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Cloning, Expression, Crystallization, and Preliminary X-Ray Analysis of Recombinant Mouse Lipocalin-type Prostaglandin D Synthase, a Somnogen-Producing Enzyme

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Lipocalin-type prostaglandin D synthase is the key enzyme for the production of prostaglandin D_2, a potent endogenous somnogen, in the brain. We cloned, produced, and crystallized the native enzyme and selenomethionyl Cys[65]Ala mutants of the recombinant mouse protein by the hanging drop vapor-diffusion method with both malonate and citrate as precipitants. The native crystals obtained with malonate belong to orthorhombic space group $P2_12_12_1$ with lattice constants a = 46.2, b = 66.8, and c = 105.3 Å. The selenomethionyl crystals obtained with citrate belong to orthorhombic space group $C222_1$ with lattice constants a = 45.5, b = 66.8, and c = 104.5 Å. The native crystals diffracted beyond 2.1 Å resolution.

Key words: crystallization, lipocalin, multiwavelength anomalous diffraction method, prostaglandin D_2, selenomethionyl protein.

Abbreviations: PG, prostaglandin; PGDS, prostaglandin D synthase; L-PGDS, lipocalin-type prostaglandin D synthase; MAD, multiwavelength anomalous diffraction.

Prostaglandin (PG) D synthase (PGDS, EC 5.3.99.2) catalyzes the isomerization of the 9,11-endoperoxide group of PGH_2, a common precursor of various prostanoids, yielding PGD_2 with 9-hydroxy and 11-keto groups, in the presence of sulfhydryl compounds. PGD_2 is actively produced in a variety of tissues as a major prostanoid, and is involved in numerous physiological and pathological events. For example, PGD_2 is known as a potent endogenous somnogen (*1*, *2*), nociceptive modulator (*3*), anticoagulant, vasodilator, bronchoconstrictor (*4*), and allergic and inflammatory mediator (*5*). PGD_2 is further converted *in vitro* to 9α,11β-PGF_2 and J series PGs, such as PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2; although the natural occurrence of these PGs *in vivo* remains to be clarified. These more recently recognized types of PGs exert various pharmacological actions different from those of other prostanoids (*6*, *7*). 15-Deoxy-$\Delta^{12,14}$-PGJ_2 was recently identified as a ligand for a nuclear receptor, peroxisome proliferator-activated receptor γ (*8*, *9*), which is involved in adipocyte differentiation, and in the regulation of macrophage and monocyte functions (*10*).

There are two evolutionally different types of PGDS (*11*); one is the lipocalin-type PGDS (L-PGDS, 12), and the other is the hematopoietic one (*13*). L-PGDS is a member of the lipocalin superfamily, which is composed of various secretory lipid-transporter proteins (*12*); it contributes to the production of PGD_2 in the brains of various mammals and in the human heart (*14*). We recently reported that transgenic mice that overexpressed the human L-PGDS gene exhibited excessive non-rapid eye movement sleep in response to the noxious stimulus of tail clipping, coupled with a significant increase in PGD_2 production in the brain (*15*). Furthermore, we also recently showed that infusion of PGD_2 into the lateral ventricle of the brain remarkably increased the amount of non-rapid eye movement sleep in wild-type mice but not at all in D type prostanoid receptor–deficient mice (*16*). Therefore, PGD_2 produced by L-PGDS is considered to play an important role in the regulation of non-rapid eye movement sleep in a DP receptor–dependent manner. In this study, we expressed and crystallized the recombinant methionyl and selenomethionyl mouse L-PGDS to determine its crystal structure by means of the multiwavelength anomalous diffraction (MAD) method (*17*).

Firstly, we cloned the L-PGDS gene (*EMBL/GenBank/DDBJ*™ accession number D83329) from mouse brains. The cDNA for mouse L-PGDS without its N-terminal signal peptide of 24 amino acids was isolated from

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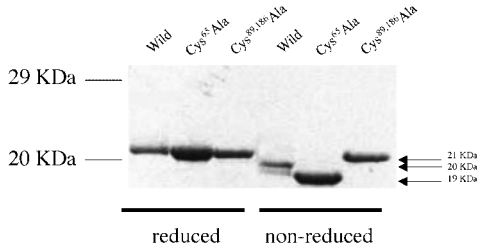


Fig. 1. **SDS-PAGE of the recombinant mouse L-PGDS**. The samples for SDS-PAGE were prepared in the presence or absence of 10 mM β-mercaptoethanol. The positions of Mr marker proteins are indicated on the left.

