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High-Quality Protein Crystal Growth of Mouse Lipocalin-Type Prostaglandin D Synthase in Microgravity

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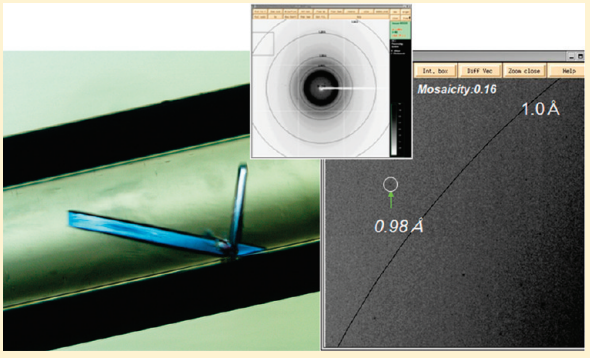
ABSTRACT: Lipocalin-type prostaglandin (PG) D synthase (L-PGDS) catalyzes the isomerization of PGH_2 to PGD_2 and is involved in the regulation of pain and of nonrapid eye movement sleep and the differentiation of male genital organs and adipocytes, etc. L-PGDS is secreted into various body fluids and binds various lipophilic compounds with high affinities, acting also as an extracellular transporter. Mouse L-PGDS with a C65A mutation was previously crystallized with citrate or malonate as a precipitant, and the X-ray crystallographic structure was determined at 2.0 Å resolution. To obtain high-quality crystals, we tried, unsuccessfully, to crystallize the C65A mutant in microgravity under the same conditions used in the previous study. After further purifying the protein and changing the precipitant to polyethylene glycol (PEG) 8000, high-quality crystals were grown in microgravity. The precipitant solution was 40% (w/v) PEG 8000, 100 mM sodium chloride, and 100 mM HEPES-NaOH (pH 7.0). Crystals grew on board the International Space Station for 11 weeks in 2007, yielding single crystals of the wild-type L-PGDS and the C65A mutant, both of which diffracted at around 1.0 Å resolution. The crystal quality was markedly improved through the use of a high-viscosity precipitant solution in microgravity, in combination with the use of a highly purified protein.



1. INTRODUCTION

Lipocalin-type prostaglandin (PG) D synthase (L-PGDS, EC 5.3.99.2) catalyzes the isomerization of PGH_2, a common precursor of various prostanoids, to produce PGD_2 and is involved in the regulation of pain[1] and of nonrapid eye movement sleep in the central nervous system,[2] the sex differentiation of male genitals,[3] the cardiovascular function in the heart and blood vessels,[4,5] the adipocyte differentiation,[6] and the modulation of renal function.[7] L-PGDS, also known as beta-trace, is the major protein in human cerebrospinal fluid; binds to various lipophilic ligands such as retinoids, thyroids, bilirubin, and biliverdin with high affinities; and is the only enzyme in the lipocalin superfamily, which is composed of various secretory lipid-transporter proteins.[8] Hematopoietic PGD synthase (H-PGDS) catalyzes the same reaction but is quite different from L-PGDS in terms of amino acid sequence, tertiary structure, cellular distribution, inhibitor sensitivity, etc.[9] Nonsteroidal anti-inflammatory drugs such as indomethacin and aspirin suppress inflammatory reactions by inhibiting PGH_2 synthase/cyclooxygenase; decrease the production of all types of prostanoids, including cytoprotective and anti-inflammatory PGs; and induce adverse effects.[10,11] An inhibitor of L-PGDS may selectively suppress the pain or drowsiness mediated by L-PGDS-catalyzed PGD_2 without various side effects.

