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Crystallization and preliminary diffraction studies of prostaglandin E_2-specific monoclonal antibody Fab fragment in the ligand complex

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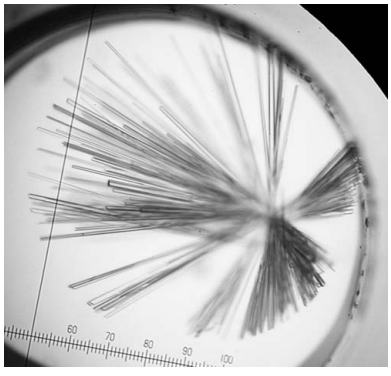

Prostaglandin E_2 is a major lipid mediator that regulates diverse biological processes. To elucidate how prostaglandin E_2 is recognized specifically by its antibody, the Fab fragment of a monoclonal anti-prostaglandin E_2 antibody was prepared and its complex with prostaglandin E_2 was crystallized. The stable Fab–prostaglandin E_2 complex was prepared by gel-filtration chromatography. Crystals were obtained by the microbatch method at 277 K using polyethylene glycol 4000 as a precipitant. A diffraction data set was collected to 2.2 Å resolution. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 70.3, b = 81.8, c = 82.2 Å. The asymmetric unit was suggested to contain one molecule of the Fab–prostaglandin E_2 complex, with a corresponding crystal volume per protein weight of 2.75 Å^3 Da^{-1}.

1. Introduction

Prostanoids such as prostaglandins (PGs) and thromboxane (TX) have numerous and diverse biological activities under various physiological and pathological conditions. In the metabolic pathway of prostanoids, dioxygenases such as cyclooxygenase catalyze the conversion of arachidonic acid to prostaglandin H_2 (PGH_2), which is a unique and unstable intermediate from which all the prostanoids (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2) are produced *via* specific synthases. PGE_2 is one of the most widely investigated prostanoids. Exhaustive studies have revealed biologically pivotal roles of PGE_2 in inflammation, pain, gastric mucosal integrity and the immune system (Willis & Cornelsen, 1973; Robert *et al.*, 1976; Arvind *et al.*, 1995). The distinct biological actions of PGE_2 are exerted by its binding to four distinct PGE_2 receptors, EP1–EP4 (Sugimoto & Narumiya, 2007).

PGE_2 (Fig. 1) is an unsaturated oxygenated product of arachidonic acid with a cyclopentane ring, α- and ω-chains attached to the ring and a carboxyl group. PGE_2 has two double bonds (5-*cis* and 13-*trans*), 11-α and 15*S* hydroxyl groups and a 9-carbonyl group.

The specific and sensitive quantification of PGE_2 in biological samples is crucial for biological and biochemical studies. Therefore, an enzyme-linked immunoassay system for PGE_2 has been developed by Shono *et al.* (1988). The anti-PGE_2 monoclonal antibody generated for this purpose was used in this study. The antibody has specific and high affinity for PGE_2 and 9-deoxy-9-methylene $PGF_{2\alpha}$, which has been utilized as a stable PGE_2 mimic to raise anti-PGE_2 antibodies (Fitzpatrick & Bundy, 1978). The antibody has lower affinity towards other analogous prostanoids such as PGD_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TXB_2 and metabolites of PGE_2 (Shono *et al.*, 1988). The mechanism by which the antibody distinguishes PGE_2 from other

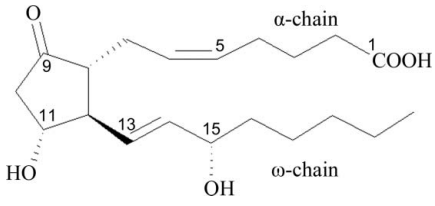

Figure 1
Chemical structure of PGE_2.

PGs and the interactions between PGs and their receptors are not well understood. Therefore, it is indispensable to determine the atomic structure of the complex of the antibody with PGE_2. Here, we report the crystallization of and preliminary crystallographic studies on the complex of PGE_2 with the Fab fragment of the antibody. The findings from this work will support ligand design for drug discovery.