a mouse brain cDNA library by means of the polymerase chain reaction (PCR) with sense primer 5′-AGCTGGATCCCAGGGCCATGACACAGTGCAGCCC-3′ and antisense primer 5′-AGCTGAATTCTTACTCTTGAATGCACTTATCCGG-3′. The cDNA was cloned into expression vector pGEX-2T (Amersham Pharmacia Biotech, Uppsala, Sweden). *Escherichia coli* DH5α cells (TOYOBO, Tokyo) were transformed with this vector, and then the recombinant L-PGDS was expressed as a glutathione (GSH) transferase-fusion protein. The recombinant L-PGDS was purified by GSH-Sepharose 4B (Amersham Pharmacia Biotech) column chromatography from the soluble fraction of *E. coli* cells. After incubation with thrombin (Sigma-Aldrich, WI, USA), the L-PGDS was further purified by column chromatography on Superdex75 in 5 mM Tris/HCl (pH 8.0), followed by Mono-S chromatography in 10 mM sodium citrate (pH 4.5), as reported previously (*18*, *19*).

The structural homogeneity of the purified wild-type L-PGDS was not sufficient for further structural studies, since the major product had an incorrect S-S linkage between Cys^{65} and Cys^{89}/Cys^{189}, and not the correct one between Cys^{89} and Cys^{189}. As judged on SDS-PAGE (Fig. 1), two isoforms of the wild type enzyme were observed under non-reducing conditions at positions corresponding to M_r of 20, 000 and 19,000. These mis-S-S linked proteins made isolation difficult. To produce a sufficient amount of the correctly folded recombinant L-PGDS for further structural studies, we constructed two mutants of L-PGDS in which Cys was substituted by Ala, *i.e.*, Cys^{65}Ala and $Cys^{89,186}$Ala, to avoid major incorrectly folded proteins due to wrong S-S cross-linking with Cys^{65}. The Cys^{65}Ala mutant with the correct disulfide bond between Cys^{89} and Cys^{186} had no PGDS activity due to mutation of putative catalytic residue Cys^{65}, and gave a single band at a position corresponding to M_r of 21,000 or 19,000 on SDS-PAGE under reduced and non-reduced conditions, respectively. The $Cys^{89,186}$Ala mutant without the disulfide bond showed PGDS activity as described below, and gave a single band at the same position, M_r 21,000, on SDS-PAGE in the presence and absence of a thiol reducing agent.

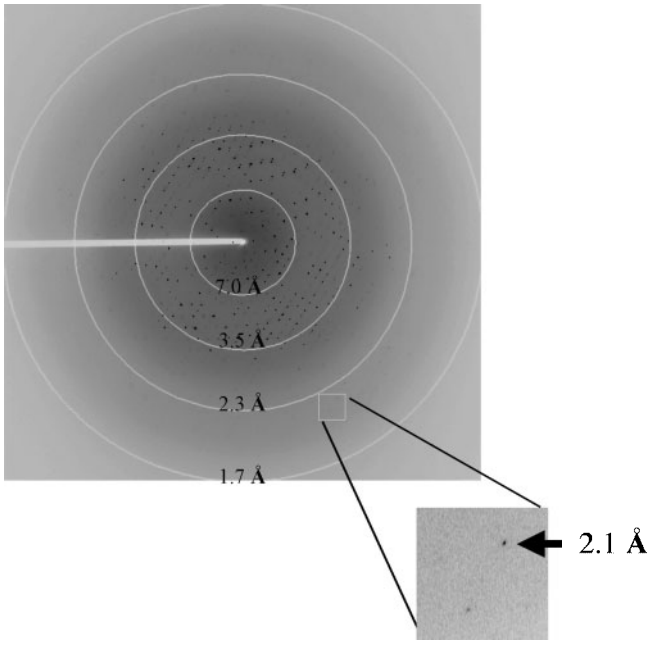
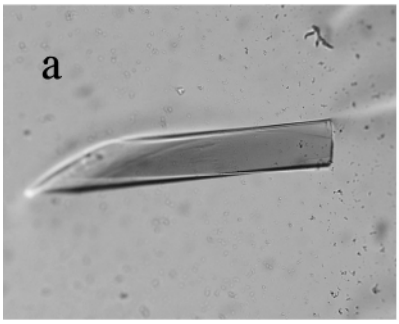


Fig. 3. **Diffraction pattern of a crystal of the native Cys^{65}Ala L-PGDS.** An oscillation photograph of a crystal of the native L-PGDS exposed using a synchrotron X-ray source. The diffraction was up to 2.1 Å resolution.

