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Three-dimensional structure of a barnase–3′GMP complex at 2.2Å resolution

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Barnase has been co-crystallized at neutral pH with its natural product the 3′-guanylic acid. The X-ray structure was solved by molecular replacement methods and refined to a final *R*-factor of 18.7%. The protein folding is essentially the same as that of the native form. The base recognition site is almost identical to that of the homologous binase–3′GMP complex, but the nucleotide is bound in a productive binding mode for a substrate with a syn glycosyl torsion angle allowing the general base Glu^{73} to hydrogen bond with the 2′O of the nucleotide as is assumed in the classical catalytic mechanism. The two molecules of the asymmetric unit form a dimer and the positions of the two nucleotides partially mimic the interaction of the RNA with the enzyme, one of the inhibitors being located in a secondary subsite.

Barnase; Complex; Nucleotide; Inhibitor; Crystal structure

1. INTRODUCTION

Barnase is an endonuclease produced and excreted by *Bacillus amyloliquefaciens*. It belongs to the large family of microbial ribonucleases which share similar structures and properties [1]. The crystal structures of several of these ribonucleases (bacterial and fungal) have been determined either in their native form or complexed with nucleotidic inhibitors. The crystal structure of barnase has been determined at 2Å resolution [2] and the solution structure obtained from NMR [3]. Recently, its complex with a deoxydinucleotide d(GpC) has also been solved [4].

Barnase hydrolyses RNA completely into mono- and small oligonucleotides. The mechanism involves 2 steps: transesterification to form a 2′3′ cyclic intermediate and then a much slower hydrolysis step to give a 3′ nucleotide. Bacterial RNases such as binase and barnase have been shown to be guanyl-specific towards short fragments of RNA as substrates, but they partially loose this specificity when hydrolysing oligonucleotides of more than 4–5 bases in length. With this decrease in specificity, which has been attributed to secondary binding sites [5], an increase in hydrolysis rate, compared with RNase T1, is observed.

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Abbreviations: RNase T1, ribonuclease T1; 3′GMP, 3′-guanylic acid; d(GpC), 2′deoxyguanylyl (3′- 5′)-2′deoxycytidine; rms deviation, root mean squares deviation.

In the case of binase, a homologous bacterial RNase (*Bacillus intermedius*) which has identical amino acids for 85% of its aligned sequence with barnase, the structure of a complex with 3′GMP has been determined [6]. In the crystal structure, the nucleotide is bound in the guanine recognition site [1,7], but adopts an anti-conformation around the glycosyl bond with the sugar showing a C2′ endo pucker, which is a non-productive binding mode for a substrate. In the structure of barnase complexed with the d(GpC), the guanine base was not located in the specific recognition site.

In order to better understand the catalytic mechanism of barnase, we have solved the crystal structure of a barnase–3′GMP complex using molecular replacement methods.

2. MATERIALS AND METHODS

2.1. *Co-Crystallization and data collection*

For crystallization, barnase was over-expressed in *E. coli* strain HB101 containing the plasmid PMT 410 [8] and purified to homogeneity as previously described [9]. The crystallization of the barnase–3′GMP complex was carried out using vapor diffusion techniques with a combination of 1.4 M ammonium sulfate and 13% polyethylene glycol 8000 as precipitants at pH 8. The hanging drops contained 0.65 mM barnase and 3.2 mM 3′GMP, in 0.5 M ammonium sulfate and 5% PEG 8000. The crystals grow as large monocrystalline prisms which belong to the orthorhombic space group $I2_12_12_1$ with cell dimensions a = 150.2 Å b = 69.9 Å c = 55.2 Å and diffract to beyond 2 Å resolution. An assumption of 2 protein molecules per asymmetric unit gives a V_m of 2.59 Å^3/Da which is in the range found for other proteins [10].

