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Crystallization of the functional domain of human thrombopoietin using an antigen-binding fragment derived from neutralizing monoclonal antibody

Ryota Kuroki,[a,b]* Masako Hirose,[a] Yoichi Kato,[a] Michael D. Feese,[a] Taro Tamada,[a,b] Hideki Shigematsu,[b] Hiroshi Watarai,[b] Yoshitake Maeda,[a,b] Tomoyuki Tahara,[b] Takashi Kato[b] and Hiroshi Miyazaki[c]

[a]Central Laboratories for Key Technology, Kirin Brewery Co. Ltd, 1-13-5 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan, [b]Pharmaceutical Research Laboratories, Kirin Brewery Co. Ltd, 3 Miyahara-cho, Takasaki 370-1295, Japan, and [c]Pharmaceutical Development Laboratories, Kirin Brewery Co. Ltd, 3 Miyahara-cho, Takasaki 370-1295, Japan

Correspondence e-mail: r-kuroki@kirin.co.jp

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Thrombopoietin (TPO) is a cytokine which primarily stimulates megakaryocytopoiesis and thrombopoiesis. The functional domain of TPO (TPO_{163}) consisting of the N-terminal 163 amino acids was prepared and crystallized. Since the crystallization of TPO_{163} was unsuccessful using the standard screening methods, a Fab fragment derived from a neutralizing monoclonal antibody was used for crystallization. It was found that the TPO_{163}–Fab complex crystallized reproducibly in 0.1 *M* potassium phosphate buffer pH 6.0 containing 20–25% polyethylene glycol 4000. Thin crystals (0.2 × 0.2 × 0.02 mm) grew in two space groups: $P2_1$, with unit-cell parameters a = 133.20, b = 46.71, c = 191.47 Å, β = 90.24°, and $C2$, with unit-cell parameters a = 131.71, b = 46.48, c = 184.63 Å, β = 90.42°. The results of a molecular-replacement analysis indicate that the Fab molecules interact with each other and provide a suitable interface for crystallization.

1. Introduction

There are several aspects that affect the crystallization of a protein (McPherson, 1999). If the crystallization of a protein is unsuccessful, the homogeneity of the sample is primarily doubted. It is, however, often still difficult to crystallize a protein because of the characteristics of the protein itself. It is empirically known that conformational instability or the existence of a flexible region such as attached sugar chains can prohibit crystallization. Therefore, if we can add a suitable interface to a protein, the chance of crystallization may increase. Thus, we considered using an immunoglobulin fragment to aid crystallization of a protein. Immunoglobulins are known to create a specific and stable complex with the epitope of the target protein and have generally been used as a tool for Enzyme Linked Immunosorbant Assay (ELISA), immunoblotting or Western blotting analysis to detect tiny amounts of protein owing to their high affinity. The tertiary structures of an antigen-binding fragment of immunoglobulin (Fab) complexed with an epitope of the antigen have been investigated in detail (Silverton *et al.*, 1984; Laver, 1990; Ostermeier *et al.*, 1995). Our approach is to utilize a Fab to add a suitable interface for the crystallization of an unstable but medically very important protein.

TPO is a haematopoietic cytokine that primarily regulates megakaryocytopoiesis and platelet production. TPO has been cloned independently by several groups (de Sauvage *et al.*, 1994; Lok *et al.*, 1994; Kaushansky *et al.*, 1994; Wendling *et al.*, 1994; Bartley *et al.*, 1994; Kuter *et al.*, 1994; Sohma *et al.*, 1994; Ogami *et al.*, 1995; Kato, Ogami *et al.*, 1995) and has been developed as an important drug candidate for platelet production. Although cloned human TPO consists of 332 amino acids, the N-terminal half (MW = 18 kDa) was found to be sufficient for biological activity (de Sauvage *et al.*, 1994; Kato, Ozawa *et al.*, 1995; Wada *et al.*, 1995; Muto *et al.*, 2000). The amino-acid sequence of the N-terminal domain has a weak similarity (about 23% identity) to that of erythropoietin (de Sauvage *et al.*, 1994) and the structure has been predicted to have a four-helix bundle fold similar to that of erythropoietin (Syed *et al.*, 1998; Cheetham *et al.*, 1998). In this paper, we show a successful example of crystallization of a new protein, human thrombopoietin, with the assistance of a Fab molecule derived from a neutralizing antibody. The crystallization of the TPO_{163}–Fab complex seems to be mainly driven by the interaction between Fab molecules.

