 75%; distilled water, 15%; and fetal bovine serum (SIGMA-Aldrich), 10%. The plasmid DNA (Toad L-PGDS-flag/pcDNA 3.1/V5-His-TOPO) was purified on Qiagen columns (QIAGEN, Hilden, Germany). For transfection, the confluent A6 cultures were split, and the cells were seeded to new 100-mm plates (7×10^5 cells/plate) at least 20 h before the transfection. The cells were transfected with Superfect reagent (QIAGEN) according to the manufacturer's instructions. In short, 10 μg of plasmid DNA per plate was used, the Superfect-DNA complex was added to the cell culture and incubated for 2 h followed by washing cells washed twice with 85% PBS and then twice with complete medium. The cells were then incubated for an additional 36 h. Purification and elution steps were carried out by using anti-FLAG M2 agarose (Sigma), according to the manufacturer's instructions. After the elution step, the recombinant cane toad protein was further purified to apparent homogeneity using of Superdex75 column chromatography (GE Healthcare UK Ltd., Buckinghamshire, England).

Purification of Cane Toad Choroid Plexus Protein Recombinantly Expressed in E. coli.—We deleted the cDNA nucleotides specifying the first 20 N-terminal amino acid residues of the signal peptide of cane toad L-PGDS homologue and then ligated into the remainder *BamH*I-*EcoR*I sites of the expression vector pGEX-2T plasmid. The recombinant enzyme was expressed as a GSH S-transferase (GST) fusion protein in *E. coli* DH5α. The fusion protein was bound to GSH-Sepharose 4B (GE Healthcare UK Ltd.) and incubated with thrombin (100 U/100 μl) to release the cane toad protein from GST. The recombinant cane toad protein was further purified to apparent homogeneity by using Superdex75 column chromatography (GE Healthcare UK Ltd.). The purified protein was dialysed against 5 mM Tris/HCl (pH 8.0).

Site-directed Mutagenesis—Site-directed mutagenesis was performed by using of a QuikChange site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. The following mutants of the cane toad L-PGDS homologue; $Gly^{39}Ser$, $Cys^{59}Ala$, $Thr^{61}Ala$, $Ala^{75}Ser$, $Ser^{140}Thr$, $Phe^{142}Tyr$, $Gly^{39}Ser/Ala^{75}Ser$, $Ser^{140}Thr/Phe^{142}Tyr$, and $Gly^{39}Ser/Ala^{75}Ser/Ser^{140}Thr/Phe^{142}Tyr$ were constructed. Both plasmids of double ($Gly^{39}Ser/Ala^{75}Ser/Ser^{140}Thr/Phe^{142}Tyr$) and quadruple ($Gly^{39}Ser/Ala^{75}Ser/Ser^{140}Thr/Phe^{142}Tyr$) mutants were constructed by repeating the above method. Introduction of mutations was confirmed by DNA sequencing.

Enzyme Assay—The PGDS activity was measured by incubating the enzyme at 25°C for 30 s with [1-^{14}C] PGH_2 in 50 μl of 0.1 M Tris/HCl (pH 8.0) containing 1 mg/ml IgG and 1 mM DTT (*12*). [1-^{14}C]PGH_2 was prepared from [1-^{14}C] arachidonic acid (2.20 GBq/mmol, Perkin-Elmer, Boston, MA, USA) as described previously (*12*). The rates of all enzymatic reactions were calculated after subtracting the blank value obtained without the enzyme.

Fluorescence Quenching Assay—Biliverdin, bilirubin, all-*trans* retinal, reverse T3, T3, and T4 were dissolved in DMSO to give a 2 mM stock solution. The concentrations were determined spectroscopically with their respective molar extinction coefficients of ε_{453} in chloroform for bilirubin = 61,700 $M^{-1}cm^{-1}$ (*14*), ε_{377} in methanol for biliverdin = 51,500 $M^{-1}cm^{-1}$ (*15*), ε_{383} in ethanol for all-*trans* retinal = 42,800 $M^{-1}cm^{-1}$ (*16*), ε_{320} at pH 10 for T3 and reverse T3 = 4,660 $M^{-1}cm^{-1}$, and ε_{325} at pH 11 for T4 = 6,180 $M^{-1}cm^{-1}$ (*17, 18*). Various concentrations of each lipophilic ligand were added to the cane toad protein in 990 μl of 5 mM Tris/HCl (pH 8.0). After incubation at 20°C for 60 min, the intrinsic tryptophan fluorescence was measured by using an FP-750 spectrofluorometer (Jasco, Tokyo, Japan) with an excitation wavelength at 282 nm and an emission wavelength at 338 nm. The quenching of tryptophan fluorescence caused by nonspecific interactions with various lipophilic ligands was corrected with 1.5 μM *N*-acetyl-L-tryptophanamide. The dissociation constant (K_d) values for binding between various kinds of lipophilic ligands and the cane toad protein were calculated by the method described earlier (*19*).

