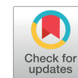
Biochemical and Biophysical Research Communications 500 (2018) 224–228

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Structural insights into the backbone-circularized granulocyte colony-stimulating factor containing a short connector



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ARTICLE INFO

Article history:
Received 3 April 2018
Accepted 6 April 2018
Available online 17 April 2018

Keywords:
Cytokine
G-CSF
Backbone circularization
X-ray crystallography
Protein engineering

ABSTRACT

Backbone circularization is a powerful approach for enhancing the structural stability of polypeptides. Herein, we present the crystal structure of the circularized variant of the granulocyte colony-stimulating factor (G-CSF) in which the terminal helical region was circularized using a short, two-amino acid connector. The structure revealed that the N- and C-termini were indeed connected by a peptide bond. The local structure of the C-terminal region transited from an α helix to 3_{10} helix with a bend close to the N-terminal region, indicating that the structural change offset the insufficient length of the connector. This is the first-ever report of a crystal structure of the backbone of a circularized protein. It will facilitate the development of backbone circularization methodology.

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1. Introduction

Backbone circularization, connecting the N- and C-termini of a polypeptide by a peptide bond, is a powerful approach for stabilizing proteins [1,2]. Since the alteration of amino acid sequence by backbone circularization (henceforth referred to as "circularization") is much smaller than that introduced by other stabilization strategies (e.g., amino acid substitution), circularization is also much less likely to induce immunogenicity. Therefore, we propose that the method might be especially applicable for the engineering of biopharmaceutical proteins. Among several technologies used for the generation of circularized polypeptides, the split intein-mediated circularization (often called SICLOPPS, the split-intein circular ligation of peptides and proteins) enables the connection of polypeptide termini with minimal alteration to sequences. Using that approach, only one residue (Cys or Ser) is inserted into the circularized product [3,4].

We have recently reported the design of circularized granulocyte colony-stimulating factor (G-CSF) [5]. G-CSF is a four-helix bundle cytokine that regulates the development of neutrophils. Recombinant G-CSF has been utilized as a biopharmaceutical [6]. We have designed, generated, and characterized the recombinant variants of circularized G-CSF harboring connecting segments of different lengths [5]. Comparison of the N- and C-terminal helical regions of G-CSF with the helix-turn-helix segments extracted from the structures deposited in the Protein Data Bank (PDB) suggested that two, five, and nine residues might be used to connect the G-CSF termini without distorting the protein structure. Specifically, we expected that the C163 variant harboring the shortest connector would be most stable, since a minimum and distortion-free connector should maximize the stabilizing effect by loop-shortening [1]. Nevertheless, although all variants were more stable than the linear G-CSF, C163 was less stable than the other circularized variants, perhaps because a two-residue connector was too short to allow maintenance of the original structure of G-CSF. On the other hand, C163 was intriguing as it was circularized without the formation of a linearized byproduct that typically arises from false *trans*-splicing reaction catalyzed by the split intein [5]. This facilitated the generation of C163 samples of high purity.

In the current report, we present the crystal structure of C163. Generation of several artificially circularized proteins has been demonstrated [7–9] but their crystal structures have not yet been reported, presumably because of the difficulty of obtaining

Abbreviations: G-CSF, Granulocyte colony-stimulating factor; PDB, Protein Data Bank; RMSD, Root mean squared deviation.

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https://doi.org/10.1016/j.bbrc.2018.04.045
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sufficient quantities of pure circularized protein. We have successfully developed the C163 preparation protocol in the previous report [5] and herein crystalized the protein to understand the effect of circularization on its structure. We determined the C163 crystal structure at a resolution of 1.65 Å and confirmed that the N- and C-termini were indeed connected by a peptide bond. We further evaluated the effect of circularization on the 3D structure, by comparing with the crystal structure of linear G-CSF. Significant structural changes were apparent in the C-terminal region of C163, namely, stretch with a transition from α helix to 3_{10} helix, and a tilt close to the N-terminal region. These structural changes suggested that the circularization forced the terminal regions to move closer to one another. In other words, the helical structure gained some flexibility to compensate for the stress associated by introducing a short connector.

2. Materials and methods

2.1. Protein expression and purification

C163 was expressed and purified as described previously [5]. Briefly, C163 harboring the split intein from *Nostoc punctiforme* was expressed in *Escherichia coli* BL21 (DE3) cells. Circularization of C163 by the split intein occurred in the bacterial cells. Upon cell sonication, the insoluble fraction was collected, washed, resolubilized, and refolded. The refolded sample was purified by using three chromatography steps, i.e., anion-exchange chromatography on HiTrapQ (GE Healthcare), size-exclusion chromatography on Superdex 75 (GE Healthcare), and anion-exchange chromatography on MonoQ (GE Healthcare).