2. Methods and results

2.1. Purification of the TPO_{163}–Fab complex

The truncated TPO (TPO_{163}) consisting of the N-terminal 163 amino acids was expressed in *Escherichia coli* as inclusion bodies and successfully refolded using redox buffer containing 10 m*M* CAPS buffer, 3 *M* urea, 30% glycerol, 0.7 m*M* cysteine and 3.5 m*M* cystamine. The details of the expression and refolding of TPO_{163} will be reported elsewhere.

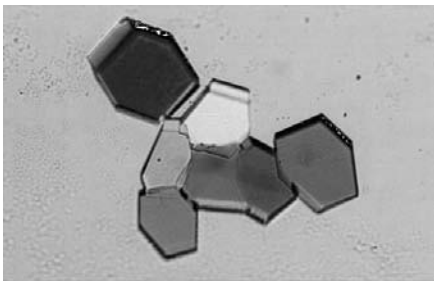

Figure 1
Crystals of TPO_{163}–Fab complex. Approximate dimensions of the crystal are 0.2 × 0.2 × 0.02 mm.

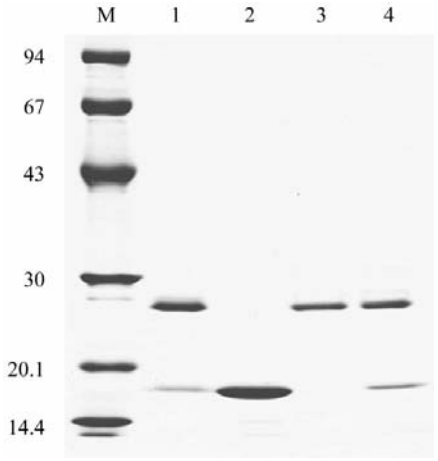

Figure 2
SDS–PAGE was performed under reducing conditions using 10/20 multi-gel (Daiichi Pure Chemicals, Japan) and stained with Coomassie Brilliant Blue. Lane 1, purified TPO_{163}–Fab complex; lane 2, truncated human thrombopoietin; lane 3, purified Fab fragment derived from TN1; lane 4, TCA-precipitated sample recovered from crystals. Lane M, molecular-weight markers (kDa).

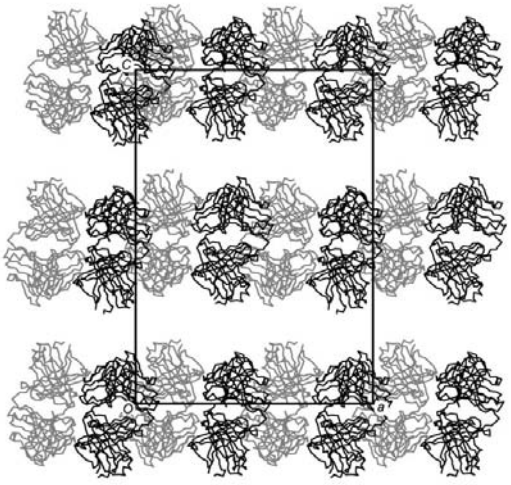

Figure 3
Location of the Fab molecules in the $P2_1$ crystal lattice of the TPO_{163}–Fab complex. Light-coloured Fab molecules and dark-coloured Fab molecules are antiparallel. The box indicates the unit cell. This figure was drawn using the program *MOLSCRIPT* (Kraulis, 1991).