RESULTS

Amino acid Sequences of Cane Toad choroid plexus proteins and mammalian L-PGDSs—Previously, we found that the major secretory protein in the choroid plexus of cane toad was homologous to mammalian L-PGDSs. This cane toad homologue was abundantly expressed and secreted from the choroid plexus of the cane toad (*4*). Figure 1 shows the multiple alignment of amino acid sequences of cane toad choroid plexus proteins and mammalian L-PGDSs. The amino acid sequences of cane toad L-PGDS homologue was 69 and 85% identical to *Xenopus* and treefrog homologues, and 43, 44, and 41% to rat, mouse, and human L-PGDSs,

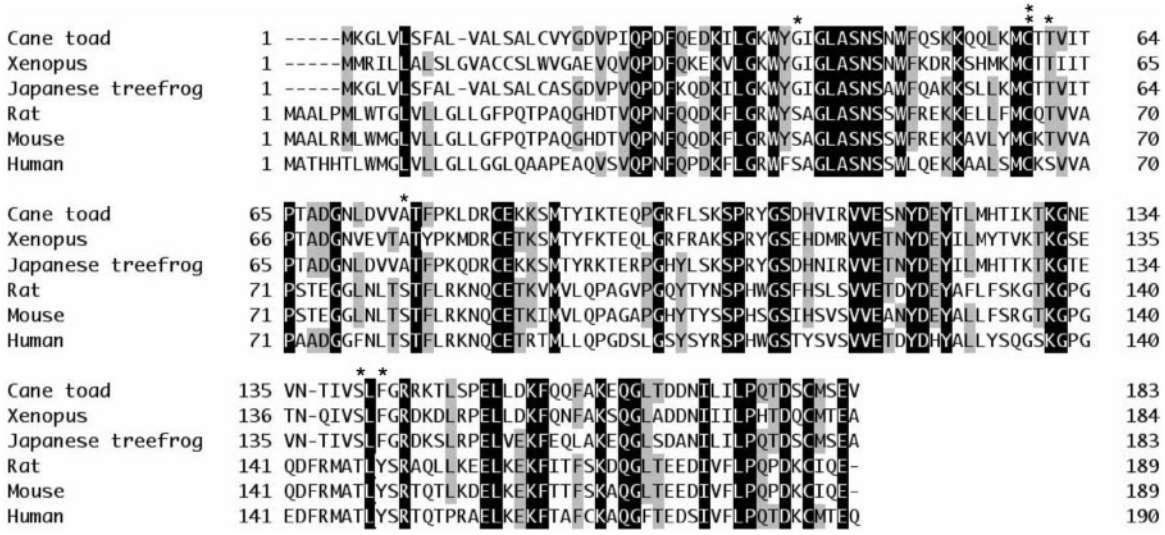

Fig. 1. **Alignment of amino acid sequences of cane toad choroid plexus protein, amphibian, and mammalian L-PGDSs.** Amino-acid sequence of the cane toad L-PGDS homologue was compared with those of *Xenopus* (DDBJ Accession Number: D83712), Japanese treefrog (DDBJ Accession Number: AB095923), rat (DDBJ Accession Number: J04488), mouse (DDBJ Accession Number: X89222), and human (DDBJ Accession Number: M61901) L-PGDSs. Dashes show the gap used to maximize the identity. Black shading indicates identical amino acid residues among the 6 proteins. Gray shading indicates more than 60% identical amino acid residues among the 6 proteins.

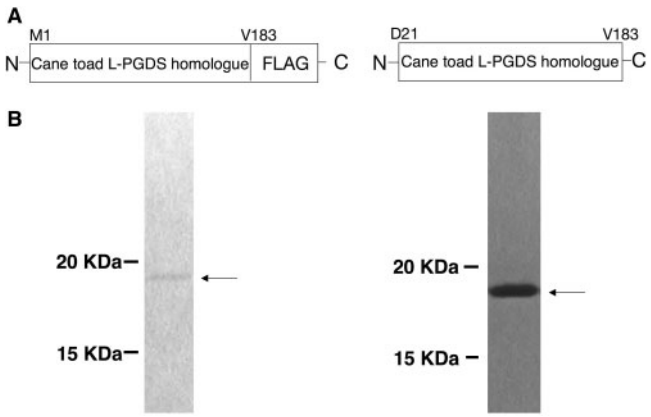

Fig. 2. **SDS-PAGE of the recombinant cane toad L-PGDS homologue**. (A) Structure of the fragments used for the expression of recombinant proteins. (B) Samples of recombinant protein, which were expressed in *Xenopus* A6 cell (left) and in *E. coli* (right), were analysed by SDS-PAGE and then stained with Coomassie Brilliant Blue. Molecular weights are indicated on the left.

