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Crystallization and preliminary X-ray investigation of the complex of RNase Sa with wild-type barstar

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Abstract

RNase Sa, an extracellular ribonuclease produced by *Streptomyces aureofaciens*, is inhibited by barstar, the natural protein inhibitor of barnase, the ribonuclease of *Bacillus amyloliquefaciens*. The complex of RNase Sa with wild-type barstar was crystallized by hanging-drop vapour diffusion. It was shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis that RNase Sa and barstar are present in equimolar proportions in the crystals. The crystals are in the hexagonal space group $P6_5$ with unit-cell dimensions $a = b = 56.95$, $c = 135.8$ Å. They diffract to 1.7 Å resolution at the DESY synchrotron source. The asymmetric unit contains one molecule of the complex.

1. Introduction

RNase Sa is an extracellular ribonuclease produced by *Streptomyces aureofaciens*. It belongs to an extended family of microbial ribonucleases. RNase Sa is a small single-chain protein with 96 amino-acid residues and one disulfide bond (Schlyapnikov *et al.*, 1986). The structures of RNase Sa and its complex with inhibitor (2′-GMP) have been determined at atomic resolution (Sevcik *et al.*, 1996).

RNase Sa has relatively low sequence similarity with the other members of the family, but all have basically the same fold (Hill *et al.*, 1983; Sevcik *et al.*, 1990). The sequence identity between RNase Sa and barnase, the ribonuclease produced by *Bacillus amyloliquefaciens*, is 23%. The active sites of the two enzymes are similar, and indeed barstar, a specific protein inhibitor of barnase, also binds to and inhibits RNase Sa. The RNase Sa–barstar complex is not as tightly bound as barnase–barstar. Its dissociation constant, about 10^{-10} *M*, is four orders higher than that of barnase–barstar. Contemporary expression of the RNase Sa gene with the gene of barstar in *E. coli* reduced the very high toxicity of the enzyme for the host cells allowing relatively high production of the enzyme (Hartley *et al.*, 1996). Complexes of barnase–barstar and RNase Sa–barstar provide good models for the study of protein–protein recognition.

Barstar is a small protein, 89 amino-acid residues, produced by *Bacillus amyloliquefaciens* (Hartley *et al.*, 1972). There are two cysteines at positions 40 and 82, which are buried in the protein and do not form a disulfide bond (Guillet *et al.*, 1993; Ramachandran & Udgaonkar, 1996). Crystals of the complex of barnase with wild-type barstar grew as aggregates. For structure determination of the complex crystals of barnase with double mutant barstar (C40, 82A) were used (Guillet *et al.*, 1993; Buckle *et al.*, 1994). It has been shown, that both the structure and activity of the double mutant barstar are very similar to those of wild-type barstar (Hartley, 1993). *S. aureofaciens* also produces a specific ribonuclease inhibitor. Isolation of the inhibitor proved to be very tedious (Krajcikova *et al.*, 1990). The gene of the inhibitor has been isolated, cloned and sequenced, and its expression is under way.

For crystallization of RNase Sa–barstar complex in our experiments wild-type barstar was used. The crystals of the complex are the first with wild-type barstar suitable for three-dimensional structure analysis. Here we report the crystallization of the complex and preliminary crystallographic characterization of the crystals.

2. Materials and methods

2.1. *Crystallization*

For crystallization trials hanging-drop vapour diffusion at room temperature was used (McPherson, 1990). A stock solution of the complex was prepared by mixing the solutions of barstar and RNase Sa in various ratios. Barstar was dissolved in 0.1–0.2 *M* cacodylate buffer (pH 6.6–8.0) to a concentration of 20 mg ml^{-1}. RNase Sa was dissolved in deionized water in the same concentration as barstar. Drops were prepared by mixing the stock solution with a precipitant solution in a 1:1 ratio to a final volume of 8 µl.