Although many biochemical studies suggest that PGD_2 and L-PGDS play important roles in a variety of physiological and pathological functions, the crystallization of wild-type L-PGDS was heretofore unsuccessful due to incorrect S−S linkages between Cys^{65} and Cys^{89}/Cys^{189} during the expression of the recombinant protein.[12] To produce a correctly folded L-PGDS with an S−S linkage between Cys^{89} and Cys^{189}, the putative catalytic residue Cys^{65} was substituted with Ala. The C65A mutant without PGDS

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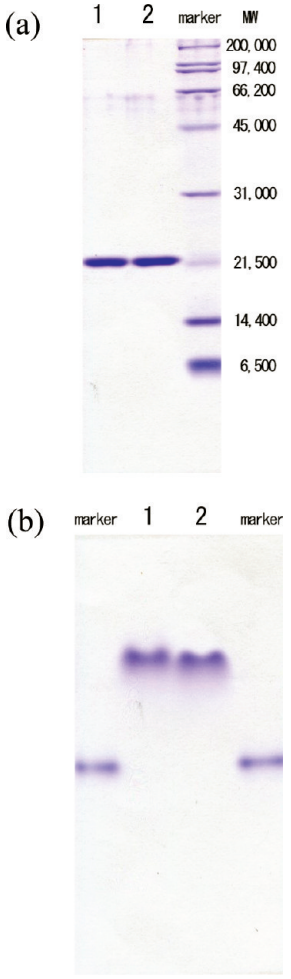

Figure 1. (a) SDS-PAGE of wild-type mouse L-PGDS (lane 1) and the C65A mutant (lane 2) under reducing conditions. The molecular weight marker proteins are indicated in the right lane. (b) Native-PAGE of wild-type mouse L-PGDS (lane 1) and the C65A mutant (lane 2) under nonreducing conditions. Bands of marker protein (pI = 4.4) are shown on both sides.

activity was used in a previous crystallization study and crystallized with malonate or citrate as a precipitant.[13] The X-ray crystallographic structure of the C65A mutant was determined at a resolution of 2.0 Å[14].

In this study, we focused on the high-quality crystallization of wild-type mouse L-PGDS. After optimization of the crystallization conditions and the sample preparation, we finally obtained high-quality crystals, which diffracted up to 0.98 Å. We also crystallized the C65A mutant of mouse L-PGDS using the same procedure and obtained crystals, which diffracted beyond 1.16 Å. Both of the crystals were grown in microgravity and were improved in quality when compared to those grown on the ground using the same crystallization conditions. These results suggested that the microgravity environment positively affected the growth of high-quality crystals of this protein.

2. EXPERIMENTAL SECTION

2.1. Protein Expression and Purification of Wild-Type Mouse L-PGDS and Its C65A Mutant. The cDNAs of wild-type mouse L-PGDS without its N-terminal signal peptide of 24 amino acids and its C65A mutant were expressed in *Escherichia coli* BL21(DE3) (Invitrogen, CA, USA) as a glutathione (GSH) transferase-fusion protein. The fusion protein was purified by GSH-Sepharose 4B (GE Healthcare, Buckinghamshire, U.K.) column chromatography from a soluble fraction of *E. coli* cells. After incubation with thrombin (Sigma-Aldrich, WI, USA), L-PGDS protein was obtained and further purified by column chromatography on Superdex 75 (GE Healthcare) in 5 mM Tris-HCl pH 8.0, followed by Mono-S HR5/5 (GE Healthcare) chromatography with a linear gradient from 0 to 500 mM sodium chloride in 20 mM Tris-HCl, pH 7.5, at 293 K. Structural homogeneities of final purified preparations of the wild-type and C65A mutant of mouse L-PGDS were evaluated by SDS-PAGE (under reducing conditions) and by native PAGE (PAGE without a denaturing reagent, such as SDS, and under nonreducing conditions) as shown in Figure 1. The wild-type L-PGDS showed the same PGDS activity as that of the C89,189A mutant.[14] They were concentrated to 40 mg mL^{-1} in 100 mM HEPES-NaOH, pH 7.0, and 100 mM sodium chloride using a Centricon YM-10 membrane (10,000 nominal molecular weight limit; Millipore, MA, USA) and stored at 277 K. The protein concentration was determined spectrophotometrically at 280 nm.

2.2. Crystallization. Wild-type mouse L-PGDS and its C65A mutant were crystallized inside the Russian TBU incubator on board the Russian Service Module on the International Space Station (ISS) for 11 weeks from August to October, 2007, as a microgravity experiment supported by the Japan Aerospace Exploration Agency (JAXA).

The temperature was kept at 293 ± 1 K in the TBU for ten weeks, but it rose to 298 ± 1 K for 90 min in the Soyuz Spacecraft on the return flight. The highest temperature was 299 K. Although the temperature became high enough to damage some protein samples, the protein in this report was sufficiently stable even at 299 K.