respectively. The Cys residue in the catalytic center (indicated by the double asterisk in Fig. 1) of L-PGDS was completely conserved among both mammalian and amphibian species, suggesting that the recombinant cane toad L-PGDS homologue might have L-PGDS-like activity.

Purification of Recombinant Cane Toad Choroid Plexus Protein—The recombinant cane toad choroid plexus protein was purified to apparent homogeneity by anti-FLAG M2 and Superdex 75 column chromatography from the culture medium of transfected *Xenopus* A6 cells (Fig. 2). The recombinant protein was efficiently expressed in *E. coli* and purified by GSH-Sepharose 4B column chromatography, incubation with thrombin, and gel filtration column chromatography (Fig. 2). Recombinant proteins from both *Xenopus* A6 cells and *E. coli* showed almost the same PGDS activity (30 nmol/min/mg protein, with 2 μM PGH_2). Therefore, we used the recombinant cane toad protein prepared from *E. coli* for further characterization.

Lipophilic Ligand-binding Activity of Cane Toad Protein—Several members of the lipocalin superfamily such as epididymal retinoic acid-binding protein (*20*), β-lactoglobulin (*21–23*), and plasma retinol-binding protein (*24*), have the ability to bind their respective ligands. Rat and mouse recombinant L-PGDSs bound the lipophilic ligands such as retinoic acid, retinal, biliverdin, bilirubin, reverse T3, T3, and T4 (*25–27*). Fluorescence quenching of the intrinsic tryptophan residue revealed that the *Xenopus* homologue of the cane toad protein bound retinal with a high affinity of $K_d = 3.5$ nM (*6*). Therefore, we examined the fluorescence quenching after incubation of the cane toad protein with various kinds of lipophilic compounds.

The recombinant cane toad protein showed fluorescence quenching in a concentration-dependent manner after the addition of biliverdin, bilirubin, all-*trans* retinal, reverse T3, T3, or T4 (Fig. 3). The fluorescence intensity decreased below 30, 30, 40, 60, 55, and 60% in the presence of 10 μM biliverdin, bilirubin, all-*trans* retinal, reverse T3, T3, and T4, respectively. The K_d values of each lipophilic ligands for cane toad protein were calculated from the quenching curves and summarized in Table 1 ($K_d = 0.17$ to 2.00 μM), which are similar to those of rat and mouse L-PGDSs [$K_d = 0.03$ to 2.08 μM; (*25–27*)]. These results indicate that the cane toad

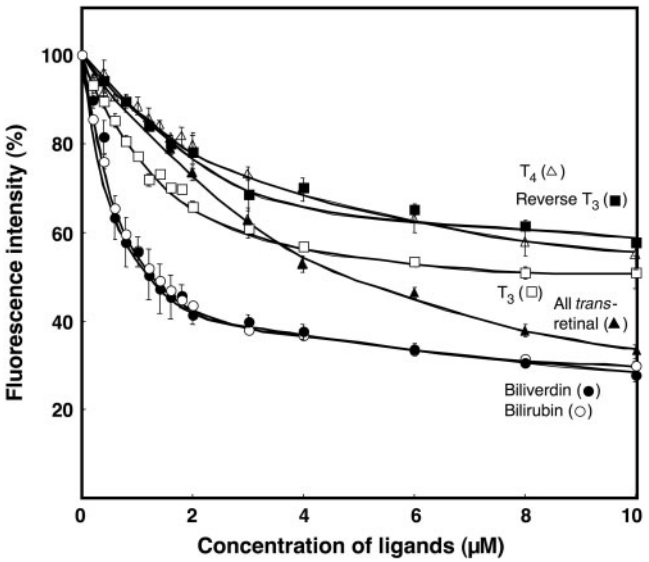

Fig. 3. **Fluorescence quenching of intrinsic tryptophan of the cane toad L-PGDS homologue by incubation with lipophilic ligands**. Cane toad protein was incubated with various concentrations of bilirubin (○), biliverdin (●), all-*trans* retinal (▲), reverse T3 (■), T3 (□), or T4 (△).

