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Site-directed mutagenesis of the base recognition loop of ribonuclease from *Bacillus intermedius* (binase)

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Abstract **Members of the microbial guanyl-specific ribonuclease family show a high level of structural homology. The structural basis for guanyl base binding by microbial ribonucleases has been established for all members of the family and the existence of a guanine recognition loop was shown. However, bacillar RNases such as binase and barnase show far less specificity towards the guanyl base in hydrolysing oligonucleotides composed of more than 4 or 5 nucleotides. Using site-directed mutagenesis we introduced a number of amino acid substitutions into the base recognition loop of binase. The donor sequence originated from the guanyl specific ribonuclease Sa. Two single, two double and one triple (entire loop substitution) mutants were constructed and overproduced in *E. coli*. The kinetic properties of the mutant variants are different from the wild-type protein. Amino acid substitutions R61V, G60S, S56Q/R61V, G60S/R61V show 3-fold, 7-fold, 4-fold and 12-fold increased guanyl specificity respectively. However, all mutants retain the ability to catalyse the hydrolysis of a poly(A) substrate.**

Key words: Ribonuclease; Binase; RNase Sa; Site-directed mutagenesis

1. Introduction

Microbial cyclizing ribonucleases have been studied over the past few years using a variety of different methods [1–4]. The enzymes within this family are specific for guanine on the 3′-side of the phosphodiester bond that is cleaved. Structural analyses identify a common recognition loop responsible for guanyl base binding in all microbial ribonucleases [4–7]. Binase is an extracellular ribonuclease produced by *Bacillus intermedius*. It is formed by a single polypeptide chain consisting of 109 amino acid residues.

The structures of several guanyl-specific ribonucleases and their complexes with 3′-GMP have been determined to high resolution [1,8,9]. The refinement of native binase alone and in complex with 3′-GMP has been carried out against data extending to 2 Å resolution (K. Polyakov, unpublished data). It was shown that the binding of guanine occurs in a base recognition loop formed by amino acid residues 55–61. Comparison of the structures and amino acid sequences of guanyl specific RNases Bi, Pb1, Sa and C2 [2,6,10] reveals a common spacial organisation together with a number of certain differences in amino acid sequences of the base recognition loop (Fig. 1).

Bacillar RNases such as binase (RNase Bi) or barnase (RNase Ba), that exibit 80% amino acid sequence homology [10], show guanyl specificity toward short fragments of RNA or cyclophosphates. However, they are far less specific in hydrolysing oligonucleotides composed of more than 4 or 5 nucleotides. In comparison, RNase T1-like ribonucleases recognise the guanyl base with high efficiency in RNA substrates of any length [5]. The high guanine specificity of RNase Sa [7] and other RNase T1-like ribonucleases was assumed to originate from the oriented interactions between a nucleobase and its protein recognition site [6]. However, previous experimental data can not explain the difference in substrate specificity between binase and RNase Sa [11–14]. The purpose of this study was to see whether a substrate recognition loop which has been shown to be involved in guanyl binding, is responsible for the different substrate specificities between the guanyl specific ribonuclease Sa and not so specific binase. Using site-directed mutagenesis, a number of RNase Bi mutants were produced, which reshape the base recognition loop to result in that of RNase Sa. The catalytic properties of the altered enzymes towards poly(I) and poly(A) substrates were studied.

2. Materials and methods

Chemically pure reagents were obtained from Merck and Sigma. Mobile phases were prepared with HPLC-grade acetonitrile (Fluka AG) as a strong solvent. The aqueous phases were prepared using Milli-Q water (Millipore). All components for growth media were from Difco Laboratories (Detroit, MI, USA). *E. coli* strains JM109 and XL-1 Blue MRF′ (Stratagene) were used for DNA manipulations and for expression of binase and its mutants.