X-ray intensities were collected from a single crystal, mounted in a glass capillary, on the Wiggler line (W32) at LURE (Université Paris-Sud, Orsay) [11] using an Image plate System. The data were processed using the MOSFLM program package [12]. 67,479 measurements

gave 15,336 independant reflections which were 99.3% complete at 2.2 Å resolution, of which 92.3% have intensities greater than three standard deviations. The R_{sym} defined as $R(I) = \Sigma | I - < I > | / \Sigma I$ is 3.9%.

2.2. *Rotation and translation search*

The barnase–3'GMP was solved by molecular replacement using the AMoRe package developped by J. Navaza [13,14]. The search model was the molecule C of the native barnase structure. A series of cross-rotation functions were calculated using several resolution ranges and integration radii, with the search model placed in a P1 orthogonal cell of dimensions 43 × 35 × 25 Å^3. The clearest contrast in the rotation functions was achieved using a resolution range from 3 to 15 Å and an outer integration radius of 24 Å; this gave two prominent peaks with correlation coefficients of 19.1 and 16.1% which were at least 6% above the next highest peaks.

A translation search was also carried out using the AMoRe package. One- and two-bodies translation functions were successively computed and allowed the location of the 2 molecules with correlation coefficients of 38.2%, 39.1% for each 1-body translation function and 63.3% for the 2-bodies one. Rigid body refinement [15] of the 2 molecules led to an R factor of 37.9% at 3 Å resolution. The crystal packing of the two molecules was checked on a PS300 graphic system revealing a dimer (Fig. 1) with a non-crystallographic two-fold axis, lying nearly in the (a–b) plane.

2.3. *Refinement of the structure*

Structure refinement was carried out by alternate steps of molecular dynamics and conventional positional refinement using the program X-PLOR [16]. The crystallographic calculations were based on 13419 reflections with $F_o \geq 2\sigma(F_o)$ in the resolution range 5–2.2 Å. Difference Fourier maps were displayed with the computer graphics program FRODO [17] allowing manual refitting of the model and addition of nucleotide and solvent atoms.

The final model of the barnase–3'GMP complex consists of 1768 protein atoms, 48 inhibitor atoms and 170 solvent atoms assigned as water molecules. The refinement converged to a crystallographic R factor of 0.187 in the resolution range 5–2.2 Å. The mean temperature factor for the two molecules A and B was 28 Å^2 and 39 Å^2, respectively, for protein atoms and, 42 Å^2 and 49 Å^2 for the inhibitor atoms. The r.m.s. deviation of bond lengths and angles compared with the ideal values is 0.017 Å and 3.034°, respectively.

3. RESULTS AND DISCUSSION

Both molecules of barnase in the asymmetric unit are essentially identical. Superimposing C_α atoms for the residues 3 to 108 of the 2 molecules gives an r.m.s. deviation between the corresponding atoms of 0.281 Å. The major deviations occur at the N and C termini. The latter deviation is due to an alternating conformation of the last two residues for molecule A while the first originates from different positions of Val^3. The first residues are not located, due to disorder as is the case in the crystal form of the free barnase.

The overall polypeptide fold of the two molecules in the barnase–3'GMP complex is almost identical to that of barnase molecules in previously solved structures. When they are superimposed on the C_α coordinates of the present complex, the mean r.m.s. deviations for the native form (three molecules) and for the barnase–d(GpC) complex (one molecule) are 0.434 ± 0.072 Å and 0.418 ± 0.015 Å, respectively, exhibiting no large conformational change upon binding.

The typical secondary structure elements are conserved with an r.m.s. deviation of 0.327 Å between the best defined molecule of both the 3'GMP complex and free barnase. They consist of three α-helices (α1: 6–18, α2: 26–34 and α3: 41–46) and a central, five stranded anti-parallel β-sheet (β1: 50–55, β2: 70–76, β3: 85–91, β4: 94–99, β5: 106–108). A larger deviation occurs in the loop region: residues 65–69 corresponding to an ill-defined zone in the native structure. In the complex structure, molecular packing stabilizes this loop which establishes contacts with a symmetry-related molecule involving this same region. Furthermore, residues 34 to 47 between α2 and β1 present a poor electron density and give C_α deviations in the range 0.4–0.9 Å between