Table 1. **K_d values for binding of lipophilic ligands to cane toad L-PGDS homologue and mammalian L-PGDS.**

Ligand	Cane toad L-PGDS homologue (μM)	Mammalian L-PGDS (μM)
Bilirubin	0.23	0.03 (*26*)
Biliverdin	0.17	0.04 (″)
All-*trans* retinal	2.00	0.10 (*25*)
T_4	1.30	0.66 (*26*)
Reverse T_3	1.60	0.82 (″)
T_3	0.88	2.08 (″)

protein captured those hydrophobic ligands with relatively high affinities.

PGDS Activity of Cane Toad Protein and Its Mutants—As described above, the recombinant cane toad protein showed PGDS activity. A typical result from the TLC analysis of the reaction products are shown in Fig. 4. The cane toad protein isomerized PGH_2 to PGD_2 in a dose-dependent manner, but did not produce PGE_2 or $PGF_{2\alpha}$.

Multiple alignment analysis of L-PGDSs indicated that Cys residues in the cane toad and *Xenopus* L-PGDS homologues at positions 59 and 60, respectively, correspond to an essential thiol, Cys^{65}, found in mammalian L-PGDS (Fig. 1). Analysis of the crystal structure of mouse L-PGDS (PDB code: 2CZT; Kumasaka *et al*., submitted) revealed that the Cys^{65} in the catalytic centre was surrounded by 3 polar residues, i.e., Ser^{45}, Thr^{67}, and Ser^{81}, in the hydrophobic cavity (Fig. 5A). These polar residues in the hydrophobic cavity were considered to be involved in the activation of the thiol group of Cys^{65}, because the substitution of these residues in mouse L-PGDS by Ala significantly decreased the PGDS activity (Kumasaka *et al*., submitted). Among

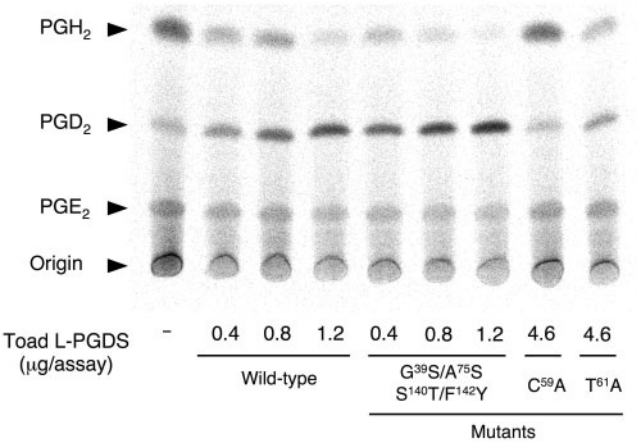

Fig. 4. **TLC profile of PGs formed from PGH_2 after incubation with the recombinant cane toad L-PGDS homologue or its mutants**. The recombinant cane toad L-PGDS homologue and various types of mutants were incubated with 10 μM PGH_2 at 25°C for 30 s in the presence of 1 mM DTT. Reaction products were extracted with diethylether: methanol: 1M citrate acid (30:4:1) and separated by TLC.

these 3 polar residues, one of them, Thr^{61}, is also conserved in the cane toad protein; whereas the other 2 residues are replaced with nonpolar residues, Gly^{39} and Ala^{75}, in the amphibian proteins (Figs. 1 and 5B). In Fig. 4, the Cys^{59}Ala mutant of the toad protein did not show any significant PGDS activity, indicating that the Cys^{59} residue is essential for the PGDS activity of the toad protein, similar to the case for the corresponding residue in other mammalian homologues of L-PGDS (*28, 29*). The Thr^{61}Ala mutant showed about 5% of the PGDS activity of the wild-type enzyme (Fig. 4), suggesting that the Thr^{61} residue is involved in the activation of the thiol group of Cys^{59}. The Gly^{39}Ser, Ala^{75}Ser, and Gly^{39}Ser/Ala^{75}Ser mutants slightly decreased the PGDS activity yet increased the affinity for the substrate PGH_2 as described later.

The crystal structure of mouse L-PGDS also revealed that 2 other polar residues, Thr^{147} and Tyr^{149}, in the hydrophobic cavity might interact with the 15-hydroxy group of PGH_2 (Fig. 5A). In the amphibian proteins, those 2 residues were replaced with Ser^{140} and Phe^{142}, respectively (Figs. 1 and 5B). Therefore, we also examined the effects of those amino acid residues of the cane toad protein on the PGDS activity by conducting site-directed mutagenesis. The Phe^{142}Tyr and Ser^{140}Thr/Phe^{142}Tyr mutants decreased the PGDS activity as described later, whereas the quadruple mutant Gly^{39}Ser/Ala^{75}Ser/Ser^{140}Thr/Phe^{142}Tyr, which contained the polar residues at the same positions as those of the mammalian L-PGDS, increased the PGDS activity as compared to wild-type enzyme (Fig. 4).

