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Structure and functional studies of the ribonuclease binase Glu43Ala/Phe81Ala mutant

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Ribonuclease from *Bacillus intermedius* (binase) is a small basic protein with antitumour activity. The three-dimensional structure of the binase mutant form Glu43Ala/Phe81Ala was determined at 1.98 Å resolution and its functional properties, such as the kinetic parameters characterizing the hydrolysis of polyinosinic acid and cytotoxicity towards Kasumi-1 cells, were investigated. In all crystal structures of binase studied previously the characteristic dimer is present, with the active site of one subunit being blocked owing to interactions within the dimer. In contrast to this, the new mutant form is not dimeric in the crystal. The catalytic efficiency of the mutant form is increased 1.7-fold and its cytotoxic properties are enhanced compared with the wild-type enzyme.

1. Introduction

Extracellular ribonuclease from the bacterium *Bacillus intermedius* (binase; EC 3.1.27.3) consists of 109 amino-acid residues. Binase is a highly cationic guanyl-specific RNase that catalyses RNA cleavage without the need for metal ions or cofactors. The cytotoxic properties of binase towards tumour cells have attracted considerable attention from researchers (Makarov *et al.*, 2008). It has been shown that binase selectively inhibits the growth of malignant cells, triggering an apoptotic response (Mitkevich *et al.*, 2010, 2011), and that the cytotoxicity of RNases is associated with their catalytic efficiency (Makarov *et al.*, 2008). Hence, modifications of binase that result in an enhancement of its catalytic properties may increase the efficiency of its cytotoxic action against tumour cells.

Binase belongs to the microbial ribonucleases (Hartley, 1980; Hill *et al.*, 1983). Within this family, three-dimensional structures of ribonucleases from bacteria, actinomycetes and fungi have been determined (Heinemann *et al.*, 2003). Previously, the following three-dimensional structures of binase have been determined: the free enzyme at 1.65 Å resolution, the enzyme in complex with sulfate at 2.0 Å resolution, the enzyme in complex with guanosine 3′-phosphate at 2.0 Å resolution (Polyakov *et al.*, 2002) and two structures of the Trp34Phe mutant at 1.1 and 1.7 Å resolution (Polyakov *et al.*, 2010). In all of the crystal structures determined previously, binase exists in a dimeric form. In these dimers, only one of the two enzyme molecules can bind a substrate molecule. The active site of the other molecule is blocked owing to intermolecular interactions. The existence of such dimers in crystals grown under various conditions and belonging to different space groups suggests that this dimeric structure might also be formed in solution. It has been hypothesized (Polyakov *et al.*, 2010) that the Glu43Ala/Phe81Ala mutant form of binase would not form dimers,

Table 1
List of the oligonucleotides used for the PCR mutagenesis.

Primers	Sequence
D43	5′-AATCTAGCAGCGGTTGCCCC-3′
R43	5′-GGGGCAACCGCTGCTAGATT-3′
D81	5′-GTCTCTGGCGCCCGAAATGC-3′
R81	5′-GCATTTCGGGCGCCAGAGAC-3′
1732-19pr	5′-GTTGCATGTACTAAGGAGG-3′
SP6	5′-GATTTAGGTGACACTATA-3′

which should lead to an increase in its enzymatic activity and cytotoxicity.

In the present study, we prepared the Glu43Ala/Phe81Ala mutant of binase, determined its structure by X-ray diffraction and studied its catalytic and cytotoxic properties. It was shown that mutation of the residues Glu43 and Phe81 that play key roles in formation of the dimeric structure of binase results in the mutant form losing the ability to form dimers. The mutant form showed a 1.7-fold higher enzymatic activity towards polyinosinic acid [poly(I)] compared with the wild-type enzyme, and the cytotoxic activity of the mutant towards acute myeloid leukaemia Kasumi-1 cells was 23% higher.

