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Crystallization and Molecular Packing Analysis of Barstar Crystals

V. Raghunathan[1], S. Khurana[1], V. Gupta[1], R. Khurana[2], J. B. Udgaonkar[2] and D. M. Salunke[1]

[1]*National Institute of Immunology*
Aruna Asaf Ali Marg, J. N. U. Complex, New Delhi 110 067, India

[2]*National Centre for Biological Sciences*
TIFR Centre, P.O. Box 1234, Indian Institute of Science Campus, Bangalore 560 012, India

Barstar, the natural inhibitor of barnase crystallizes in many different crystal forms under almost identical conditions. Although barstar is a monomeric protein, it crystallizes with four molecules in the asymmetric unit in two crystal forms, rhombohedral (space group $R3$; $a = b = 118.0$ Å; $c = 75.5$ Å) and tetragonal (space group $P4$; $a = b = 105.1$ Å; $c = 36.0$ Å), which exist simultaneously under identical crystallization conditions. The relation between the four molecules in the asymmetric unit of the crystals belonging to space group $P4$ can be interpreted in terms of a small distortion in the crystallographic symmetry of the higher symmetry space group $P422$.

Keywords: barstar; crystallization; rotation function; non-crystallographic symmetry; molecular packing

Barstar is an intracellular protein inhibitor of barnase, an endoribonuclease secreted by *Bacillus amyloliquefaciens* (Hartley, 1988, 1993). The inhibitor is specific for barnase although *in vitro* it cross-reacts with binase, a highly homologous ribonuclease from *Bacillus intermedius* (Pavlovsky *et al.*, 1983). *In vitro*, the inhibition of barnase by barstar is stoichiometric, it involves formation of a tight 1:1 complex and does not require a cofactor (Hartley & Smeaton, 1973). Although no complex *in vivo* of barstar with barnase has been detected so far there is indirect evidence of its existence. It is thought that the role of barstar is to neutralize the toxic effects of barnase to the host cell by inhibiting the barnase molecules that have folded before secretion (Hartley, 1988). The three-dimensional structure of barnase as well as the barnase–barstar 1:1 complex has been determined (Baudet & Janin, 1991; Bycroft *et al.*, 1991; Guillet *et al.*, 1993*a*). Here we report the characterization of a new crystal form and the analysis of the molecular packing in different crystals of recombinant barstar.

Barstar was expressed in *Escherichia coli* strain MM294 containing the plasmid pMT316 (Khurana & Udgaonkar, 1994). A one-litre culture of *E. coli* containing the barstar expression plasmid was harvested after 24 hours of growth, and lysed by sonication. The ammonium sulphate precipitation (the 40% to 80% cut contained barstar), gel permeation chromatography (G-75 Sephadex) and ion-exchange chromatography (DEAE Sephadex) were the three main steps involved in the purification. The final pure protein solution was desalted by ultrafiltration (using an Amicon YM-3 membrane), and freeze-dried. The purity of the protein was found to be greater than 99% by tricine SDS polyacrylamide gel electrophoresis.

Crystals of the recombinant barstar were grown at 24°C by the hanging drop vapour diffusion method (McPherson, 1990). A 22 mg/ml stock solution of the protein in 0.05 M phosphate buffer (pH 6.5) was used with ammonium sulphate as the precipitant. The effective protein concentration in an 8 µl crystallization drop was about 11 mg/ml. Crystals began appearing within one week and grew to their maximum size within three weeks. Although the best crystals were grown under the above conditions, they could be obtained at a wide range of pH (pH 5.5 to 8.5) using phosphate as well as Tris·HCl buffers. Two morphologically well-defined crystal forms, rhombohedral and tetragonal, were identified in the drops. Both forms co-exist in a drop over a range of pH and ammonium sulphate concentrations, although rhombohedral crystals are more frequent than the tetragonal ones.

Both the crystal forms diffract beyond 2.5 Å resolution and are fairly stable in the X-ray beam. Precession photographs were recorded on a Huber precession camera using a rotating anode X-ray generator with nickel-filtered CuK_α radiation. The

Correspondence: D. M. Salunke, National Institute of Immunology, Aruna Asaf Ali Marg, J. N. U. Complex, New Delhi 110 067, India.