Enzymatic Characteristics of Cane Toad Protein and Its Mutants—Comparison of kinetic parameters of the L-PGDS activity for the cane toad L-PGDS, its mutants and rat L-PGDS are summarized in Table 2. Figure 6A showed the PGH_2 dependency of the PGDS activity catalysed by the wild-type and the quadruple mutant

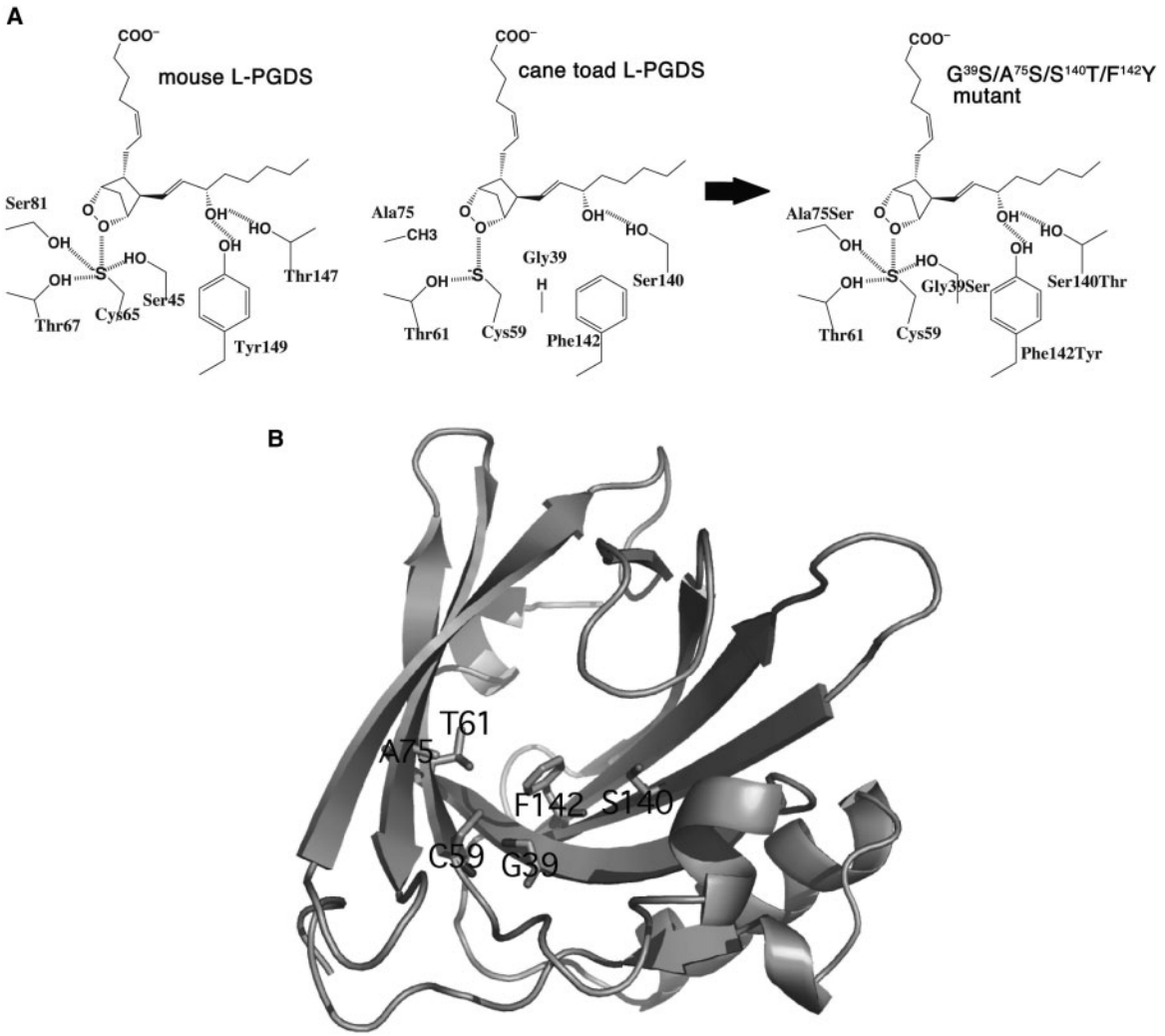

Fig. 5. **Structure surrounding catalysis site and model structure of cane toad L-PGDS**. (A) Schematic representation of the interaction between PGH_2 and the catalysis-related amino acid residues in the hydrophobic cavity of the wild-type cane toad protein, its quadruple mutant, and mouse L-PGDS. (B) The model structure of cane toad L-PGDS. The ribbon diagram was draw by 'Pymol' using the cane toad L-PGDS model based on mouse L-PGDS structure (PDB code; 2CZT). Cane toad L-PGDS was indicated in gray. Gly39, Cys59, Thr61, Ala75, Phe142 and Ser140 were represented in stick model.