2. Materials and methods

2.1. Introduction of the Glu43Ala and Phe81Ala mutations into the RNase binase gene

The construction of the plasmid pPBi for expression of the RNase binase gene in *Escherichia coli* has been described previously (Shulga *et al.*, 1994). The two mutations Glu43Ala and Phe81Ala were introduced into the binase gene by consecutive PCRs. Firstly, the regions of the gene that flank the Glu43Ala mutation site were accumulated by PCR. To perform this, the two complementary mutagenic primers D43 and R43 as well as the additional primers 1732-19pr and SP6, which anneal at the 5′ and 3′ ends of the expression cassette, respectively, were used (Table 1). One of the reaction mixtures was supplemented with the D43 and SP6 primers, while the R43 and 1732-19pr primers were added to the other reaction mixture. The pPBi plasmid DNA was used as a template. PCR products were fractionated by electrophoresis in agarose gel and the DNA fragments were then isolated from the agarose gel using a MinElute Gel Extraction Kit (Qiagen, USA). Two fragments were recombined by overlapping sites by PCR using the 1732-19pr and SP6 primers. The resultant DNA fragment containing the mutant Glu43Ala binase gene was isolated from the agarose gel using a MinElute PCR Purification Kit (Qiagen, USA) and used as a DNA template in the second round of PCR mutagenesis, which was carried out according to the same scheme as before. The regions of the gene that flank the Phe81Ala mutation site were elaborated by PCRs using the primer pairs D81/SP6 and R81/1732-19pr (Table 1). The isolated overlapping fragments were recombined by PCR using the primers SP6 and 1732-19pr. The resultant DNA fragment, which contained both desired mutations, was cleaved by the *Bam*HI and *Hin*dIII restriction enzymes. The products of hydrolysis were fractionated by electrophoresis in agarose gel. DNA fragments of the desired length were isolated from the gel using a MinElute Gel Extraction Kit (Qiagen) and cloned into the pPBa plasmid between *Bam*HI and *Hin*dIII restriction sites. This plasmid is similar to pPBi but was designed for the expression of the RNase barnase, which is related to binase. The clones containing the binase gene were selected by restriction analysis with *Eco*RV as the binase gene contains a unique site for this enzyme, unlike the barnase gene. The presence of the Glu43Ala and Phe81Ala mutations in the binase gene was confirmed by DNA sequencing using ABI PRISM Big Dye Terminator v.3.1 reagents on an ABI PRISM 3100 Avant automatic sequencer. To be more accurate, the DNA sequencing was carried out by two strands using the 1732-19pr and SP6 primers.

2.2. Cultivation of the strains producing RNase binase and its Glu43Ala/Phe81Ala mutant form

Cultivation of the strains that produce RNase binase and its Glu43Ala/Phe81Ala mutant form was carried out in a Biostat B2 laboratory fermenter (Sartorius BBI, Germany) with a total volume of 2.5 l. To prepare for inoculation, 40 ml YT medium (8 g l^{-1} Bacto Tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl) was poured into a 300 ml flask and ampicillin was added to 300 µg ml^{-1}. An approximately 100 µl portion of the producer-strain glycerol stock was thawed at 277 K and added to the medium. The inoculation material was incubated at 301 K overnight with constant shaking at 250 rev min^{-1}. The cultivation in the fermenter was carried out in TB medium (12 g l^{-1} Bacto Tryptone, 24 g l^{-1} yeast extract, 5 g l^{-1} glycerol, 2.31 g l^{-1} KH_2PO_4, 12.54 g l^{-1} K_2HPO_4) supplemented with 300 µg ml^{-1} ampicillin in a total volume of 1.7 l. During cultivation, the following conditions were maintained: the temperature before and after induction was 301 and 310 K, respectively, air delivery was at approximately 2 l min^{-1}, the oxygen partial pressure (p_{O_2}) was at least 30% of the saturation threshold and the stirring rate was adjusted using a regulatory device according to the p_{O_2} (500–1000 rev min^{-1}). Recombinant protein synthesis was induced by increasing the incubation temperature to 310 K when the optical density of the suspension reached 1.5–2.0 units, and incubation then proceeded for a further 20 h. When the incubation was finished, the samples were centrifuged in 50 ml tubes at 9000*g* and 277 K for 10 min. The obtained supernatant was collected in a separate flask and used for protein isolation.

