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Purification, crystallization and preliminary X-ray analysis of a complex between granulocyte colony-stimulating factor and its soluble receptor

Masaharu Aritomi,† Naoki Kunishima, Nahoko Okitsu, Masato Shimizu, Yoshimi Ota‡ and Kosuke Morikawa*

Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita-city, Osaka 565-0842, Japan

† Present address: Asahi Chemical Industry Co. Ltd, 2-1 Samejima, Fuji-city, Shizuoka 416-8501, Japan.
‡ Present address: Toray Industrial Inc., 1111 Tebiro, Kamakura-city, Kanagawa 248-0036, Japan.

Correspondence e-mail: morikawa@beri.co.jp

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Crystals of the complex between granulocyte colony-stimulating factor and its soluble receptor were obtained by a vapour-diffusion method using ammonium sulfate as a precipitant. Addition of 1,4-dioxane was critical in order to grow the crystals to sufficient sizes. Cryoprotection was essential in order to collect diffraction data at atomic resolution. Two kinds of crystal forms were obtained depending on the cryoprotectants. In a cryosolvent with the same salt concentration as in the crystallization conditions, the crystal belonged to the space group $I4_122$. At higher salt concentrations, the crystal was converted to a different space group $P4_12_12$ ($P4_32_12$) with the same unit-cell parameters.

1. Introduction

Granulocyte colony-stimulating factor (G-CSF) belongs the cytokine superfamily, which consists of growth hormone (GH), erythropoietin (EPO) and several other cytokines (Wells & de Vos, 1996). G-CSF regulates the maturation and proliferation of the precursor cells of neutrophilic granulocytes (Metcalf, 1989). The physiological activities of G-CSF are mediated through binding to its receptor (G-CSF-R), which is expressed on the target-cell surface (Nagata & Fukunaga, 1991). The mature G-CSF-R consists of an immunoglobulin-like (Ig-like) domain, a cytokine-receptor homology (CRH) region, three fibronectin type III-like (FNIII-like) domains, a transmembrane domain and a cytoplasmic domain (Fukunaga *et al.*, 1990; Nagata & Fukunaga, 1993). The CRH region of G-CSF (gs-CRH) was found to be most critical for ligand binding and biological activity, indicating that the gs-CRH is a minimal extracellular segment essential to mediate the signal (Fukunaga *et al.*, 1991).

Previous biochemical studies revealed that Ig-like gs-CRH forms a 1:1 complex with G-CSF (Hiraoka, Anaguchi & Ota, 1995), while the Ig-like domain of gs-CRH forms either 2:2 (Horan *et al.*, 1997) or 4:4 complexes (Hiraoka *et al.*, 1994) and the entire extracellular region forms a 2:2 complex (Horan *et al.*, 1996). Although these results are not consistent with each other, the G-CSF:G-CSF-R complex is likely to function through unknown oligomerization schemes which differ from the 1:2 stoichiometry of the ligand–receptor complexes of GH:GH-R (Cunningham *et al.*, 1991; de Vos *et al.*, 1992) and EPO:EPO-R (Syed *et al.*, 1998). To clarify the binding scheme between G-CSF and G-CSF-R, we have initiated an X-ray crystallographic study of the G-CSF:gs-CRH complex.

2. Materials and methods

2.1. Sample preparation

Recombinant human G-CSF was provided by Kirin Brewery Co. Ltd. It contains 175 amino-acid residues, including an extra methionine residue at the N-terminus. Recombinant mouse gs-CRH was expressed in insect cells as reported previously (Hiraoka, Anaguchi, Asakura *et al.*, 1995). The secreted protein was purified by G-CSF immobilized affinity and cation-exchange (Mono-S, Pharmacia) column chromatography. The complex was produced by mixing a slight excess of G-CSF with gs-CRH at room temperature for 10 min. The mixture was then purified by gel filtration (HiLoad Superdex 200, Pharmacia) using an eluent consisting of 0.1 *M* NaCl, 10 m*M* MES–NaOH pH 6.0. Exchange between the human and mouse sequences yields essentially no difference in either the K_d values or in the biological activities of the mixed ligand–receptor complex (Fukunaga *et al.*, 1991).