Table 2. **Comparison of kinetic parameters of the L-PGDS activity for the cane toad L-PGDS, its mutants, and rat L-PGDS.**

	Toad L-PGDS								Rat L-PGDS[a]
	Wild	$Thr^{61}Ala$	$Gly^{39}Ser$	$Ala^{75}Ser$	$Phe^{142}Tyr$	$Gly^{39}Ser/Ala^{75}Ser$	$Ser^{140}Thr/Phe^{142}Tyr$	$Gly^{39}Ser/Ala^{75}Ser/Ser^{140}Thr/Phe^{142}Tyr$	
Turnover number (min^{-1})	6.1	–	3	3	3	3	4	9.8	170
K_m for PGH_2 (μM)	17	–	2	2	26	2	12	24	14
Optimum pH	9	>10	–	–	–	–	–	8.5	9.5

[a]Values from (*12*).

of toad L-PGDS measured in the presence of 1 mM DTT. Figure 6B showed the Lineweaver–Burk plots of the PGH_2 dependency. The apparent K_m value for PGH_2 (17 μM) of the toad L-PGDS was almost the same as that of rat L-PGDS (14 μM), while the turnover number (6.1 min^{-1}) was 28 fold lower than that of rat L-PGDS [170 min^{-1}, (*10*)]. As compared with the wild-type toad L-PGDS, the $Gly^{39}Ser$, $Ala^{75}Ser$, $Phe^{142}Tyr$, $Gly^{39}Ser/Ala^{75}Ser$, and $Ser^{140}Thr/Phe^{142}Tyr$ mutants decreased the turnover number (3.0–4.0 min^{-1})