2.3. Purification and isolation of RNase binase and its Glu43Ala/Phe81Ala mutant form

The supernatant obtained after cultivation of the producer strains was supplemented with glacial acetic acid to bring the pH to 4.5, mixed for 60 min at 277 K and centrifuged under the previously described conditions. The obtained supernatant was diluted fivefold with Milli-Q water and loaded onto a P-cellulose column (Sigma). The column was washed and the protein was eluted with 50 m*M* Na_2HPO_4, 1.7 *M* ammonium sulfate pH 7.0. The obtained eluate was concentrated using an Amicon Ultra-15 with molecular-weight cutoff 3000

Table 2
Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P4_3$	$P4_322$
Unit-cell parameters (Å)	a = 50.36, c = 196.12	a = 50.36, c = 196.12
Resolution (Å)	50–1.89 (2.01–1.89)	50–1.89 (2.01–1.89)
No. of observed reflections	124585	124869
No. of unique reflections	37967	20881
Completeness (%)	97.7 (89.8)	98.2 (91.8)
$\langle I/\sigma(I)\rangle$	12.1 (2.5)	16.1 (3.3)
$R_{\rm meas}$ (%)	9.1 (59.8)	9.2 (59.8)
B factor from Wilson plot (Å^2)	25.3	25.3
R factor (%)	17.7	20.1
$R_{\rm free}$ (%)	20.5	26.2
Twinning fraction	0.525/0.475	
No. of protein atoms	3424	1712
No. of waters	264	161
R.m.s.d. from ideal values		
Bond lengths (Å)	0.016	0.021
Bond angles (°)	1.8	1.8

(Millipore, USA). The concentrated sample was supplemented with 3.4 *M* ammonium sulfate in a 3:2 ratio and loaded onto Phenyl Sepharose FF (GE Healthcare, USA). The protein was eluted from the column with a decreasing gradient of 2.0–0 *M* ammonium sulfate and transferred into 20 m*M* sodium acetate pH 4.5 by dialysis. Subsequent fractionation of proteins was carried out on a Mono S HR 10/100 column (GE Healthcare, USA) according to a standard protocol. The purified protein was dialyzed against 0.01 m*M* EDTA pH 7.0 and then lyophilized.

2.4. Measurement of the kinetic parameters of poly(I) hydrolysis by binase and its Glu43Ala/Phe81Ala mutant

Steady-state kinetic studies of ribonucleases catalyzing the hydrolysis of poly(I) were performed at 298 K and pH 6.2. The buffer used was 0.1 *M* sodium citrate. The concentrations of binase and its mutant form were determined spectrophotometrically using ε_{280} = 27 411 M^{-1} cm^{-1} (Mitkevich *et al.*, 2003). The poly(I) concentration was measured using ε_{248} = 10 000 M^{-1} cm^{-1} at pH 7.8 (Chamberlin & Patterson, 1965). The initial reaction rates were determined by recording the changes in absorption at 248 nm using the difference molar extinction coefficient value of 1330 M^{-1} cm^{-1} (Yakovlev *et al.*, 1992). To obtain the kinetic parameters, the initial rates were measured for 7–8 substrate concentrations. The rate of poly(I) hydrolysis changed hyperbolically as the substrate concentration increased. The values of $k_{\rm cat}$ and $K_{\rm M}$ were determined using the nonlinear regression method.

2.5. Determination of cell viability

Human acute myeloid leukaemia Kasumi-1 cells obtained from the Heinrich-Pette Institute Leibniz Institute for Experimental Virology, Hamburg, Germany were used. Kasumi-1 cells were grown on RPMI-1640 medium containing 20% FCS, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 1 m*M* sodium pyruvate at 310 K in a humid atmosphere with 5% CO_2. Cellular viability was assessed using a WST-1-based test (Roche Diagnostics) as described previously (Mitkevich *et al.*, 2011). The cells were plated onto 96-well plates (3×10^4 cells per well) and cultured for 24 h at 310 K. The cells were then treated with binase. After 72 h of binase treatment the cells were incubated with the WST-1 reagent for 60 min at 310 K. The absorbance of the samples was measured using an Anthos 2020 microplate reader (Anthos Labtech Instruments GmbH) at 450 nm. The reference wavelength was 620 nm. A mixture of cell-free medium and the WST-1 reagent was used as a background control. The viability of the untreated cells was taken as 100%. All reported values are the means of three independent measurements with standard deviations.

2.6. Determination of the denaturation temperature

Denaturation temperature ($T_{\rm d}$) measurements of binase and its mutant form were carried out using a VP-DSC microcalorimeter (MicroCal, USA) at a heating rate of 1 K min^{-1} for 0.5 mg ml^{-1} protein solutions as described elsewhere (Mitkevich *et al.*, 2003). To analyze the excess heat capacity, the *Origin* DSC software package was used. The buffer used was 0.1 *M* sodium citrate pH 6.2.