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Table 1

Comparison of the crystal parameters of barstar in various crystal forms

Crystal form	Space group	a (Å)	b (Å)	c (Å)	α (°)	β (°)	γ (°)	N†	Crystal density (g/cm^3)	Solvent content (%)
nii1	$R3$	118.0	118.0	75.5	90	90	120	4	1.182	48
nii2	$P4$	105.1	105.1	36.0	90	90	90	4	1.190	46
cnrs1	$P4$	109.0	109.0	37.4	90	90	90	4	—	—
cnrs2	$P422$	76.8	76.8	37.4	90	90	90	1	—	—
cnrs3	$P6$	143.6	143.6	35.6	90	90	120	4	—	52

nii1 and nii2 were obtained in this laboratory whereas cnrs1, cnrs2 and cnrs3 were from Guillet *et al.* (1993).

† N, the number of molecules per asymmetric unit.

crystal densities were measured in a Ficoll gradient (Westbrook, 1976, 1985) calibrated against 2 μl drops of toluene-CCl_4 mixtures of known densities. The X-ray diffraction intensity data were collected on the Nicolet area detector equipped with a rotating anode X-ray generator. A single barstar crystal belonging to tetragonal morphology was mounted in a glass capillary and the data were collected to 3.0 Å resolution in two different orientations 90° to each other. A total of 450 frames were collected in each orientation with an ω scan of 0.2 degree per frame and processed using the XENGEN package (Howard *et al.*, 1987). The final $R_{(\text{merge})}$ value was 12%. Only partial intensity data were recorded for the crystals with rhombohedral morphology.

The analysis of the precession photographs and the intensity data recorded on the area detector showed that the crystals with rhombohedral morphology (nii1) belong to the space group $R3$ (with the hexagonal unit cell defined by $a = b = 118.8$ Å and $c = 75.5$ Å) and those with tetragonal morphology (nii2) to the space group $P4$ ($a = b = 105.1$ Å and $c = 36.0$ Å). The 3-fold and the 4-fold symmetry axes were evident in the precession photographs, corresponding to $hk0$ and $hk1$ zones of the respective crystals. The crystal densities of $R3$ and $P4$ crystals were measured to be 1.182 gm/cm^3 and 1.190 g/cm^3, respectively. The solvent content (V_{sol}) was thus estimated to be 48% for the rhombohedral and 46% for the tetragonal form. Assuming the partial specific volume of the protein to be 0.74 cm^3/g (Matthews, 1968), the Matthews number V_m is 2.36 Å^3/dalton for the rhombohedral crystals, indicating that there are four molecules of barstar in the asymmetric unit. Similarly the V_m for the tetragonal crystals is 2.28 Å^3/dalton, also giving four molecules of barstar per asymmetric unit.

While this paper was under preparation, a preliminary report on barstar crystallization (Guillet *et al.*, 1993*b*) appeared showing three crystal forms: cnrs1, cnrs2 and cnrs3, corresponding to space groups $P4$, $P422$ and $P6$, respectively. Our tetragonal crystals (nii2) grown using ammonium sulphate as precipitant closely resemble cnrs1 crystals obtained using either potassium phosphate or sodium citrate as seen from Table 1. However, the cnrs1 crystals diffract very poorly. Two of the crystal forms reported by Guillet *et al.* (1993*b*), cnrs1 and cnrs3 (belonging to space groups $P4$ and $P6$, respectively), also have four molecules of barstar per asymmetric unit. Thus, three out of the four independent crystal forms have four molecules in the asymmetric unit. This suggests the possibility of a molecular association between barstar monomers.

The existence of an oligomeric state of barstar was analysed by gel permeation chromatography. Less than 5% of the protein oligomerizes to a dimeric state, which upon addition of a 20 molar excess of DTT, readily revert back to the monomer, suggesting a role of cystein residues in its formation. A mutant of barstar (BSCCAA) with both cystein residues replaced by alanine shows no oligomerization under oxidizing conditions. Thus, barstar is a monomeric protein in non-denaturing solutions, implying that the crystals of barstar belonging to the space group $P4$, $P6$ and $R3$ contain four independent molecules in the asymmetric unit.

The packing of the non-crystallographically related barstar molecules in the tetragonal crystals was analysed by self-rotation function using the MERLOT program (Fitzgerald, 1988). A self-rotation function search was performed using the data in the 10 to 4.5 Å resolution range with the radius of integration equal to 16 Å. Reflections with $I/\sigma(I) > 2.0$ were used. The results of the self-rotation function calculations ($\kappa = 180°$) of the P_4 crystals are given in Table 2. The self-rotation function at $\kappa = 180°$ shows four distinct peaks significantly above the background level. These peaks correspond to four 2-fold axes at $\varphi = 90.0°$ and $\phi = 10.0°$, 35.0°, 55.0° and 80.0°, respectively. The common φ value of 90.0° suggests that all the four axes are in the ab-plane with 45.0° related pairs (10.0° and 55.0°; 35.0° and 80.0°).

