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Mutational analysis of the active site of RNase of *Bacillus intermedius* (BINASE)

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Abstract To elucidate the functional role of some residues in the active site of binase, the extracellular ribonuclease of *Bacillus intermedius*, we used site-directed mutagenesis. On cleavage of various substrates the catalytic activity of binase mutant His[101]Glu is 2.0–2.7% of that for native enzyme. The decrease in activity is determined mainly by the decrease in molecular rate constant k_{cat}, with almost unchanged affinity of the enzyme for the substrate, characterized by K_M. This is the expected result if His[101] acts as an general acid, donating a proton to the leaving group on cleavage of a phosphodiester bond. The replacement of Lys[26] by Ala causes a reduction in the enzyme activity to 13–33%, depending on the substrate. The activity decreases are due to changes in both k_{cat} and K_M for poly(I) and poly(A) but in k_{cat} alone for GpA. In the latter case the effect is far less than that seen in the homologous mutation in the closely related enzyme, barnase.

Key words: Ribonuclease; Catalytic property; Site-directed mutagenesis

1. Introduction

Binase is a small ribonuclease produced and secreted by *Bacillus intermedius*. The enzyme consists of a single polypeptide chain of 109 amino acids with no disulfide bridges [1]. It belongs to a large family of homologous microbial ribonucleases [2,3]. Binase hydrolyses RNA into mono- and small oligonucleotides. It has been shown to be guanyl-specific towards nucleotide-2′,3′-cyclophosphates and purine-preferential towards dinucleoside-phosphates and polynucleotides [4]. The chemical mechanism of the *trans*-esterification reaction includes a nucleophilic attack of the ribose 2′OH group on the phosphate group, formation of the transition state with a pentacovalent phosphorus and cleavage of the P-O5′ bond with the formation of 2′,3′-cyclic phosphate. X-ray studies of the binase complex with 3′-GMP showed that there are five amino acid residues with ionizing side chains at the catalytic site of the enzyme [5]. According to the X-ray structure of the complex one can assume that the carboxyl group of Glu72 as a general base accepts a proton of the ribose 2′-OH group and in doing so facilitates a nucleophilic attack of the 2′-OH group on the phosphorus atom. The role of the remaining residues is not clear. To elucidate the functional roles of two of the other residues of the active site, we have used site-directed mutagenesis. This study reports the effect on the enzymic properties of binase on substitution of His[101] by glutamic acid or of Lys[27] by alanine.

2. Materials and methods

Wild-type binase and its mutants were purified by the procedure described in [6] from the culture medium of *E. coli* (JM107) carrying the appropriate expression plasmids. The cloning and sequence of the binase gene have been reported [7]. The plasmids used in this work are based on an independent cloning of the gene by one of us (E.B.C.). Except for the structural gene coding for the ribonuclease, pML163 (wild-type binase) and pML164 (binase Lys[26]Ala) are identical to the barnase expression plasmid pMT416 [8] with the enzyme on a *tac* promoter and *E. coli pho*A signal sequence and with the inhibitor barstar on its own promoter. The expression vector for binase His[102]Glu is the analagous derivative of the functionally equivalent pMT702 [9]. Mutants were prepared by the recombinant circle PCR method of Jones and Howard [10]. Codon changes were AAA to GCA for Lys[26]Ala and CAT to GAG for His[102]Glu. Dinucleoside phosphate GpA was purchased from Serva. Polynucleotides poly(I) and poly(A) were synthesized using polynucleotide phosphorylase (NPO Vector, Novosibirsk, Russian Federation) according to the procedure described in [11] and were fractionated on a G-50 Sephadex column (100 × 1 cm). The fractions with lengths of more than 100 nucleotides were used for measurements. The concentrations of binase and its mutants and substrates were determined spectrophotometrically. The following extinction coefficients were used: binase and its mutants Δe_{280} = 22,500 $M^{-1} \cdot cm^{-1}$ [12]; GpA Δe_{280} = 9760 $M^{-1} \cdot cm^{-1}$ at pH 7 [13]; poly(I) Δe_{248} = 10,000 $M^{-1} \cdot cm^{-1}$ at pH 7.8 [14]; poly(A) e_{257} = 10,000 $M^{-1} \cdot cm^{-1}$ at pH 7.5 [15]. For determination of the initial velocities of cleavage of substrates, the following difference molar coefficients were used at pH 6.2 and 25°C: GpA Δe_{280} = 930 $M^{-1} \cdot cm$ [16]; poly(I) Δe_{248} = 1330 $M^{-1} \cdot cm$ [4]; poly(A) Δe_{260} = 5000 $M^{-1} \cdot cm$ [4]. In kinetic measurements a buffer containing 0.1 M sodium citrate and 0.1 M NaCl was used. The pH was adjusted by the addition of NaOH. Spectral and kinetic measurements were performed using a Specord M-40 spectrophotometer (Carl Zeiss, Germany). The spectral width of the optical slit was 0.6–1.2 nm. Cells having an optical path length 0.2–1 cm were used and were thermostatted at 25°C. To determine kinetic parameters for a given enzyme and substrate, initial rates were measured for 6–7 concentrations of