2.7. Crystallization

Screening for crystallization conditions of the Glu43Ala/Phe81Ala mutant form of binase was performed using crystallization screening kits from Hampton Research, USA. Crystallization was carried out by the hanging-drop vapour-diffusion method using lyophilized protein dissolved in Milli-Q water (at a concentration of 10 mg ml^{-1}) at room temperature. Crystals were obtained using a reservoir solution consisting of 0.1 *M* citric acid pH 3.5, 3 *M* sodium chloride.

2.8. Data collection and processing

An X-ray data set was collected to 1.9 Å resolution from one crystal with dimensions 0.3 × 0.25 × 0.1 mm on the K4.4 beamline at the Kurchatov Synchrotron Radiation Source, Moscow at 100 K. Before X-ray data collection, the crystal was placed in a cryosolution consisting of 0.1 *M* citric acid pH 3.5, 3 *M* sodium chloride, 25% glycerol for a few seconds. The crystal belonged to the tetragonal system, with unit-cell parameters a = 50.36, c = 196.12 Å. X-ray diffraction data were collected to 1.89 Å resolution. The systematic absences indicated four possible space groups: $P4_1$, $P4_3$, $P4_122$ or $P4_322$. The X-ray data were processed using the *XDS* program (Kabsch, 2010). The X-ray data-collection statistics are given in Table 2.

2.9. Structure solution and refinement

The *CCP*4 software suite was used for structure solution (Winn *et al.*, 2011). Initially, the structure of the mutant form (Glu43Ala/Phe81Ala) of binase was solved by the molecular-replacement method in space group $P4_322$ using the structure of native binase (Polyakov *et al.*, 2002; PDB entry 1gou) as the starting model. Structure solution was performed with the *MOLREP* program (Vagin & Teplyakov, 2010) using a radius of integration of 25 Å and diffraction data to 4.0 Å resolution.

The resulting model contained two protein molecules in the asymmetric unit. Structure refinement was performed with the *REFMAC*5 program (Murshudov *et al.*, 2011) to an *R* factor of 20.1% (R_{free} = 26.2%) based on data at 1.89 Å resolution. Taking the rather high values of the *R* factors into account, we performed subsequent calculations in the lower symmetry space group $P4_3$. For this purpose, the coordinates of two additional protein molecules were generated from the coordinates of two crystallographically independent molecules refined in space group $P4_322$ by noncrystallographic axes which coincide with the crystallographic twofold axes perpendicular to the *c* axis in space group $P4_322$. The statistical properties of the diffraction intensities are indicative of possible twinning (Fig. 1). This made it possible to refine the model consisting of four independent molecules per asymmetric unit using the *REFMAC*5 program, taking twinning into account. The twin refinement converged to an *R* factor of 17.7% (R_{free} = 20.5%). In the course of the refinement, we performed repeated manual corrections of the model using *Coot* (Emsley & Cowtan, 2004). The structure-refinement statistics are given in Table 2. A fragment of the electron density of the regions containing the mutations is shown in Fig. 2. The coordinates and structure factors of the binase Glu43Ala/Phe81Ala mutant form have been deposited in the Protein Data Bank (PDB entry 4haa).