The refolded TPO_{163} was purified by two steps of cation-exchange chromatography. A 26 × 100 mm CM-Sepharose (Pharmacia) column was used for the first step. The protein was eluted with 20 m*M* sodium phosphate buffer pH 7.2 containing 0.5 *M* NaCl. The second step was performed by HPLC with a 4.6 × 75 mm column of Tosoh SP-5PW (Tosoh, Japan). The protein was eluted with a linear gradient from 20 m*M* Tris–phosphate buffer pH 7.2 to the same buffer containing 0.5 *M* NaCl in 30 min at a flow rate of 1 ml min^{-1}.

The mouse IgG1 (TN1), which neutralizes human TPO activity, was obtained as reported previously (Tahara *et al.*, 1996). The Fab fragment was prepared by papain digestion at the hinge region of the IgG to remove the Fc region. IgG molecules were incubated for 1 h under reducing conditions (1 m*M* cysteine) and incubation continued with activated papain for 4 h. Because of the amino-acid sequence at the hinge region of the heavy chain of mouse IgG1, the digested Fab fragment has a free cysteine residue in the C-terminal part of Fd fragment. This free cysteine was blocked with iodoacetamide. The Fab was purified by cation-exchange HPLC using a Tosoh SP-5PW column (4.5 × 75 mm, Tosoh, Japan). The protein was eluted with a linear gradient from 20 m*M* sodium acetate buffer pH 5.5 to the same buffer containing 0.5 *M* NaCl. After adding an equimolar amount of TPO_{163} to the purified Fab fragment, the antigen–Fab complex was also purified using a Tosoh SP-5PW column with a linear gradient from 20 m*M* MES buffer pH 6.5 to the same buffer containing 0.5 *M* KCl at a flow rate of 1 ml min^{-1}. All other chemicals were analytical grade commercial products.

2.2. Crystallization

Screening of crystallization conditions for the TPO_{163}–Fab complex was performed using the hanging-drop vapour-diffusion method using two approaches. The first was the standard screening method (Jancarik & Kim, 1991; Cudney *et al.*, 1994) using Crystal Screen kits I and II which are commercially available from Hampton Research; the second was a custom screen based on previously reported conditions for Fab crystallization (Fischmann *et al.*, 1991; Love *et al.*, 1993; Chang, Jeffrey *et al.*, 1994; Chang, Whitaker *et al.*, 1994; Pokkuluri *et al.*, 1994; Liu *et al.*, 1996). Polyethylene glycol (PEG) 4000 has often been used for the crystallization of Fab fragments. Two buffer systems, potassium phosphate and imidazole, were used to adjust the pH. The protein concentration used was between 10 and 20 mg ml^{-1}. All screening was performed at both 277 and 295 K.

The most suitable crystals for X-ray diffraction study were grown at 295 K in 5 µl drops containing equal volumes of 18–20 mg ml^{-1} complex (in 20 m*M* imidazole pH 7.0) and reservoir solution [19–21%(*w*/*v*) PEG 3350, 100 m*M* potassium phosphate pH 6.0]. This condition is similar to the crystallization conditions for the FabD1.3–lysozyme complex (Fischmann *et al.*, 1991). Thin plate crystals grew to dimensions of 0.2 × 0.2 × 0.02 mm within one week (Fig. 1).

In order to confirm that the crystal contains both TPO_{163} and the Fab fragment, the protein sample was recovered from the crystal. The crystals were washed with reservoir solution followed by precipitation by 5% trichloroacetic acid and SDS–PAGE analysis (Fig. 2). For comparison, the sample after purification by cation-exchange chromatography (used for crystallization) was loaded in lane 1. The pattern from the recovered protein showed that there are at least two components in the crystal (lane 4). One component has a molecular weight of 18 kDa, corresponding to TPO_{163}, and the other has a molecular weight of 25 kDa, corresponding to both the light chain and Fd fragment digested by papain, which was quite similar to the pattern before crystallization. Moreover, each component was analyzed by Western blotting using anti-TPO monoclonal antibody and anti-mouse IgG (H+L). The 18 kDa and 25 kDa protein bands were attributed to TPO_{163} and the mixture of light chain and Fd fragment, respectively. Thus, it was confirmed that the crystal contains a 1:1 complex of the TPO_{163} and the Fab fragment.