Hexagonal bipyramidal crystals were obtained using precipitant solution containing 30–34% PEG 6000 and solid ammonium sulfate in 0.1 *M* concentration. Crystals appeared within three to four weeks and grew over the pH range 6.6–8.0.

Using saturated ammonium sulfate solution in concentration 28–30% as the only precipitant, crystals of the same shape appeared within 4–7 d. They grew over the same pH range as above and at various RNase Sa–barstar ratios. With RNase Sa in excess, there was no precipitate in the drops, but the crystals were very small. An excess of the inhibitor resulted in the presence of precipitate in the drops, but the crystals were larger.

Relatively good crystals were obtained from a solution of RNase Sa and barstar mixed in a 1:1 ratio in 0.1 *M* cacodylate buffer, pH 7.0–7.4 and 28–30% saturated ammonium sulfate as precipitant. The concentration of the complex in the stock solution was 20 mg ml^{-1}. The relatively small crystals, with largest dimension about 0.2 mm, diffracted after cryogenic freezing (100 K) only to 2 Å resolution and were not used for data collection.

Under the same conditions, but with protein concentration increased to 40 mg ml^{-1}, the crystals grew larger (0.2 × 0.2 × 0.8 mm) as hexagonal needles (Fig. 1). They diffracted to 1.7 Å resolution and were used for data collection.

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Crystals were obtained without purification of the complex. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed, that the crystals contain RNase Sa and barstar in a 1:1 ratio, Fig. 2.

2.2. *X-ray data collection*

For data collection the EMBL X31 beamline at the DORIS storage ring, DESY, Hamburg, with an MAR Research (Hamburg) imaging-plate scanner was used. All data were measured at room temperature from a single crystal grown at a protein concentration 40 mg ml^{-1}. Two sets of data were collected to 1.7 and 3.0 Å resolution. For both sets a total of 54° of rotation was covered. The wavelength was 0.95 Å. Total collection time was 25 h. The data were processed by *DENZO* (Otwinowski, 1993).

3. Results and discussion

The largest crystals diffracted to 1.7 Å resolution on the synchrotron source at room temperature. The space group is $P6_5$ with unit-cell parameters $a = b = 56.95$, $c = 135.80$ Å, $\gamma = 120°$. A summary of data collection is given in Table 1. The unit-cell dimensions, molecular weight of the proteins (10 194 for barstar and 10 540 for RNase Sa) and space group give a V_M of 3.0 Å^3 Da^{-1} which is in the expected range (Matthews, 1968). The asymmetric unit contains one molecule of RNase Sa–barstar complex with a solvent content of 59%.

Fig. 1. Crystal of the complex of RNase Sa with barstar. The largest dimension of the crystal is 0.8 mm.

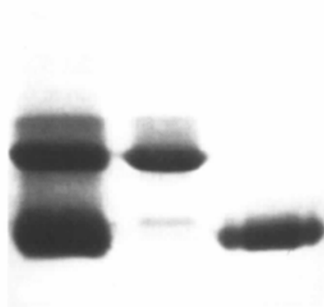

Fig. 2. Sodium dodecyl sulfate gel electrophoresis of crystals (left), RNase Sa (middle) and barstar (right).

Table 1. *Data-collection parameters*

Site	EMBL
Source	DORIS
Wavelength (Å)	0.95
Resolution range (Å)	24.7–1.7
Fully recorded	118438
Partially recorded	22844
Unique reflections	27354
Completeness (%)	100.0
$R(I)_{merge}$ (%)	4.6

The crystal structure of the barnase–barstar complex and recognition between these two proteins are known. Barnase and its natural inhibitor, barstar, are produced by the same organism. In the RNase Sa–barstar complex the two interacting proteins are produced by distinct organisms, therefore the recognition and inhibition processes are even more interesting. It is hoped, that the structure will contribute to better understanding of conformational changes which play a role in protein–protein complex formation and explain why the dissociation constants for the two complexes are so different.

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