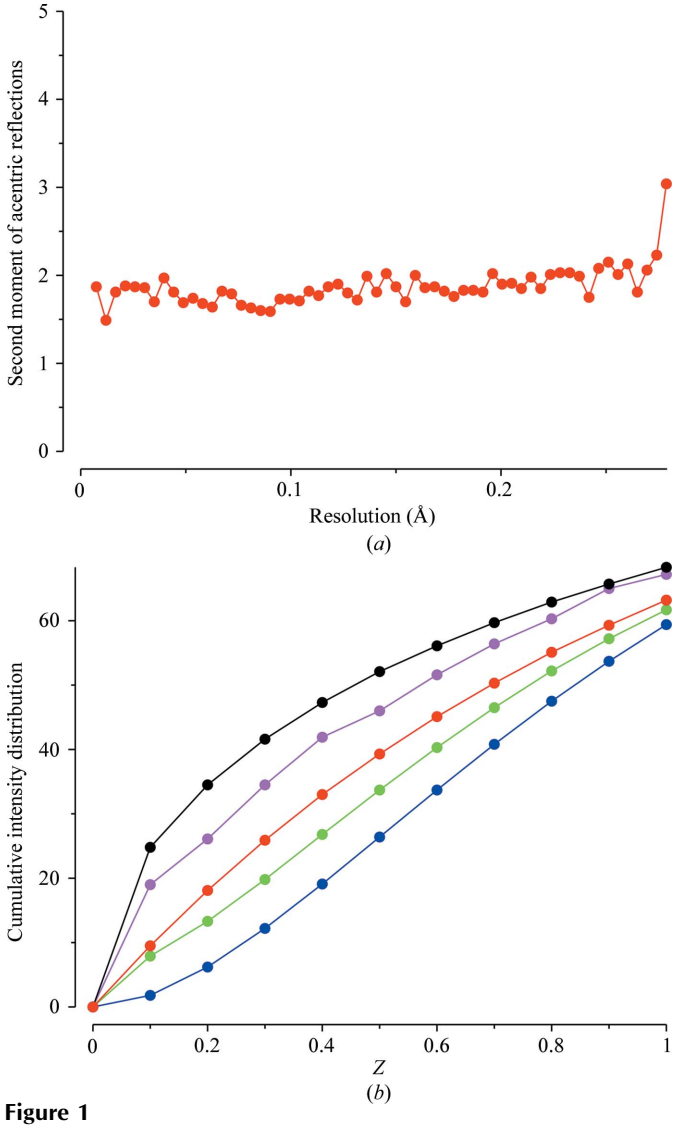

Figure 1
(*a*) The experimental second moment of *Z* for acentric reflections *versus* resolution. The expected value for an untwinned case is 2 and that for a perfect twin is 1.5. (*b*) The cumulative distribution of *Z* for observed centric reflections (magenta), observed acentric reflections (green), theoretical centric reflections in the untwinned case (black), theoretical centric reflections in the case of perfect hemihedral twinning and acentric reflections in the untwinned case (red) and theoretical acentric reflections in the case of perfect hemihedral twinning (blue).

3. Results and discussion

All four crystallographically independent molecules of the binase mutant form have very similar structures and they are also similar to previously determined structures of binase molecules. The r.m.s.d.s for the superimposed C^{α} atoms are in the range 0.3–0.5 Å. Substantial differences were found in the molecular packing of the mutant form compared with previously determined crystal structures of the enzyme.

In all crystal structures of binase determined previously the strongest intermolecular contacts are identical. Crystals grown under different conditions and belonging to different space groups contained dimers of the same type (Fig. 3). In these dimers, the active site of one molecule is blocked owing to the following interactions within the dimer. Firstly, the side chain of Phe81 of the second molecule in the dimer is involved in a stacking interaction with the side chain of the catalytic residue Tyr102 of the first molecule and partially overlaps the guanyl-binding region of the first molecule. Secondly, the side chain of Arg58 of the first molecule (which is involved in a stacking interaction with the guanyl in the structure of binase in complex with substrate) points outwards from the active site and forms a salt bridge to the Glu43 side chain of the second molecule. This dimeric structure is characterized by an accessible surface area (interface area) of about 400 $Å^2$. Although according to *PISA* predictions this dimer is unstable in solution, it cannot be ruled out that under some conditions a substantial portion of binase molecules may exist as such

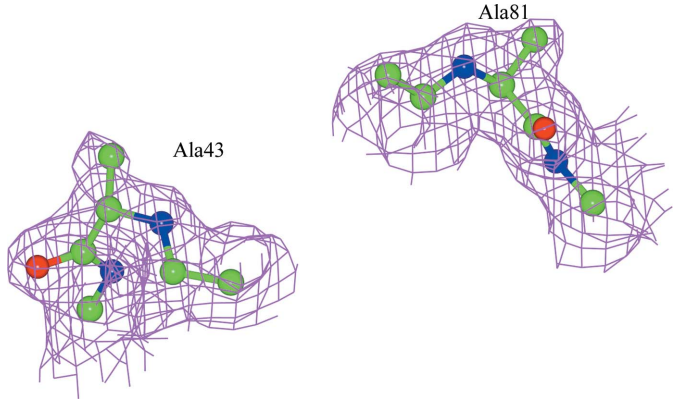

Figure 2
Fragment of electron density ($2F_o - F_c$, 1σ) for Ala43 and Ala81 in the structure of the Glu43Ala/Phe81Ala mutant form of binase.