Table 2

Polar co-ordinates and the peak heights (in arbitrary units) in the self-rotation function ($\kappa = 180°$)

Peak	ϕ	φ	Peak height
I	0.0	0.0	2.6
II	10.0	90.0	2.2
III	35.0	90.0	2.4
IV	55.0	90.0	2.2
V	80.0	90.0	2.4

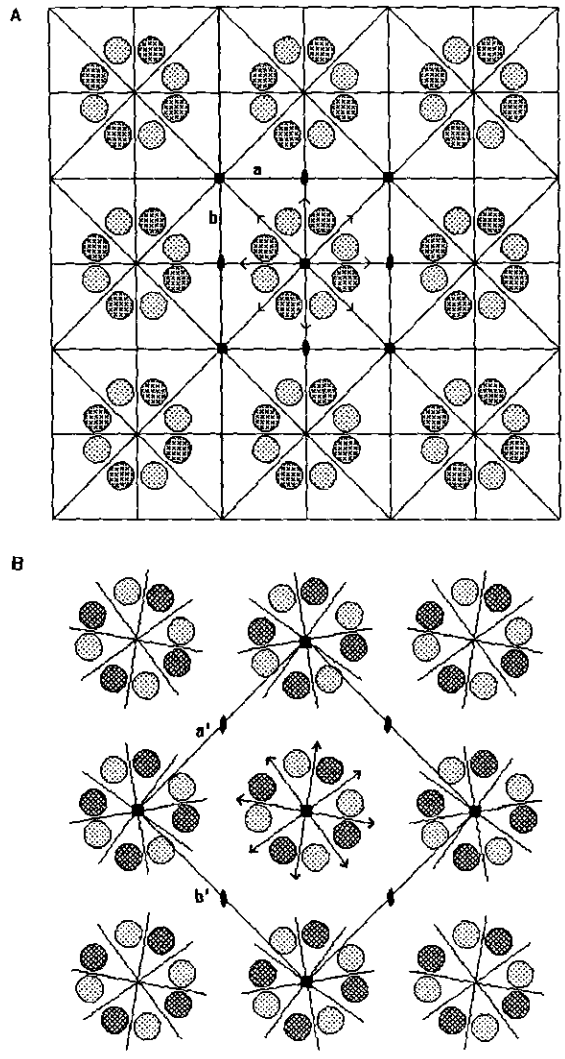

Figure 1. The crystal packing in space group $P422$ (A) and $P4$(B) with 4 molecules in the asymmetric unit. The barstar monomers are shown by filled circles. The dots and crosses used for filling circles distinguish the positive and negative z-co-ordinate. The crystallographic symbols have been marked for only one unit cell in the lattice.

These peaks are in addition to the origin peak which coincides with the crystallographic 4-fold rotation axis. The self-rotation function calculations were also carried out at $\kappa = 90°$ and 120°. No peaks were observed with $\kappa = 120°$ and a peak at origin coinciding with the crystallographic 4-fold axis was observed with $\kappa = 90°$.

A comparison of the unit cell parameters and symmetry of the $P4$ and $P422$ crystals (Table 1) leads to the possibility that the two crystal forms are closely related, as also suggested by Guillet *et al.* (1993*b*). The c-axis is almost identical in these two crystals and the a-axis of $P4$ crystals corresponds to the face-diagonal in the ab-plane of $P422$ crystals. Thus, the similarity in the molecular packing of the two crystal forms is evident. Figure 1 provides the packing relationship between the two crystal forms on the basis of the self-rotation function analysis. In the packing diagram of $P422$ crystals (Figure 1A), a super lattice with unit cell defined by the face-diagonal for the ab-plane as the a-axis can be constructed. The new unit cell has dimensions similar to the $P4$ crystals. Furthermore, rotation of the crystallographic 2-fold axes in this plane by $\pm 10°$ satisfies the observed rotation function results. Therefore, a combination of the unit cell transformation and 10° adjustment in the rotation axes relates the two crystal forms. These changes result in the rotation of the molecular cluster at 1/2 1/2 0 with respect to that at 0 0 0 by 20°, thus making it a pseudo C-centring type of arrangement (Figure 1B). This interpretation is further confirmed by the intensity data for $P4$ crystals, which indicate systematic absences corresponding to C-centred lattice with about 10% reflections violating the condition. The $P6$ crystals also have a c-axis identical and the hexagonal unit cell of the $R3$ crystals shows that the c-axis is double that in the $P4$ crystals. It is likely that the packing in these crystals is also similarly related to that in $P422$ and $P4$ crystals.

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