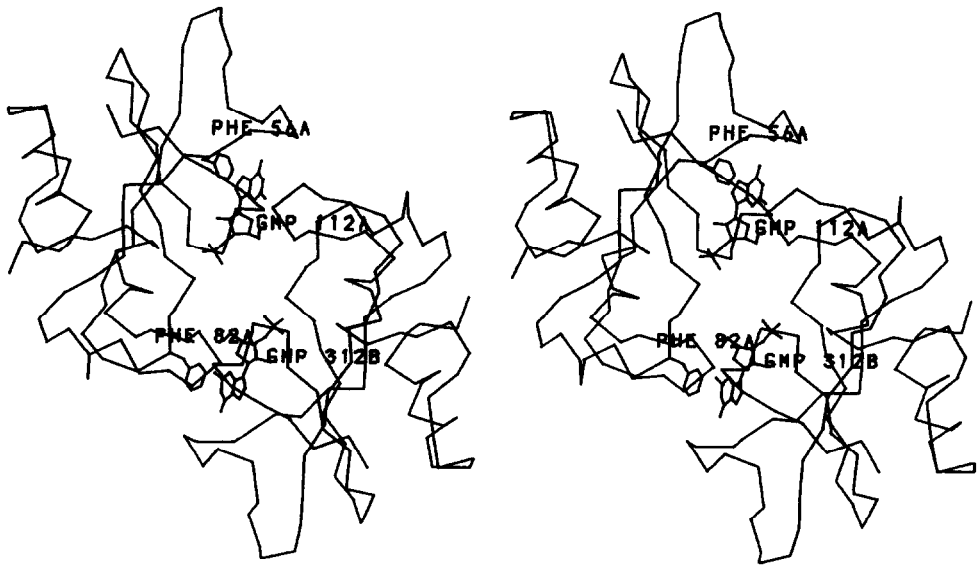

Fig. 1. Steroscopic C_α drawings of the barnase-3'GMP dimer (molecules A and B). The nucleotide molecules are shown with the residues Phe^{56} and Phe^{82} of molecule A which correspond to the primary and a possible secondary binding site on barnase respectively.

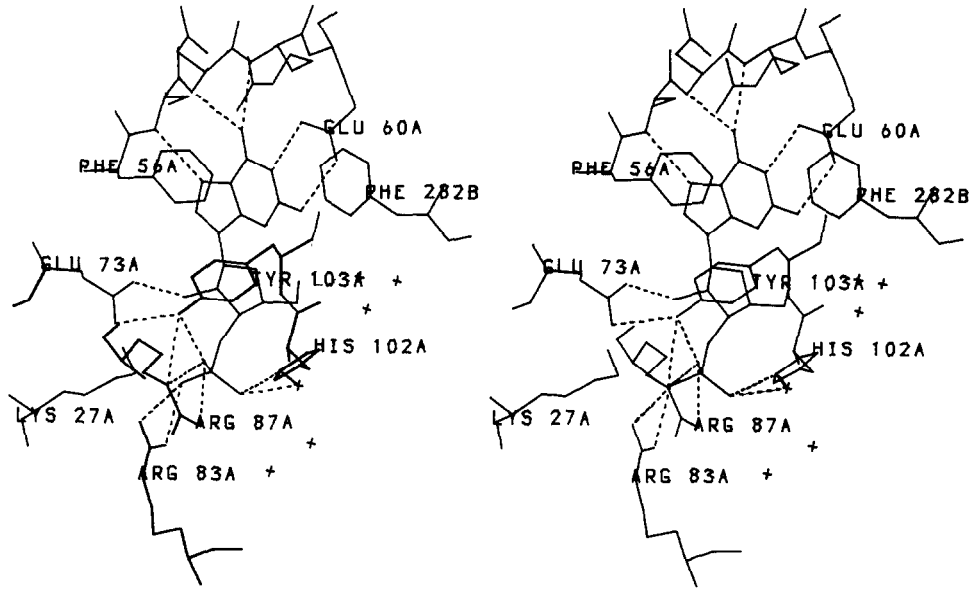

Fig. 2. Steroscopic drawings of the nucleotide binding site in the barnase–3′GMP complex. Hydrogen bonds are represented as broken lines and water molecules with crosses.