Table 3
Functional properties of binase and its Glu43Ala/Phe81Ala mutant form: kinetic parameters characterizing the cleavage of poly(I), the temperature of denaturation and cytotoxicity towards Kasumi-1 cells.

RNase	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}\,s^{-1}$)	T_d (K)	IC_{50} (μM)
Binase	188 ± 16	77 ± 6	2441558	56.5 ± 0.1	0.56 ± 0.06†
Mutant	286 ± 22	70 ± 6	4085714	51.9 ± 0.1	0.43 ± 0.05

† From Mitkevich *et al.* (2011).

dimers in solution. In the crystal structure of the Glu43Ala/Phe81Ala mutant form the dimer that is responsible for self-inhibition is absent. It should be noted that binase dimers have not been detected in solution previously (Makarov *et al.*, 1993). We performed additional experiments in order to identify the dimeric form of binase in solution by gel filtration and fluorescence polarization (concentration range 0.1–5 mg ml^{-1}). The presence of the dimeric form was not detected in these experiments.

Table 3 provides functional characteristics of binase and its Glu43Ala/Phe81Ala mutant form: kinetic parameters characterizing the hydrolysis of poly(I), cytotoxicity towards Kasumi-1 cells and denaturation temperature. The efficiency of the hydrolysis (k_{cat}/K_m) of the model substrate poly(I) by the Glu43Ala/Phe81Ala mutant form of binase is almost 60% higher than that by the native protein (Table 3). These enzymes have similar affinities for substrate (K_m) and the observed increase in the efficiency is achieved owing to an increase in the catalytic constant (k_{cat}).

The higher catalytic activity of the binase mutant form compared with the wild-type enzyme may be explained by the inability of the mutant enzyme to form the self-inhibited dimeric structure or by the lower stability of this dimer. Previously, an inverse linear dependence between enzymatic activity and thermal stability has been shown for binase and its mutant forms (Schulga *et al.*, 1998). Hence, the observed decreased stability of the Glu43Ala/Phe81Ala mutant enzyme may be considered as a possible explanation for the higher catalytic activity of this form compared with the wild-type form (Table 3).

The enhanced efficiency of mutant binase correlates with its higher cytotoxicity. The value of IC_{50} for mutant binase (the concentration of the protein at which 50% of cells survive) estimated for Kasumi-1 cells is 23% lower than that for the native enzyme (Table 3). The weaker increase in the cytotoxicity of binase compared with the increase in its catalytic activity may be associated with the fact that the less stable protein is more sensitive to the effect of intracellular proteases (Klink & Raines, 2000).

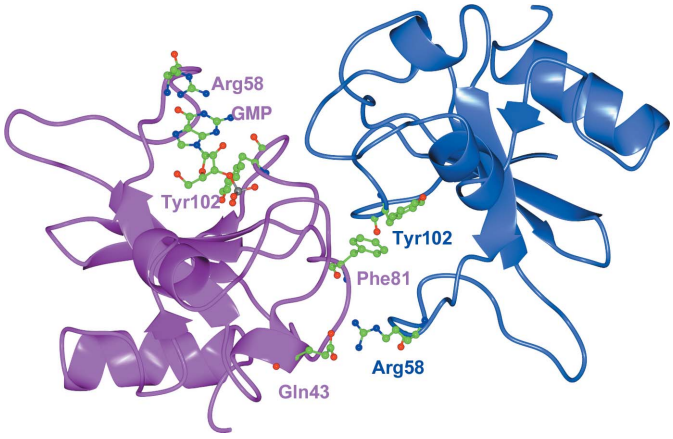

Figure 3
The dimeric structure formed by two crystallographically independent molecules (*A* is coloured magenta and *B* is coloured blue) in the structure of the complex of binase with 3′-GMP (PDB entry 1goy; Polyakov *et al.*, 2002). The 3′-GMP molecule only binds to binase molecule *A*. In molecule *B* the potential 3′-GMP-binding region is occupied by the Phe81 side chain of molecule *A*. The Arg58 side chain of molecule *A* takes part in binding of 3′-GMP, and Arg58 of molecule *B* forms a salt bridge to the Glu43 side chain of molecule *A*.

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