A gel-tube method,[15] modified from the original counterdiffusion method,[16−18] was used for crystallization, as described.[19] Briefly, the protein solution (8 μL, 40 mg mL^{-1} protein, 100 mM sodium chloride, and 100 mM HEPES-NaOH, pH 7.0) was added to a glass capillary (47 mm length, 0.5 mm diameter) to a length of 40 mm. The end of the capillary was attached to a plastic tube (gel-tube, 3 mm length), which contained 1% polymerized agarose gel presoaked in 20% (w/v) PEG 8000, 100 mM sodium chloride, and 100 mM HEPES-NaOH, pH 7.0. The top of the capillary was sealed with clay and epoxy adhesive. The agarose-end of the capillary was placed in a tube, which contained the precipitant solution of 40% PEG 8000, 100 mM sodium chloride, and 100 mM HEPES-NaOH, pH 7.0. These conditions were fixed to start crystallization after the samples had been placed in the microgravity environment by adjusting the precipitant concentration, the gel-tube length, and the composition of the presoaked solution of the gel-tube. The same crystallization conditions were used in the control experiment on the ground.

2.3. Data Collection and Analysis. Diffraction data were collected at 100 K with an X-ray wavelength of 0.9 Å on the BL41XU beamline at SPring-8, Harima, Japan, using an ADSC315 detector system. No additional cryoprotectant was needed, since the concentration of PEG 8000 in the mother liquor was high enough to act as a cryoprotectant. The method of harvesting crystals from the capillaries has been reported previously.[20] Briefly, to harvest a crystal, the section of the capillary tube containing the desired crystal was cut out with a capillary cutting stone. Then a gentle flow of harvest solution from a micropipet was used to wash the crystal out of the capillary segment into harvest solution in a concave slide glass with a single depression well. One crystal was extracted with a nylon loop and plunged directly into a nitrogen-gas stream at 100 K. A total of 180 frames were collected using a crystal-to-detector distance of 130 mm with 1° oscillation. The diffraction images were integrated and scaled using the programs *DENZO* and *SCALEPACK* from the *HKL*-2000 suite.[21]