2.1. Mutagenesis and production of the proteins

The binase variants were obtained by site-directed mutagenesis according to the manufacturer's specifications (Promega). The DNA fragment of vector pBIT36 [15], containing binase and barstar structural genes was subcloned into the pSELECT-1 vector. The following primers were used to introduce single amino acid mutations within RNase Bi: G60S 5′-C TCT AAC CGG GAG TCG CGA CTT CCT TCA GCA AGC-3′; R61V 5′-C CGG GAG GGA GTA CTT CCT TCA GC-3′; S56Q 5′-GGA GAT GTT TTC CAA AAC CGG GAG-3′ and for the introduction of a double amino acid substitution: G60S/R61V 5′-CT AAC CGG GAG TCA GTA CTT CCT TCA GCA AG-3′

All mutations within the RNase Bi gene were verified by DNA sequencing. Recombinant binase and the mutant variants were subcloned into the vector pBIT36. The binase gene and mutant constructs in pBIT36 were also sequenced after protein expression. A previously described system for barnase and binase expression [15,16], which includes the P_R promoter of *E. coli* phage λ under cI_{857} regulation and co-expression of RNAse and its specific protein inhibitor barstar [17], was used to produce the binase mutants in *E. coli*. Fusion to the

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*pho*A signal peptide directed the expressed ribonucleases to the cell periplasm. The recombinant RNase Bi and its mutant derivatives were purified by reverse phase chromatography [18] to approximately 99% homogenity. Protein yields ranged between 10 mg and 50 mg of pure protein/l cell culture for the different RNase Bi mutants.

2.2. RNase activity assay

RNase activity was assayed at pH 8.0 in 120 μl of a buffer containing 0.1 M Tris-HCl, 0.1 M NaCl and 1.6 mg/ml of yeast ribonucleic acid (Serva). After incubation at 37°C for 15 min, 300 μl of 2-propanol was added with vigorous mixing. The samples were incubated for 20 min at −20°C and then centrifuged for 10 min at $14000 \times g$. The supernatant was removed and diluted a hundred-fold in water and the absorbance at 260 nm was measured. The absorbance of the supernatant was a linear function of added enzyme up to an A_{260} of at least 1.5. One unit of ribonuclease activity in 120 μl reaction volume will produce alcohol-soluble oligonucleotides equivalent to a ΔA_{260} of 1.0 in 30 min at pH 7.5 and 37°C.

2.3. Kinetic measurements

Experiments were performed with an Uvicon 1800 Spectrophotometer (Contron Instruments). Thermostated cells (25°C) having an optical path length of 10 mm were used in kinetic measurements. The concentrations of binase, its mutant derivatives, and the nucleic acid substrates were determined spectrophotometrically. The following extinction coefficients were used: binase and its mutants ε_{280} = 22 500 M^{-1} cm^{-1} [19]; poly(I) ε_{248} = 10 000 M^{-1} cm^{-1} at pH 7.8 [20]; poly(A) ε_{257} = 10 000 M^{-1} cm^{-1} at pH 7.5 [21]. For determination of the initial velocities of substrate cleavage the following difference molar coefficients were used at pH 6.2 and 25°C: poly(I) $\Delta\varepsilon_{248}$ = 1330 M^{-1} cm [22], poly(A) $\Delta\varepsilon_{260}$ = 5000 M^{-1} cm [22]. The buffer conditions used for kinetic measurements contained 0.1 M sodium citrate and 0.1 M NaCl. The pH was adjusted by the addition of NaOH. To determine kinetic parameters for a given enzyme and substrate, initial rates were measured for six different concentrations of substrate. The kinetic constants were calculated from Lineweaver-Burk plots using the program ENZFITTER [23].