2.2. Biochemical analyses

N-terminal amino-acid sequences were analyzed using a peptide sequencer (492A, Applied Biosystems). The sedimentation equilibrium experiment was performed using a Beckman Optima XL-A centrifuge at 293 K. MALDI–TOF MS was carried out on a

Table 1
Comparison of the diffraction data between the two different cryosolvents.

Cryosolvent†	High salt	Low salt
Space group	$P4_12_12$ ($P4_32_12$)	$I4_122$
Unit-cell parameters		
a, b‡ (Å)	125.7 (0.2)	125.5 (0.3)
c‡ (Å)	373.3 (0.5)	372.8 (0.9)
Beamline	Photon Factory BL6B	SPring-8 BL41XU
Resolution§ (Å)	3.5	2.8
Unique reflections	26681	35565
Measured reflections	173168	169036
Completeness (%)	69.1	95.4
$R_{\rm merge}$¶ (%)	19.8	5.7

† High salt: 2.0 *M* ammonium sulfate, 30%(*v*/*v*) glycerol, 100 m*M* HEPES–NaOH pH 7.5. Low salt: 50%(*w*/*v*) sucrose, 1.2 *M* ammonium sulfate, 100 m*M* HEPES–NaOH pH 7.5. ‡ The figures in parentheses are the standard deviations. § Both crystals diffracted X-rays to about 2.8 Å resolution. However, the resolution limit for the structure determination of the *P* tetragonal form should be 3.5 Å owing to the high $R_{\rm merge}$ value of the reflections in the high-resolution range. ¶ $R_{\rm merge} = \sum_i \sum_j |\langle I_i \rangle - I_{ij}| / \sum_i \sum_j I_{ij}$, where $\langle I_i \rangle$ is the mean intensity of the ith unique reflection and I_{ij} is the intensity of the jth observation.

Voyager Elite mass spectrometer (PerSeptive Biosystems). The sugar contents were analyzed at Toray Research Institute by high-performance liquid chromatography (HPLC) using the post-label fluorescence method.

2.3. X-ray diffraction

The diffraction data were collected using the macromolecular-oriented Weissenberg camera (Sakabe, 1991) installed at beamline BL-6B at the Photon Factory (PF) of the National Laboratory for High Energy Physics, Tsukuba, Japan and the R-AXIS IV detector installed at beamline BL-41XU at SPring-8, Hyogo, Japan. The crystal was mounted in a nylon-fibre loop and was flash-cooled in a nitrogen-gas stream at 100 K. Diffraction data were integrated and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

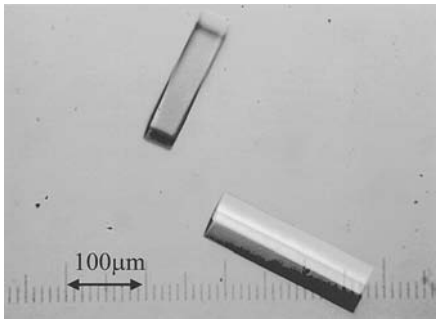

Figure 1
Crystals of the G-CSF:gs-CRH complex obtained by the hanging-drop vapour-diffusion method.

3. Results and discussion

3.1. N-terminal heterogeneity of gs-CRH

The gs-CRH purified by cation-exchange chromatography was heterogeneous and consisted of at least three components. Analyses of the N-terminal residues revealed that they mainly arise from different cleavage of the N-terminal signal sequence. The major fraction (peak 1) corresponded to the intact sequence, in agreement with the construction (Val85–Ala309, with five extra residues at the N-terminus), while the second (peak 2) and third (peak 3) fractions were found to contain proteins lacking nine and 15 N-terminal residues, respectively.