2. Materials and methods

2.1. Preparation of anti-PGE_2 antibody Fab fragment

Monoclonal anti-PGE_2 antibody was produced using a mouse hybridoma cell line established by Shono *et al.* (1988) in GIT serum-free medium (Nihon Pharmaceutical). The antibody that accumulated in the culture medium was collected and purified using Protein A Sepharose CL4B column chromatography (GE Healthcare). The collected fractions were concentrated to 1 mg ml^{-1} protein in PBS buffer (10 m*M* sodium phosphate pH 7.4, 140 m*M* NaCl) using ultracentrifugation. The light-chain subtype of the mouse antibody was determined to be $IgG1_\kappa$ using the Isotyping Monoclonal Antibodies Kit (GE Healthcare). The Fab fragment was prepared by papain digestion of the antibody (5 mg ml^{-1}) with 1:1000(*w*:*w*) papain (Calbiochem) for 3 h at 310 K in 75 m*M* sodium phosphate buffer pH 7.0 containing 20 m*M* cysteine–HCl, 75 m*M* NaCl, 2 m*M* EDTA and 5 m*M* NaN_3. The reaction was stopped by the addition of *N*-ethylmaleimide to a concentration of 20 m*M*. After incubation for 30 min at room temperature in the dark, the digested product was adsorbed onto HiTrap Protein G HP (GE Healthcare) equilibrated with 20 m*M* sodium phosphate buffer pH 7.0. After washing with the same buffer, the Fab fragment was eluted with 0.1 *M* glycine–HCl buffer pH 2.75. The collected Fab fraction was concentrated in Fab buffer (10 m*M* sodium phosphate pH 7.0, 140 m*M* NaCl) to a protein concentration of 10 mg ml^{-1} with an ultrafiltration apparatus (Vivaspin 20, Sartorius).

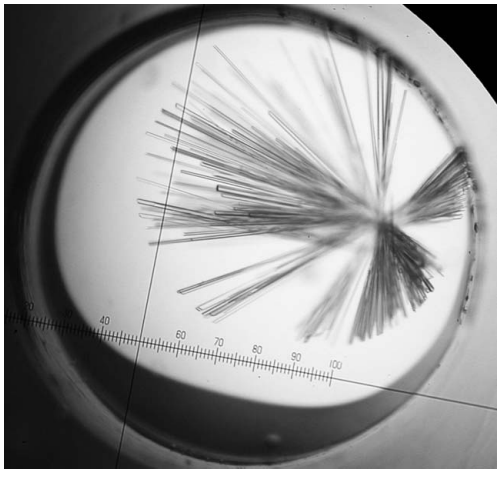

Figure 2
Needle-shaped crystals of the Fab–PGE_2 complex. Nine graduations on the scale in this photograph correspond to 0.1 mm.

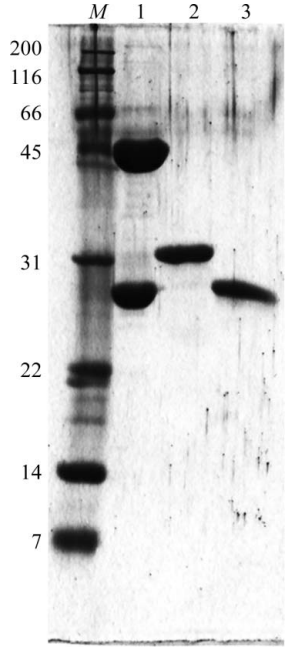

Figure 3
SDS–PAGE (12.5%) of the purified monoclonal anti-PGE_2 antibody (lane 1), Fc fragment (lane 2) and Fab fragment (lane 3). Lane *M*, molecular-weight markers (labelled in kDa). The samples were prepared prior to electrophoresis by heating in the presence of 1 m*M* 2-mercaptoethanol.

2.2. Crystallization and data collection of the Fab–PGE_2 complex

20 µl 1 mg ml^{-1} PGE_2 in ethanol (Cayman) was dispensed and dried in 1.5 ml sample tubes under a nitrogen-gas stream. After mixing the Fab fragment with a twofold molar excess of PGE_2, the Fab–PGE_2 complex was purified by Superose12 HR 10/30 gel-filtration column chromatography (GE Healthcare) equilibrated with Fab buffer. The eluted proteins were monitored by UV absorbance at 280 nm. The following standard proteins were used as molecular-weight markers (their molecular weights and elution volumes are