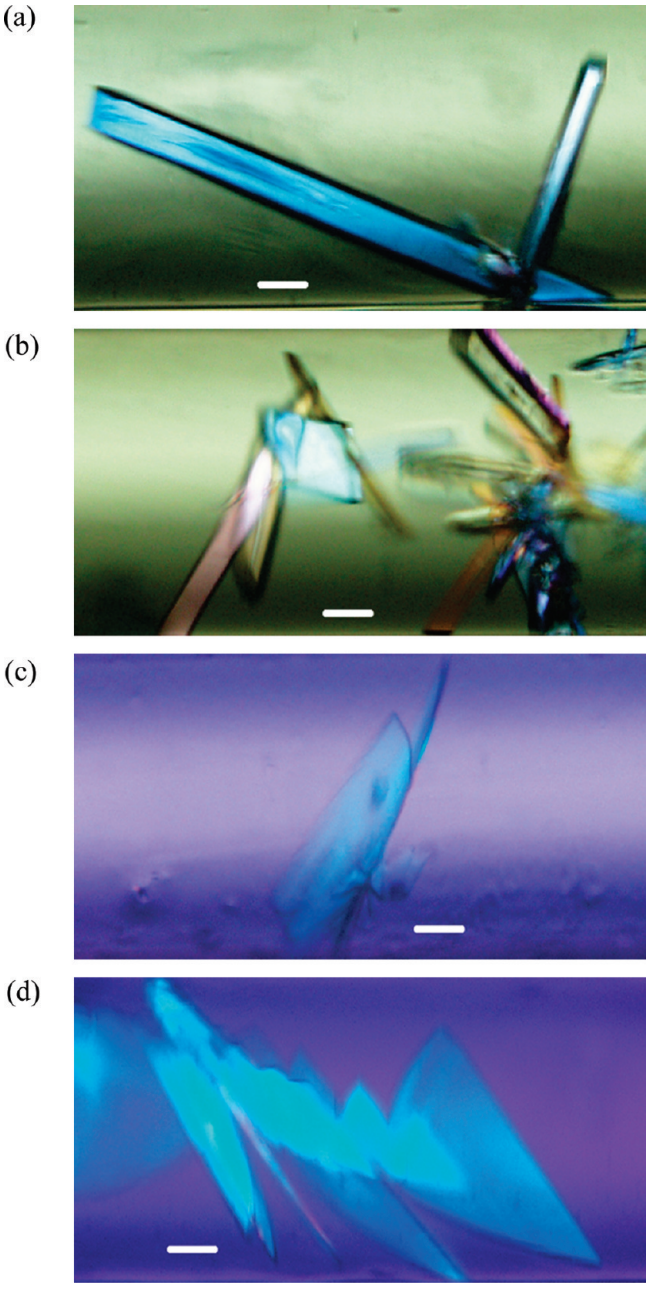

Figure 2. (a) Crystals of wild-type L-PGDS grown in microgravity. (b) Crystals of the C65A mutant grown in microgravity. (c) Crystals of wild-type L-PGDS grown on the ground. (d) Crystals of the C65A mutant grown on the ground. The scale bars correspond to 100 μm.

3. RESULTS AND DISCUSSION

Native-PAGE of the wild-type L-PGDS, purified by the previously reported method,[13] showed a minor band with an incorrect S−S linkage. Therefore, we changed the elution condition of the recombinant protein through Mono-S chromatography and obtained highly purified wild-type L-PGDS and the C65A mutant. Figure 1 shows SDS-PAGE and native-PAGE of the final purified homogeneous samples used for crystallization.

Crystals started growing in the capillaries 2 to 3 weeks after sample loading on the ground. The crystals we obtained are shown in Figure 2. In microgravity, we obtained rodlike crystals of the wild-type L-PGDS ($1.0 \times 0.1 \times 0.1$ mm^3, Figure 2a) and the C65A mutant ($0.5 \times 0.1 \times 0.1$ mm^3, Figure 2b) along the capillaries. While, on the ground, thin, platelike crystals of the wild-type L-PGDS ($0.4 \times 0.1 \times 0.05$ mm^3, Figure 2c) and the C65A mutant ($0.5 \times 0.3 \times 0.05$ mm^3, Figure 2d) were grown in the capillaries. Crystals of wild-type mouse L-PGDS were obtained for the first time.

For X-ray diffraction data collection, we selected crystals grown on the opposite side of the gel-tube in the capillaries, because crystals of better quality tend to grow on this side, where the precipitant concentration changes slowly.[22] All crystals chosen for X-ray diffraction data collection were judged to be of sufficient size and of good quality by visual inspection. To avoid fluctuations in the data caused by differences in the crystal size, we chose crystals similar in size. However, crystals of the C65A mutant grown on the ground were too thin and fragile to carry out the diffraction experiment even at the synchrotron site.

The X-ray diffraction data are summarized in Table 1. Crystals of wild-type L-PGDS obtained with PEG 8000 as a precipitant in microgravity belonged to the primitive orthorhombic space group $P2_12_12$, with unit-cell dimensions of a = 62.3, b = 67.0, and c = 35.7 Å. We collected a diffraction data set up to 1.06 Å (the maximum resolution of approximately 0.98 Å). Crystals of the wild-type enzyme grown on the ground belonged to the same space group as those grown in microgravity, with unit-cell dimensions of a = 62.2, b = 67.0, and c = 35.6 Å. We collected a diffraction data set up to 1.30 Å (the maximum resolution of approximately 1.29 Å). The mosaicity of the crystals grown in microgravity was reduced to 0.16 when compared to the mosaicity of the crystals grown on the ground (0.35). Crystals of the C65A mutant obtained with PEG 8000 as a precipitant in microgravity belonged to the same space group as those of the wild-type enzyme, with unit-cell dimensions of a = 62.2, b = 66.2, and c = 35.5 Å. We collected a diffraction data set up to 1.16 Å (mosaicity: 0.20).

There are four variables in this report: the crystallization method (counterdiffusion vs vapor-diffusion); the protein sample homogeneiety (high vs comparatively low); the precipitant (PEG 8000 vs sodium malonate); and the crystallization environment (space vs ground). All of these may contribute to the improvement of crystal quality. In this study, we elucidated that the variables positively work to obtain high quality crystals.