the native and complexed forms. Residues Ser^{38} and Lys^{39} of molecule B form hydrogen bonds with the sidechain of Arg^{59} of molecule A contributing to the stability of the dimer. Arg^{59} has a different sidechain conformation in molecules A and B.

The 3′GMP is well defined by the electron density allowing a clear interpretation in the two independant molecules (Fig. 2). The conformation of both nucleotides is the same in that the ribose displays a C1′ exo puckering and a glycosyl torsion angle in the syn range. This brings the Glu^{73} sidechain within a hydrogen bond distance (2.7 Å) of the 2′O of the nucleoside. That is in agreement with the classical catalytic mechanism for the first deprotonation step. The positively charged Arg^{83} and Arg^{87} form salt bridges with two oxygens of the phosphate group and the catalytic His^{102} NE2 is hydrogen bonded to the third oxygen. Tyr^{103} makes contacts with residues of the active site in a similar manner as the native barnase and forms an additional hydrogen bond with an oxygen of the phosphate group.

The Lys^{27} has been shown to play an important role in stabilizing the transition state by binding the 3′ phosphate. In the mutation Lys^{27}- > Ala of barnase, indeed, only 1.3% of the wild-type activity against RNA remains [5]. It is suggested as well that the structural equivalent of His^{40} sidechain in RNase T1 would be Lys^{27} sidechain in barnase [18]. In our structure, the Lys^{27} NZ is within close proximity to the phosphate moiety, but its orientation is such that no hydrogen bond is possible, although the distances are 3.42 Å and 3.13 Å in molecules A and B, respectively. For the latter, Lys^{27} is hydrogen bonded to the Glu^{73} OE1 via 2 water molecules which are not clearly located in the first one.

The interactions between the guanine base and the barnase are essentially identical to those observed in the binase–3′GMP complex. There are three hydrogen bonds involving the protein main chain atoms ((a) Ser^{57} NH to N7Gua (b) Asn^{58}NH to O6Gua (c) Arg^{59} NH to O6Gua) and two hydrogen bonds involving the sidechain of Glu^{60} ((d) Glu^{60} OE1 to HN1Gua (e) Glu^{60} OE2 to HN2Gua). Furthermore, hydrophobic interactions are observed, involving the guanine base and enzymatic aromatic sidechains, one of them being the Phe^{56}, as it is shown in the Fig. 2. The base is also stabilized by the Phe^{82} sidechain belonging to the other molecule of the dimer (282B residue). In solution, this role is supposed to be taken by Arg^{59} [19], as is the case in the crystal structure of the binase 3′GMP complex [6]. In our crystal structure, this residue does not establish any Van der Waals contact with the base. It is in the same position as the free form for molecule A, but, for molecule B, it is displaced making contacts with a symmetry related molecule.

The existence in barnase of a series of subsites for binding its substrate has been shown by kinetic studies [5,20]. Secondary base subsites could explain both the higher activity towards long RNA compared to Rnase T1 and the relaxation of guanyl-specificity when hydrolysing RNA rather than dinucleotides. A molecular modelling study of barnase with a ribonucleotide: 5′3′ (ApAp2′3′GMPApAp) [18] confirmed these results indicating that base subsites, other than the primary guanine recognition site could extend over the active site, Phe^{82} stacking against the last Adenine base. As observed in our crystal structure (Fig. 1), the guanine in molecule B is stacked against the Phe^{82} and takes the space occupied by the nucleotide base at the 3′ end of

the pentanucleotide. This lends some support to the hypothesis that the Phe^{82} is a good candidate for a secondary base subsite.

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