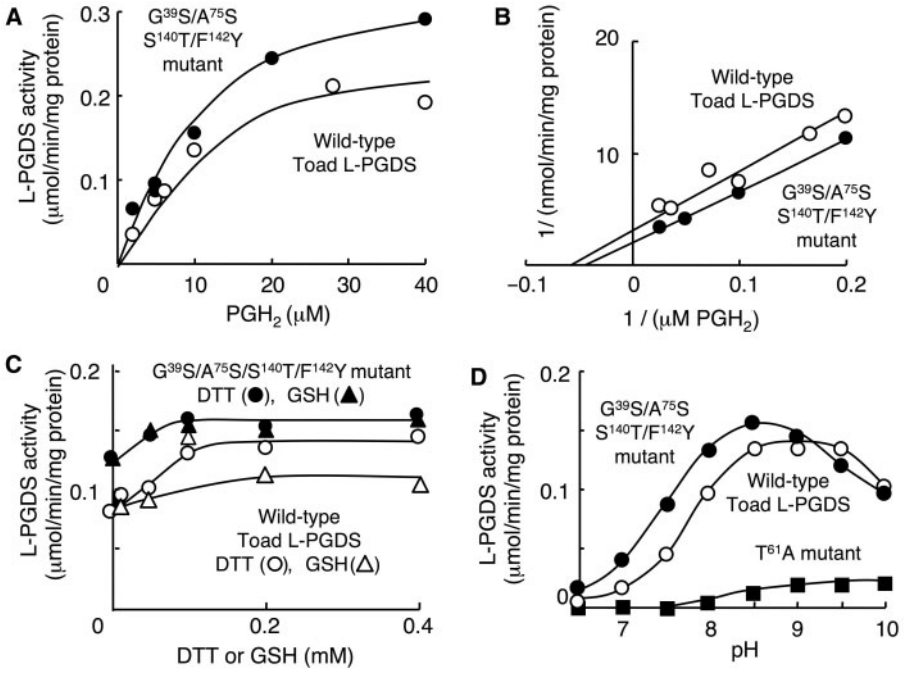

Fig. 6. **Enzymatic properties of the cane toad L-PGDS homologue**. The data showed a representative of three independent experiments. (A) Dependency of L-PGDS activity on PGH_2. The wild-type cane toad L-PGDS homologue (○) and its quadruple mutant (●) were incubated with various concentrations of PGH_2 and 1 mM DTT. (B) Lineweaver–Burk plot of the PGH_2 dependency of the wild-type cane toad L-PGDS homologue (○) and its quadruple mutant (●). (C) Dependency of L-PGDS activity on DTT (○, ●) and GSH (△, ▲). The wild-type cane toad protein (○, △) and its quadruple mutant (●, ▲) were incubated at 25°C for 30 s with 10 μM PGH_2 and various concentrations of GSH or DTT. (D) pH dependency of L-PGDS activity. The cane toad protein (○) and its quadruple (●) and $Thr^{61}Ala$ mutants (■) were incubated with 10 μM PGH_2 and 1 mM DTT. The buffers used were as follow (final conc. 0.1 M): pH 6.5, Bis-Tris/HCl; pH 7 and pH 7.5, Hepes-NaOH; pH 8, pH 8.5, pH 9, and pH 9.5, Tris/HCl; pH 10, Glycine/NaOH.

but the quadruple mutant increased 1.6 fold (9.8 min^{-1}). The K_m values were decreased in the $Gly^{39}Ser$, $Ala^{75}Ser$, $Gly^{39}Ser/Ala^{75}Ser$ (2 μM) and $Ser^{140}Thr/Phe^{142}Tyr$ (12 μM) mutants but increased in the $Phe^{142}Tyr$ (26 μM) and quadruple (24 μM) mutants.

The PGDS activity of toad L-PGDS was increased 30 and 60% by GSH and DTT, respectively, in a concentration-dependent manner up to 0.2 mM (Fig. 6C). The activation was remarkably weaker than that of mammalian L-PGDS, because the activity of mouse L-PGDS increased from 0.1 to 2 and 3 μmol/min/mg protein by adding 1mM GSH and 1 mM DTT, respectively. On the other hand, the quadruple mutant was only weakly (<20%) activated by exogenous SH compounds.

The pH dependency of the PGDS activity of toad L-PGDS was shown in Fig. 6D, where the maximum activity was observed at pH 9. When the pH dependency of the PGDS activity catalysed by the $Thr^{61}Ala$ mutant was examined, the optimum pH was shifted to above 10, being consistent with the proposed mechanism that the Thr^{61} residue is involved in the activation of the thiol group of Cys^{59}. The $Gly^{39}Ser/Ala^{75}Ser/Ser^{140}Thr/Phe^{142}Tyr$ mutant shifted the optimum pH from 9.0 to 8.5 for the wild-type enzyme. These results are in good agreement with the hypothesis that those hydrophilic and polar amino acid residues within the hydrophobic cavity of L-PGDS are involved in the activation of thiol group of Cys^{59} at neutral pH and also in the recognition of substrate PGH_2. These results also suggest that the cane toad protein is a primitive type of L-PGDS; because, in the cane toad protein, the number of polar residues surrounding the active centre containing Cys was lower than that for the mammalian protein.

DISCUSSION

In the present study, by monitoring fluorescence quenching, we demonstrated that the cane toad choroid plexus protein bound bilirubin, biliverdin, all-*trans* retinal and thyroid hormones with high affinities as indicated by the K_d values of 0.17–2.00 μM (Fig. 3, Table I). These data suggest that this protein may transport retinoids and thyroid hormones and also scavenge hydrophobic harmful compounds including bile pigments. Xlcpl1, that is the *Xenopus* homologue of the toad protein, bound all-*trans* retinal with a high affinity, as indicated by a K_d value of 3.5 nM (*5, 6*), which is a much lower value than that of the cane toad L-PGDS ($K_d = 2$ μM). Moreover, the binding affinities of the cane toad protein for these lipophilic substances were distinct from those of mammalian L-PGDSs, suggesting that the ligand selectivity as a transporter protein was divergent between amphibians and mammalians. Therefore, the

transporter activity of the cane toad protein may be different from that of the *Xenopus* protein.

Thyroid hormones are important for the regulation of brain differentiation and function (*1*). Transthyretin is recognized as the major thyroxine-binding protein in the mammalian, bird, and marsupial central nervous systems (*1*). Although transthyretin is actively produced in the mammalian choroid plexus and secreted into the cerebrospinal fluid to be a major cerebrospinal fluid protein, the amphibian choroid plexus does not produce transthyretin (*4*). Therefore, the transporter protein for thyroid hormones in amphibian central nervous system remains to be identified. As shown in this study, the binding affinities of the cane toad protein for thyroid hormones ($K_d = 0.88–1.60\,\mu M$) were almost the same as those of mammalian L-PGDSs ($K_d = 0.66–2.08\,\mu M$). Thus, in this study, we identified, for the first time, that the cane toad protein may act as a thyroid-transporter protein in the amphibian central nervous system.