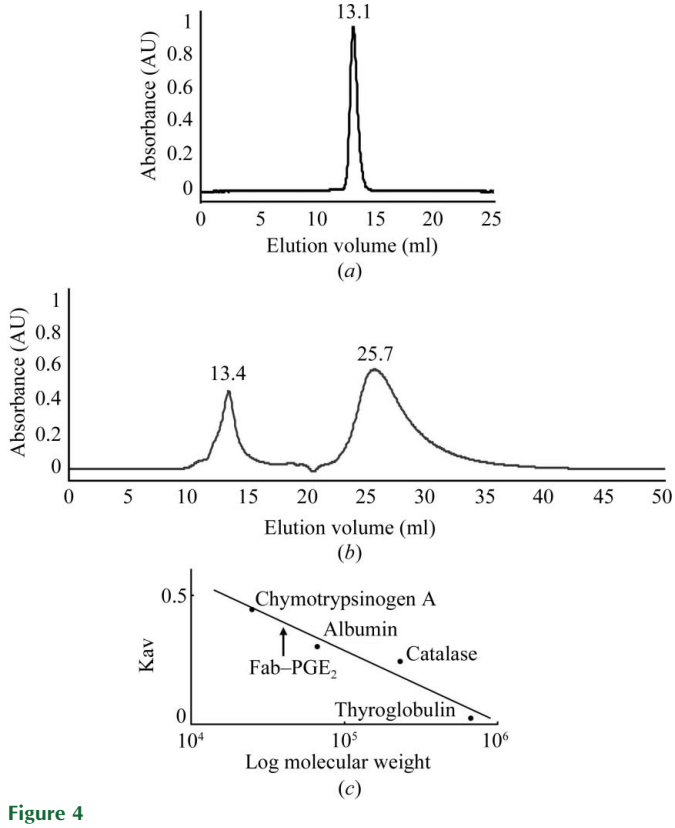

Figure 4
Gel-filtration chromatograms. (*a*) Fab–PGE_2 complex, (*b*) apo Fab. (*c*) Graph of elution time *versus* molecular weight for molecular-weight markers and the Fab–PGE_2 complex.

given in parentheses): chymotrypsinogen A (25 000 Da, 14.8 ml), albumin (67 000 Da, 12.5 ml), catalase (232 000 Da, 11.6 ml) and thyroglobulin (669 000 Da, 8.14 ml) (GE Healthcare). The eluted Fab–PGE_2 complex fraction was concentrated using a centrifugal concentrator (Vivaspin 20, Sartorius). Initial crystallization was carried out by the oil-microbatch method (Chayen *et al.*, 1990) using a screening kit designed for high-throughput protein crystallization (Sugahara & Miyano, 2002). TR plates (Takeda Rika Kogyo) were used in which 0.5 µl of each screening solution was mixed with 0.5 µl protein solution (38 mg ml^{-1} protein in Fab buffer) and the mixture was covered with 15 µl paraffin oil; this was followed by incubation at 277 K. During optimization of the initial crystallization conditions, needle-shaped crystals appeared after a few days from a screening solution composed of 12.5%(*w*/*v*) PEG 4000, 50 m*M* $MgCl_2$ in 0.1 *M* Tris–HCl buffer pH 8.2. Thick needle-shaped crystals (Fig. 2) were transferred using a nylon loop (Hampton Research) from the crystallization drop into a cryoprotectant comprised of Paratone N and 10%(*v*/*v*) glycerol (Kwong & Liu, 1999). After removal of the aqueous solution from around the crystal, it was flash-frozen using a liquid-nitrogen gas stream at 100 K. X-ray diffraction data were collected on an ADSC Q210 CCD detector using synchrotron radiation on the RIKEN beamline BL44B2 at SPring-8. The native diffraction data were collected as 180 oscillation images with an exposure time of 20 s and an oscillation range of 1° using a camera distance of 200 mm. Images were processed using the programs *MOSFLM* (Leslie, 1992) and *SCALA* and the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Monoclonal anti-PGE_2 antibody was produced on a large scale by culturing an established hybridoma cell line (Shono *et al.*, 1988) in serum-free medium. Approximately 7–8 mg IgG was obtained from 400 ml culture medium. 2 mg of the Fab fragment was purified from 20 mg IgG protein (Fig. 3). As shown in Fig. 4(*a*), the Fab complexed with PGE_2 showed a sharp elution peak at 43 kDa (Fig. 4*c*) in gel-filtration column chromatography. In sharp contrast, the apo Fab fragment eluted in two broad peaks from the same column (Fig. 4*b*), with the ratio of the peak heights varying from experiment to experiment. These eluates migrated at the same distance as the Fab–PGE_2 complex on SDS–PAGE (data not shown). Presumably, the purified Fab fragment exists in at least two forms hydrodynamically and the addition of PGE_2 converts these multiple forms to a single form which gives a single sharp peak as in Fig. 4(*a*). We attempted to crystallize both apo Fab and the Fab–PGE_2 complex. Crystals of the Fab–PGE_2 complex were obtained with dimensions of 0.3 × 0.01 × 0.01 mm after two weeks (Fig. 2), but the apo Fab could not be crystallized despite exhaustive trials. The unsuccessful crystallization of apo Fab may be a consequence of its multiple forms as mentioned above.