For MAD phasing, the selenomethionyl Cys^{65}Ala L-PGDS mutant was produced using *E. coli* B834 (DE3) cells (Novagene, WI, USA). We modified the LeMaster medium (*20*) to increase the growth of the *E. coli* cells, as reported previously (*21*). Purification of the selenomethionyl L-PGDS was performed in the same way as for the native one. The PGDS activity of the selenomethionyl $Cys^{89,186}$Ala mutant (5.0 μmol/min/mg of protein) was almost the same as that of the wild-type $Cys^{89,186}$Ala mutant (2.9 μmol/min/mg of protein, Ref. *18*) or that of the purified L-PGDS from rat brain (4.0 μmol/min/mg of protein), human seminal plasma (4.5 μmol/min/mg of



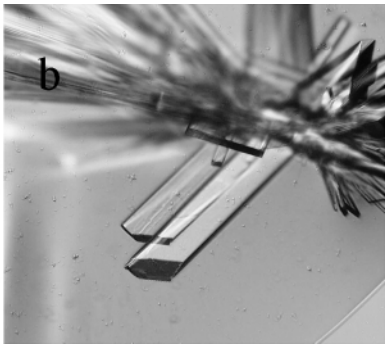


Fig. 2. **Crystals of the native Cys^{65}Ala mutant of mouse L-PGDS.** The crystals were prepared by the hanging drop vapor-diffusion method with malonate (a) or citrate (b) as the precipitant.

protein), or bovine seminal plasma (4.5 μmol/min/mg of protein, Ref. *12*). Furthermore, the elution profiles of both the $Cys^{89,186}$Ala and Cys^{65}Ala mutants from the Superdex75 and Mono-S columns were essentially indistinguishable for the native and selenomethionyl proteins. These results suggest that the catalytically essential structure of L-PGDS is not affected by the presence or absence of the correctly linked S-S bridge between Cys^{89} and Cys^{186}, and also indicate that the overall architecture of selenomethionyl L-PGDS is the same as that of the native enzyme.

For crystallization trials, both the native and selenomethionyl L-PGDS mutant proteins were dialyzed against 5 mM Tris/HCl (pH 8; crystallization stock buffer) containing 10 μM all-*trans* retinoic acid (Sigma-Aldrich), and then concentrated to 10 mg/ml by ultrafiltration with a YM-3 membrane (Millipore, Bedford, MA). Initial screening for the crystallization conditions was carried out at 22.5°C by the hanging drop vapor-diffusion method using 24-well tissue culture trays (ICN, OH, USA) and a screening solution set (Hampton Research, Laguna, CA, USA; Ref. *22*). After refinement of the crystallization conditions, a crystal of the native Cys^{65}Ala L-PGDS suitable for the diffraction study was obtained by mixing the protein solution (2 μl) with an equal volume of a reservoir solution (1 ml) of 0.1 M Tris/HCl (pH 8.0) containing 2.0 M sodium malonate and 10% (v/v) 1,4-dioxane (Fig. 2a). The native Cys^{65}Ala L-PGDS was also crystallized using the reservoir solution of 0.1 M Tris/HCl (pH 8) containing 1.25 M sodium citrate and 10% (v/v) 1,4-dioxane (Fig. 2b). The selenomethionyl Cys^{65}Ala L-PGDS was crystallized by using a reservoir solution of 0.1 M Tris/HCl (pH 9.5) containing 1.25 M sodium citrate, 10% (v/v) 1,4-dioxane and 2% Triton X-405. However, the crystallization of the native enzyme and selenomethionyl $Cys^{89,186}$Ala mutants was not successful.

Diffraction studies on the crystals were carried out at BL45XU-PX, SPring-8 (*23*), with a Rigaku R-axis IV imaging plate system at a cryogenic temperature (100 K) using the flash frozen method. The cryoprotectant was the crystallization mother solution containing 0.5 M trehalose. A crystal of the native Cys^{65}Ala mutant obtained with malonate belonged to primitive orthorhombic space group $P2_12_12_1$, with unit-cell dimensions of a = 46.2, b = 66.8, and c = 105.3 Å. Assuming two L-PGDS molecules per asymmetric unit, the V_M value and solvent content were calculated to be 2.0 $Å^3$/Da and 0.728, respectively. We collected a diffraction data set up to 2.1 Å (Fig. 3). The crystallographic parameters of a selenomethionyl crystal were face-centered orthorhombic space group $C222_1$ with unit-cell dimensions of a = 45.5, b = 66.8, and c = 104.5 Å; and the V_M value and solvent content were 2.2 $Å^3$/Da and 0.44, respectively, with one molecule per asymmetric unit, respectively. We collected a MAD data set up to 2.5 Å. Structural determination of L-PGDS is currently in progress.

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