The enzymatic properties of the purified cane toad protein were essentially the same as those of rat L-PGDS (*8, 12*), in terms of the optimum pH, activation of the reaction by the sulfhydryl compounds, and the K_m value for PGH_2. However, the turnover number of the cane toad protein was about 28 fold lower than that of the rat L-PGDS (Fig. 6A, Table 2). Moreover, L-PGDS homologues from non-mammalian species such as zebrafish and chicken were recently identified (*30*). Chicken L-PGDS homologue showed very weak PGDS activity, but the zebrafish one did not exhibit any activity. Thus, the cane toad protein is a primitive type of L-PGDS. In addition, the *Xenopus* homologue, Xlcpl1 protein was also associated with the L-PGDS activity just like cane toad L-PGDS (D.I. and Y.U., unpublished results). Furthermore, the cane toad L-PGDS and mammalian L-PGDSs share several common features in terms of their tissue distribution; i.e., both proteins are selectively expressed in the choroid plexus and secreted into the cerebrospinal fluid. Therefore, the cane toad protein is considered to act as the amphibian counterpart of the mammalian L-PGDS, acting as a bifunctional protein that serves as an enzyme and as a transporter protein in the central nervous system of amphibians.

The mutational analysis of cane toad L-PGDS identified Cys^{59} as an active centre for the PGDS activity (Fig. 1). Thr^{61} was also shown to be important for the catalysis probably in order to activate the thiol group of Cys^{59} (Fig. 5), as evidenced by the facts that the Thr^{61}Ala mutant showed almost negligible PGDS activity and the pH dependency of the activity by the Thr^{61}Ala mutant was similar to the ionization profile of the inactive Cys residue (Figs. 4 and 6). The Gly^{39}Ser, Ala^{75}Ser, and Gly^{39}Ser/Ala^{75}Ser mutants showed lower K_m value for PGH_2, suggesting that the introduction of hydroxyl-bearing amino acid residues at these positions within a hydrophobic pocket were effective for recognition of the substrate, PGH_2 (Fig. 5). The Phe^{142}Tyr mutant showed a slightly increased K_m value for PGH_2 ($26\,\mu M$) and a decreased turnover number ($3.0\,min^{-1}$) as compared with the wild-type enzyme, suggesting that this mutant was effective in the release of the product, PGD_2. However, the quadruple mutant Gly^{39}Ser/Ala^{75}Ser/Ser^{140}Thr/Phe^{142}Tyr, which contained the polar residues at the same positions as those of the mammalian L-PGDS, showed an increased turnover number and increased K_m value for PGH_2 ($24\,\mu M$), indicating that the substitution of hydroxyl amino acid residues further promoted the formation of the thiolate anion of Cys^{59} and the release of the product PGD_2 from cane toad L-PGDS (Fig. 5). Due to these effects, the specific activity of the quadruple mutant Gly^{39}Ser/Ala^{75}Ser/Ser^{140}Thr/Phe^{142}Tyr increased 1.6 fold as compared with that of the wild-type enzyme, and the optimum pH was shifted from 9 to 8.5 (Fig. 6). In amphibian L-PGDS, these positions of Gly^{39} and Ala^{75} may also be involved in the further activation of Cys^{59} at neutral pH. Our results suggest these polar amino acids in the mammalian L-PGDS are also crucial for the activation of the Cys^{59} and the recognition of PGH_2 (Fig. 5). Recently, the cDNA for a homologue of prostaglandin G/H synthase (cyclooxygenase) and that of other arachidonic cascade-related enzymes such as microsomal PGE synthase and leukotriene C_4 synthase were isolated from EST clones from *Xenopus* (*31*). Therefore, the cane toad L-PGDS may be coupled to the cane toad cyclooxygenase to produce PGD_2.

In conclusion, our results have demonstrated that the cane toad choroid plexus protein binds to small lipophilic substances, such as bile pigment, vitamin A, and thyroid hormones and also catalyses the isomerization of PGH_2 to produce PGD_2. Although the cane toad protein showed affinity for those lipophilic ligands and a K_m value for PGH_2 comparable to those of the mammalian L-PGDS, the turnover number of the PGDS activity was one order or more lower than that of the mammalian enzyme, indicating that the toad protein is a prototype of L-PGDS, having a highly functional ligand-binding pocket and yet retaining primitive catalytic pocket.

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