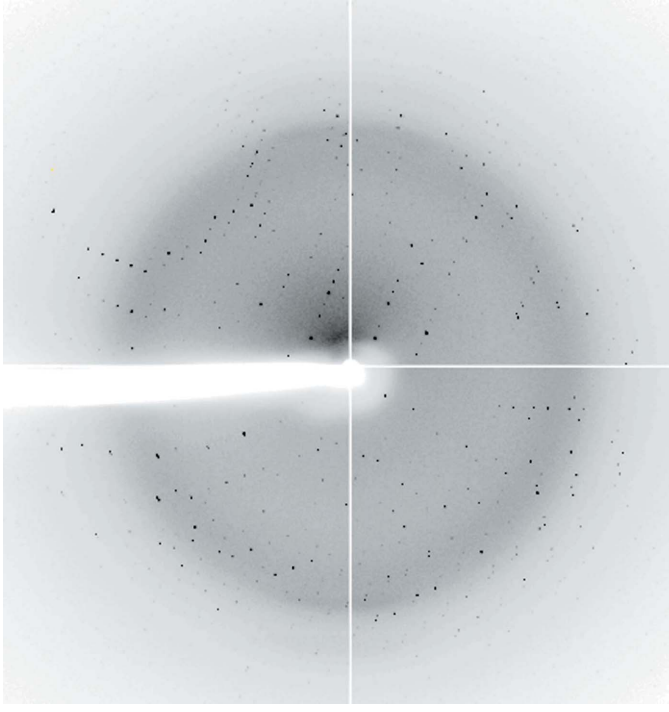

Figure 5
Diffraction image of the Fab–PGE_2 complex crystal.

Table 1
Crystal parameters and data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Synchrotron (BL44B2 at SPring-8)
Wavelength (Å)	1.0000
Detector	CCD camera (ADSC Q210)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	
a	70.3
b	81.8
c	82.2
Mosaicity (°)	0.52
Wilson *B* factor ($Å^2$)	15.8
Resolution range (Å)	58–2.2 (2.32–2.2)
Total observations	178036 (25785)
Unique reflections	24732 (3523)
Multiplicity	7.2 (7.3)
Completeness (%)	100.0 (100.0)
Mean $I/\sigma(I)$	12.7 (5.0)
R_{merge}† (%)	11.4 (42.1)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity of the *i*th measurement and the mean intensity of the reflection with indices *hkl*, respectively.

The collected diffraction images (Fig. 5) were reduced to 24 732 reflections (the total number of reflections was 178 036) in the resolution range 58–2.2 Å with an R_{merge} of 11.4% (Table 1). Crystals of the Fab–PGE_2 complex belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 70.3, b = 81.8, c = 82.2 Å. The Matthews coefficient of 2.75 $Å^3$ Da^{-1} suggested the presence of one molecule of the Fab–PGE_2 complex (43 kDa) per asymmetric unit, corresponding to a solvent content of 55.3% (Matthews, 1968). As described above, we could obtain crystals from mixture of Fab and PGE_2 which gave a single sharp peak when subjected to gel filtration. The preparation was referred to as 'PGE_2–Fab complex'. Our crystallographic analyses indicated the presence of PGE_2 in the crystal and a manuscript describing observations on the PGE_2–Fab complex structure is now in preparation.

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