2.2. Crystal structure determination

C163 was concentrated to 7 mg/mL and used in crystallization trials. After screening using Crystal Screen (HAMPTON REARCH), suitable crystals were obtained in 0.4 M ammonium phosphate buffer. Diffraction data were collected using the beamline AR-NW12 at the Photon Factory (Tsukuba, Japan) under cryogenic conditions (100 K). Diffraction images were processed and scaled using programs MOSFLM [10] and SCALA [11], respectively. The C163 structure was determined by the molecular replacement method using PHASER [12] and the coordinates of human G-CSF (PDB 2D9Q, chain A [13]) as the search model. Coordinate refinement was performed using programs REFMAC5 [14] and COOT [15]. No additional constraints were applied except for the permission to create bonds between proximal residues. Model quality was assessed using PROCHECK [16]. Secondary structure content was evaluated using STRIDE [17]. Molecular graphics were created using the UCSF Chimera package [18]. C163 atomic coordinates and structure factors have been deposited in the PDB, under the entry code 5GW9.

3. Results

3.1. The overall C163 structure

We determined the crystal structure of C163 at a 1.65 Å resolution (Fig. 1, Table 1). All (652) residues comprising four molecules of C163 in an asymmetric unit were modeled. Considerable structural differences between the four molecules were not apparent; the Cα atoms of these molecules superimposed with the root mean squared deviation (RMSD) of less than 0.10 Å. The connection between Gly163 and Ser1 was clearly visible in the electron density map, indicating that the structure of a circularized protein was

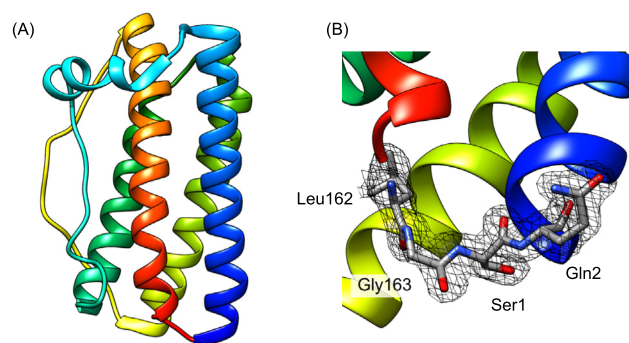

Fig. 1. Crystal structure of C163. (A) The overall C163 structure presented in rainbow colors. (B) Close-up of the connected terminal region. Two residues at the connected site (Gly163 and Ser1) and their adjacent residues (Leu162 and Gln2) are shown as sticks using CPK colors. The electron density corresponds to the σ_A-weighted $2F_o-F_c$ map contoured at 1.5 σ.

Table 1
Data collection and refinement statistics.

	C163
Data collection	
Space group	$P3_2$
Unit cell	
Dimensions (Å)	a = b = 60.94, c = 178.51
Angles (°)	α = β = 90, γ = 120
Wavelength (Å)	1.0000
Resolution range (Å)	50.0–1.65
Total observations	508,514
Unique observations	89,146
I/σ (I)	35.5 (3.2)
Completeness (%)	99.9 (100)
R_{merge} (%)[a]	6.2 (63.6)
Multiplicity	5.7 (5.6)
Refinement	
R_{work}/R_{free} (%)[b]	19.4/22.9
Protein chains	4
Protein residues	652
Protein atoms	5325
Ligands	0
Ligand atoms	0
Water molecules	464
B-factor, protein (Å^2)	28.1
B-factor, ligands (Å^2)	–
B-factor, water (Å^2)	38.0
RMSD bonds (Å)	0.022
RMSD bonds (°)	2.22
Coordinate error (Å)	0.07
Ramachandran plot	
Preferred regions (%)	97.1
Allowed regions (%)	2.7
Outliers (%)	0.2
PDB code	5WG9

[a] $R_{merge.} = \Sigma_{hkl}\ \Sigma_i|I(hkl)_i - [I(hkl)]|/\Sigma_{hkl}\ \Sigma_i\ I(hkl)$.
[b] $R_{work} = \Sigma_{hkl}\ |F(hkl)_o - [F(hkl)_c]|/\Sigma_{hkl}\ F(hkl)_o$; R_{free} was calculated as R_{work}, where $F(hkl)_o$ values were taken from 5% of data not included in the refinement.

indeed determined (Fig. 1B). Most parameters for the peptide bond geometry between Gly163 and Ser1 were almost the same as the ideal values from the International Tables Online site hosted by the International Union of Crystallography [19] (Fig. 2). The C-N distance (1.29 Å) and the C-N-Cα bond angle (116°) appeared to be far from the ideal values (1.34 Å and 122°, respectively). However, we did not consider these differences to be significant since they fell within the distribution of numbers calculated from all (652) bonds in the structure (Supplementary Fig. S1, Fig. S2).

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