3. Results and discussion

A comparison of the amino acid sequences of the base recognition loops within binase and ribonuclease Sa is shown in Fig. 1. One can see the differences in amino acid identity at positions 56, 60 and 61 for binase. However, superposition of the substrate recognition loops of several guanyl specific ribonucleases [6] shows that the overall protein fold is highly conserved and does not provide evidence for differences in substrate specificity. Previous analyses have not clearly identified the determinants of substrate specificity [24–27]. In the present study, the properties of site-directed mutants of binase containing substitutions in the base recognition loop were determined. Five mutant proteins were expressed and purified: G60S, R61V, S56Q/R61V, G60S/R61V and S56Q/G60S/R61V. Kinetic parameters for the cleavage of poly(I) and poly(A) substrates by binase and its mutants and the relative activity with yeast tRNA substrate are presented in Tables 1 and 2, respectively. One can see the difference in catalytic parameters of the mutant variants relative to wild-type binase. For the single substitution mutant R61V the catalytic activity is greatly increased with the poly(I) substrate. In contrast, the kinetic parameters for the poly(A) substrate are similar to those of the wild-type binase. Due to the higher K_M for the poly(I) substrate, the final calculated (k_{cat}/K_M)poly(I))/(k_{cat}/K_M)poly(A) ratio, (poly(I)/poly(A)), is just 3-fold higher than that of wild-type binase. Another single substitution mutant, G60S, shows marked decrease in activity for both substrates. However, the reduced activity is more pronounced with the poly(A) substrate than with the poly(I) substrate. Interestingly, the Michaelis constants for binding to both substrates do not change as significantly as the k_{cat}. The result is a poly(I)/poly(A) ratio 7-fold greater than for wild-type binase.

Enzyme	amino acid sequence of recognition loop	I/A ratio
	55 56 57 58 59 60 61	
binase	F-S-N-R-E-G-R	5.8
barnase	F-S-N-R-E-G-K	440
RNase Sa	F-Q-N-R-E-S-V	4.8×10^6
RNase T1	Y-N-N-Y-E-G-F	2.9×10^5
RNase Pb1	Y-H-N-Y-E-G-F	1.06×10^6

Fig. 1. The amino acid sequence comparison of the base recognition loops of binase, barnase, ribonuclease Sa, T1 and Pb1. The numbering is according to the binase amino acid sequence. The I/A ratio is calculated as (k_{cat}/K_M) poly(I)/(k_{cat}/K_M) poly(A).

The double substitution mutant S56Q/R61V displays a similar catalytic activity to that of wild-type binase. This double mutant is 2-fold more active on the poly(I) substrate and the poly(I)/poly(A) ratio is only 4-fold higher. This similarity in activity may result from compensatory changes produced by the individual amino acid mutations.

The double mutant G60S/R61V exibits similar K_M values with the poly(I) and poly(A) substrates and a similar k_{cat} value with the poly(I) substrate to those of wild-type binase. However, a 20-fold lower activity is seen with the poly(A)

Table 1
Kinetic parameters for the cleavage of polynucleotides by wild-type binase, mutant binase variants and RNase Sa. The I/A ratio is calculated as (k_{cat}/K_M) poly(I) (k_{cat}/K_M) poly(A)

Protein	Substrate	K_M(μM)	k_{cat}(s^{-1})	k_{cat}/K_M(s^{-1} M^{-1})	I/Aratio
binase wt	poly(A)	150	28	1.88×10^5	
	poly(I)	29	30	1.1×10^6	5.8
Bi R61V	poly(A)	150	15	1.0×10^5	
	poly(I)	110	203	1.8×10^6	18
Bi G60S	poly(A)	230	0.71	3.13×10^3	
	poly(I)	56	7.2	1.3×10^5	45
Bi S56Q,R61V	poly(A)	140	23.7	1.64×10^5	
	poly(I)	150	66	4.4×10^6	27
Bi G60S,R61V	poly (A)	130	1.55	1.2×10^4	
	poly (I)	46	40	8.7×10^5	72
Bi S56Q,G60S, R61V	poly (A)	184	0.54	2.9×10^3	
	poly (I)	86	7	8.1×10^4	27
Sa, wt [22]	poly(A)	2500	1.5×10^{-3}	0.6	
	poly(I)	133	385	2.9×10^6	4.8×10^6

substrate, resulting in a poly(I)/poly(A) ratio 12-fold greater than wild-type binase.

Finally, the triple mutant S56Q/G60S/R61V which replaces the entire recognition loop of binase with that of RNase Sa, was assayed for enzymatic activity. The resulting mutant was less active towards both nucleic acid substrates and retained substrate binding abilities similar to wild-type binase. The poly(I)/poly(A) ratio was slightly shifted in favour of poly(I) and was only 4.5-fold greater than for binase.