2.3. Data collection

Since the crystals obtained were quite sensitive to X-ray exposure, the diffraction data were collected at 100 K. The crystals were soaked in cryoprotectant solution containing 20%(*w*/*v*) PEG 3350, 100 m*M* potassium phosphate pH 6.0 and 8% glycerol and then flash-frozen in a nitrogen-gas stream. The data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and determined to arise from two similar space groups, $P2_1$ and *C*2. The $P2_1$ data set was collected at beamline BL6B

Table 1
Data collection and reduction statistics of TPO_{163}–Fab complex.

Values in parentheses are for the highest resolution shell (2.96–2.90 Å for the $P2_1$ crystal and 2.59–2.50 Å resolution for the $C2$ crystal).

Crystal form	$P2_1$	$C2$
Resolution (Å)	57.7–2.9	33.0–2.5
Observations used in scaling	113572	273678
Unique reflections	52318	35210
$\langle I/\sigma(I)\rangle$ overall	3.8 (0.6)	6.5 (2.1)
Completeness for $F/\sigma(F) > 2.0$ (%)	93 (84)	90 (90)
Multiplicity	2.17	2.9
Mosaicity	0.414	0.635
Wilson B factor	32.1	42.6
$R_{\rm merge}$† (%)	14.0 (66.0)	9.2 (33.3)

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$, where I is the integrated intensity of a given reflection.

of the Photon Factory, KEK, Japan and processed to 2.9 Å resolution with an overall $R_{\rm merge}$ of 14%. The unit-cell parameters were determined to be $a = 133.20$, $b = 46.71$, $c = 191.47$ Å, $\beta = 90.24^\circ$. The $V_{\rm M}$ value was calculated to be 2.31 Å^3 Da^{-1}, suggesting four molecules in the asymmetric unit. The $C2$ data set was collected at beamline BL41-XU of SPring-8, Japan. After processing the data set to 2.5 Å resolution with an overall $R_{\rm merge}$ of 9%, the crystal was determined to have unit-cell parameters $a = 131.71$, $b = 46.48$, $c = 184.63$ Å, $\beta = 90.42^\circ$. The $V_{\rm M}$ value was calculated to be 2.19 Å^3 Da^{-1}, suggesting two molecules in the asymmetric unit. The β angles in both crystal forms are almost 90°; however, neither crystal form belongs to the orthorhombic system judging from the high $R_{\rm merge}$ values (55.6% for $P222$ and 50.9% for $C222$, respectively). The reason for the two different crystal forms ($P2_1$ and $C2$) may be attributed to the shrinkage of the molecules during flash-freezing. It is, however, impossible to confirm this as we could not collect diffraction data at room temperature. Data-collection and reduction statistics are shown in Table 1.

Molecular-replacement analysis was performed using the program *AMoRe* (Navaza, 1994) in the 10–4 Å resolution range using the coordinates of the Fab structure (PDB code 1afv) as an initial search model. The locations of the Fab molecules in the $P2_1$ and $C2$ crystals were identified with correlations F of 0.396 and 0.542 and crystallographic R values of 47.0 and 48.1%, respectively. All values were distinct from those of other solutions. It was found that Fab molecules in both the $P2_1$ and $C2$ crystal lattices were aligned in planes across the unit cell. The $P2_1$ and $C2$ crystal forms, having grown under identical conditions with identical morphology and extremely similar unit-cell parameters, almost certainly have nearly identical packing of TPO_{163} and Fab molecules in the unit cell. Indeed, the transformation from $C2$ symmetry to $P2_1$ symmetry is achieved merely by disrupting the exact translational operation along the a unit-cell edge. In Fig. 3, the location of Fab molecules in the $P2_1$ crystal lattice is shown. Although the density belonging to TPO_{163} is not yet clear, these findings suggest that interactions between Fab molecules are the main driving force for the crystallization of the TPO_{163}–Fab complex.

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