3.2. Crystallization conditions

Various crystallization conditions for the G-CSF:gs-CRH (peak 1–3) complexes were examined using a vapour-diffusion method with a matrix of ammonium sulfate concentration *versus* pH. The peak 3 fraction yielded tetragonal prismatic crystals after incubation in 1.1–1.2 *M* ammonium sulfate solution (pH 7–8) at 293 K for 4 d, whereas no crystals were obtained from the peak 1 and 2 fractions. In spite of further attempts to refine the crystallization conditions, we could not grow crystals larger than 20 × 20 × 150 μm, presumably because of a high frequency of nucleation. Successive seeding failed to grow single crystals because of serious secondary growth. Therefore, additives were tested to control the nucleation. Consequently, a crystal with a sufficient size for X-ray diffraction study (maximum dimensions 60 × 60 × 500 μm) was obtained from a 1.2 *M* ammonium sulfate solution (pH 7.5) with 5%(*v*/*v*) 1,4-dioxane (Fig. 1).

3.3. Sugar-chain analysis of gs-CRH

The sugar-content analysis showed that gs-CRH contained 4.3 equivalents of mannose, 2.3 equivalents of fucose and 2.9 equivalents of *N*-acetyl glucosamine, while *N*-acetyl galactosamine and neuraminic acid were not detected. The sugar chain should be classified as N-linked as it contains *N*-acetyl glucosamine but not *N*-acetyl galactosamine. MALDI–TOF mass spectrometry of peak 3 showed that the molecular mass was 26 825 ± 9 Da, which is 1217 Da higher than the value calculated from the amino-acid sequence. The spectrum showed a shoulder in a high molecular-mass region, presumably owing to the sugar-residue heterogeneity. However, it showed a sharp band in comparison with the spectra of peaks 1 and 2 (data not shown). A typical N-linked sugar chain contains at least two residues of *N*-acetyl glucosamine and three residues of mannose and thus the minimum calculated molecular mass for the sugar chain should be 892 Da. Therefore, only a single N-linked sugar chain would be acceptable for peak 3. Furthermore, the N-terminal analysis showed that Asn104, one of the potential N-glycosylation sites, was not assigned as a free amino acid, implying that the gs-CRH of peak 3 is N-glycosylated at Asn104. These results are consistent with the crystal structure of gs-CRH (Aritomi *et al.*, 1999).

3.4. Data collection

Cryoprotection was essential to collect sufficient diffraction data, as the crystal was sensitive to X-ray radiation damage. Interestingly, two distinct crystal forms were obtained depending on the cryoprotectants (Table 1). As shown in Table 1, the *I* tetragonal form allowed a structural determination at 2.8 Å resolution, although the effective resolution of the *P* tetragonal form was 3.5 Å. It is noteworthy that the conversion from the *I* tetragonal form to the *P* tetragonal form took place within a few hours, implying smooth rearrangements of the protein molecules in the crystal lattice.

3.5. Stoichiometry of the complex

The stoichiometric study of the G-CSF:gs-CRH complex by sedimentation analysis showed that the proteins form a 1:1 complex in a solution containing 0.1 *M* NaCl and 10 m*M* MES pH 6.0, even at a high protein concentration of 10 mg ml^{-1} (data not shown). HPLC analyses also demonstrated the formation of a 1:1 complex under similar conditions (Hiraoka, Anaguchi & Ota, 1995). On the other hand, the V_m value of the *I* tetragonal crystal was calculated to be either 4.24 or 2.83 Å^3 Da^{-1}, assuming the asymmetric unit to contain two or three complex molecules with a 1:1 stoichiometry of the ligand to gs-CRH, respectively. It is likely that the crystal contains two or three 1:1 complexes, as both values are in the theoretically possible range for protein crystals (Matthews, 1968). Interestingly, the sample aggregated in solutions at pH 7.5, as revealed by sedimentation analyses. Thus, higher order oligomerization may occur at neutral pH. In fact, the 2:2 complex was observed in a crystal which was grown at pH 7.5 (Aritomi *et al.*, 1999).

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