The greatest effect on binase activity is due to the mutations Rr61V and G60S. Both of these amino acids are located at the end of the substrate recognition loop and are not directly involved in enzymatic catalysis or substrate binding (Figs. 2 and 3). An arginine to valine substitution at position 61 in binase might therefore be expected to have little or no effect on the catalytic activity. However, the k_{cat} for this mutant is 7-fold greater than for wild-type binase. This suggests that the effects of the arginine 61 substitution may be due to loss of hydrogen bonds between Arg61(NE) and Ala103(O) and Arg61(NH1) and Tyr102(O) (Fig. 3), which are present in the complex of wild-type binase and 3′-GMP, but not in free binase (K. Polyakov, unpublished data). These bonds could stabilize the wild-type conformation of the active centre. The removal of these hydrogen bonds in the Arg61Val mutant may induce a shift in the position of tyrosine 102 and the adjacent catalytic residue, histidine 101, resulting in a 7-fold increase in the k_{cat} but a nearly 3-fold lower K_M in binding of poly(I). The mutation did not affect K_M for poly(A) hydrolysis because poly(A) binding depends on nonspecific, in most part, electrostatic interactions.

Changes in enzymatic activity for mutant binase could also be induced by local structural changes within the recognition loop itself. The substitution of glycine for serine at position 60 could disrupt hydrogen bonds formed between Gly60(N) and Asn57(O) and Arg61(N) and Asn57(OD1) (Fig. 3). This localised change in the hydrogen bonding pattern may be the cause of its decrease in the k_{cat} as seen for G60S and G60S/R61V substitutions with the poly(A) substrate. However, the effects on catalytic function by the mutations are not additive in the double mutant G60S/R61V, as compared to the effects from the individual single mutations (Table 1). Substitution of glycine 60 to serine might affect the conformation of the active centre by reducing loop flexibility. These results provide additional support for the importance of maintaining an intact hydrogen bond network between the catalytic subsites within the enzyme active site.

A general model exists for the mechanism of NpN-type substrate discrimination by guanyl-specific ribonucleases [5,26–28]. For dinucleotide substrates binding occurs at three

Table 2
The relative activity of binase mutants on yeast RNA substrates.

Protein	Relative activity (%)*
Bi, w.t.	100
Bi, R61V	280
Bi, G60S	20
Bi, G60S/R61V	180
Bi, S56Q/R61V	212
Bi, S56Q/G60S/R61V	25

*One unit of ribonuclease activity in 120 μl reaction volume will produce alcohol-soluble oligonucleotides equivalent to a ΔA_{260} of 1.0 in 30 min at pH 7.5 and 37°C.

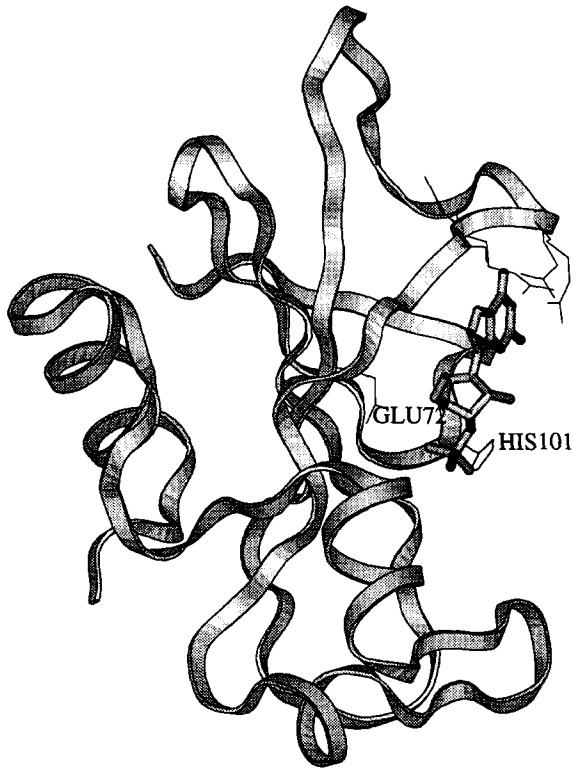

Fig. 2. Ribbon diagram of wild-type binase in complex with 3′-GMP. Amino residues Ser56, Asn57, Arg58, Glu59, Gly60 and Arg61 are shown forming the guanyl recognition loop. Catalytic residues Glu72 and His101 are also present.