In the previously reported method, 2 M sodium malonate was used as a precipitant with a less homogeneous protein sample of mouse L-PGDS C65A mutant using the vapor-diffusion method on the ground. However, the crystallization probability was low, and even when we obtained a crystal, it diffracted X-rays up to only 2.0 Å.[14] When we applied the same crystallization conditions to the counterdiffusion method on the ground, it yielded similar results of low crystallization probability, although the counterdiffusion method has been reported to improve crystal quality in some cases.[23] We also used these crystallization conditions in JAXA's high-quality protein crystallization project (JAXA-GCF) in microgravity three times in 2003, 2004, and 2005, but no crystal was obtained.

We have previously demonstrated in the crystallization of H-PGDS[19,24] that a low diffusion coefficient (D) and a high kinetic constant for crystal growth (β) enhance the microgravity effect on crystallization. Thus, we further purified the L-PGDS sample used for crystallization to increase the β index[25] by reducing impurities which interfere with the growth of crystals. The purified sample was used for the crystallization experiment with sodium malonate as a precipitant both on the ground and in

Table 1. Summary of X-ray Diffraction Experiments on L-PGDS Crystals[a]

L-PGDS	space		ground	
	wild type	C65A	wild type	C65A[b]
space group	$P2_12_12$	$P2_12_12$	$P2_12_12$	$C222_1$
cell dimensions (a, b, c) (Å)	62.3, 67.0, 35.7	62.2, 66.2, 35.5	62.2, 67.0, 35.6	46.3, 67.1, 104.6
resolution (Å)	50.0−1.06 (1.08−1.06)	50.0−1.16 (1.18−1.16)	50.0−1.30 (1.32−1.30)	10.0−2.0
mosaicity	0.16	0.20	0.35	
completeness (%)	98.8 (97.6)	97.6 (88.2)	99.5 (99.2)	77.2
$I/\sigma(I)$	10.2 (1.55)	13.8 (1.83)	10.2 (2.0)	
R_{merge}	0.064 (0.554)	0.065 (0.426)	0.078 (0.563)	0.052
precipitant	PEG 8000	PEG 8000	PEG 8000	malonate
synchrotron	SPring-8 BL41XU	SPring-8 BL41XU	SPring-8 BL41XU	SPring-8 BL45XU
crystallization method	counterdiffusion	counterdiffusion	counterdiffusion	vapor-diffusion

[a] Values in parentheses are for the highest resolution shell. The data were processed to the resolution range at which $I/\sigma(I) > 2$ and $R_{merge} < 50\%$. [b] Reference 14.

microgravity in the same flight as the following experiment, yet the crystals grown in both environments diffracted X-rays up to 2.3 Å.

We then increased the viscosity of the crystallization solution by the usage of PEG 8000 as a precipitant to decrease the D index.[26] Using PEG 8000 instead of sodium malonate with an optimum salt concentration[27] remarkably improved the quality of the crystal grown on the ground (maximum resolution 1.30 Å, mosaicity 0.35), probably because the space group of the crystal grown in the PEG 8000 solution ($P2_12_12$) was different from the one grown in sodium malonate solution ($C222_1$). The crystallization probability also increased to nearly 100% in the PEG solution, which ensured successful crystallization in microgravity.

The use of PEG 8000 as a precipitant also significantly improved the crystal quality in microgravity. So far to date, we have obtained the best wild-type mouse L-PGDS crystals for this protein indexed by the maximum resolution (beyond 1.0 Å) and the minimum mosaicity (0.16). Structural refinement of this diffraction data is currently in progress.

Our results suggested that crystal quality was improved, mainly due to the use of PEG 8000 solution, especially in microgravity, in combination with the usage of the purified protein in this study. The quality improvement is thought to be the result of the filtering effects of the concentration depletion zones of protein and impurity around the crystals,[28−30] which were strongly enhanced by a high-viscosity PEG solution in a convection-free environment such as microgravity.[26,31]

There are some reports which introduce the opinion that the addition of agarose gel to a crystallization solution can mimic the effects of microgravity.[32] Therefore, we tried a crystallization experiment in this gel. However, the optimization of the crystallization condition was not successful and we could not obtain any crystals.

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