main positions. The 5′-base of the substrate binds within the recognition loop, which exibits a spacial preference for guanine. The phosphate group interacts with the catalytic centre of the enzyme by contacting positively charged amino acid residues. These interactions provide the majority of the binding energy in the K_M value. Finally, the second base stacks against the active centre histidine (His101 for binase) [4,29]. One can see from the kinetic properties of binase and its mutants that the first two interactions described above between substrate and ribonuclease are sufficient for conformational stabilisation of ribose in the active centre and efficient catalysis. The same discrimination mechanism may also be used for poly(N) type substrates. Indeed, the ratio of k_{cat} in the reaction of RNase Sa with poly(I) and poly(A) substrates is 10^5, while the K_M ratio is 18.7 [14]. For RNases binase and barnase the k_{cat} ratio is 40 (barnase) and 20 (binase) and the difference in K_M less than 10-fold [12,22].

However, the main result of our work is that all mutants retain the ability to catalyse the hydrolysis of a poly(A) substrate. Since the partial and complete substitution of the recognition loop of binase by that of RNase Sa does not result in a complete shift in substrate specificity, there may be a second nucleo-base binding centre as seen for RNase A [32]. There is also biochemical evidence for a second phosphate binding centre [29,33]. This is supported by experimental data which show a decrease in the K_M for binase and barnase toward the polynucleotides [14]. Our results also correlate with the data obtained for the E60Q mutant of barnase [31]. This conservative residue is involved in hydrogen bonding with guanyl base of the substrate [26,29]. The mutation affects only hydrolysis of the dinucleotides, not of the polysubstrates.

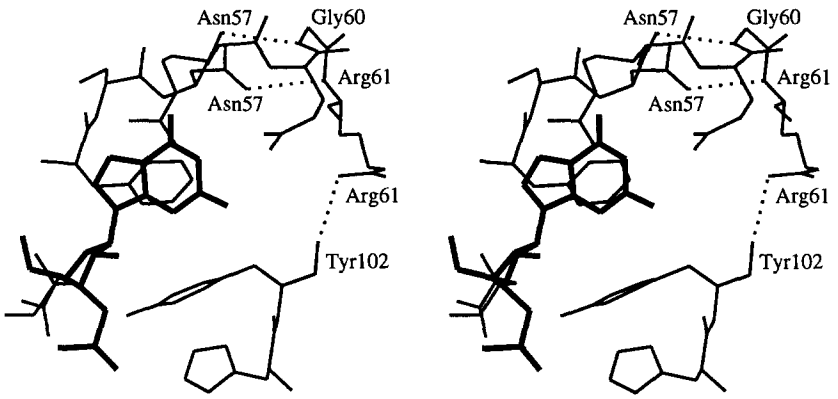

Fig. 3. A schematic representation of the active site of binase. The substrate analog 3′-GMP is shown in thick lines. Hydrogen bonds formed from Arg61(NH1) to (O)Tyr102, Gly60(N) to (O)Asn57, and Arg61(N) to (OD1)Asn57 are shown.

In our work we show that the base recognition loop of binase is not only the main component which takes part in the substrate binding as in T1 RNase family and RNase Sa. Together with the base recognition loop there is another factor which challenges the substrate binding mechanism through a 'G-specific' recognition loop and provides the ability to bind another purine base as a substrate in the case of long substrates. Potentially this factor could be a second phosphate binding centre.

The structurally similar family of microbial ribonucleases can be used as a model system for a comparative analysis of substrate recognition mechanisms. The present results show the contribution certain amino acids within the recognition loop of binase make towards substrate specificity and catalytic activity. Determination of the structural basis of substrate recognition by ribonucleases could help in the design of enzymes with altered specificity and/or increased enzymatic activity.Unlinked BIB's List:[30]

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