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Abbreviations: 3′GMP, guanosine 3′-phosphate; GpA, guanylyl-(3′-5′)adenosine; poly(I), polyinosinic acid; poly(A), polyadenylic acid. Enzymes: ribonucleases binase from *Bacillus intermedius* and barnase from *Bacillus amyloliquefaciens* (EC 3.1.4.23), ribonuclease A, bovine pancreatic ribonuclease (EC 3.1.27.5).

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substrate. The rate of hydrolysis for all substrates changed hyperbolically with increase in substrate concentration. Kinetic parameters were determined from Lineweaver–Burk plots. These data were analyzed using a weighted least-square procedure.

3. Results and discussion

Kinetic parameters of the cleavage reaction of poly(I), poly(A) and GpA by binase and its mutants are presented in Table 1. One can see that for cleavage of various substrates the catalytic activity of binase $His^{101}Glu$ is 2.0–2.7% of that of the native enzyme. The decrease in activity is determined mainly by the decrease in the molecular rate constant k_{cat} with almost unchanged enzyme–substrate affinity, characterized by K_M. This is the result expected if His^{101} acts as a general acid, donating a proton to the leaving group in the course of enzymatic cleavage of a phosphodiester bond. An enzyme activity dependent on the extent of protonization of a residue with definite pK is proportional to the factor $1/(1+10^{pH-pK})$ [17]. Therefore, on replacing of such a residue with pK_1 by a residue with pK_2 the ratio of activities A_1 and A_2 equals:

$$A_1/A_2 = (1+10^{pH-pK_2})/(1+10^{pH-pK_1})$$

The pK value for His^{101} in binase found from the pH-dependence of k_{cat}/K_M is 6.5 [18]. The expected pK of the carboxyl group of the glutamic acid residue in the $His^{101}Glu$ mutant equals 4.2–4.5. Then the expected relative activity of binase on substitution of Glu for His^{101} is 1.5–2.9% that of the wild-type enzyme. The observed decrease in the enzyme activity thus corresponds to the expected value. This role of His^{101} as a proton donor corresponds to that for the homologous His^{102} of barnase as reported by Mossakowska et al. [19].

On replacement of the binase Lys^{26} residue by Ala the enzyme activity is reduced to 13–33%, depending on the type of substrate. This relatively modest (3–7 fold) activity decrease is due to the changes in both k_{cat} and K_M for the polymeric substrates, and in k_{cat} alone in the case of GpA. For the homologous mutation in barnase, $Lys^{27}Ala$, the reported effect [19] is much more striking, particularly with regard to the k_{cat} for GpA which is decreased by a factor of 2700, the authors concluding that Lys^{27} strongly affects the transition state of the barnase–substrate complex. This conclusion is supported by the findings of Meiering et al. [20] that this mutation produces minimal change in surrounding structures as seen by NMR. The difference in the response of the GpA k_{cat} to this mutation in the two enzymes is curious but, if we adopt the same mechanism for binase, it might be explained by only a very small difference in the distances between Lys-26/-27 and the phosphate group of the bound substrate. This would require a compensating subtle difference elsewhere, perhaps in positioning of one of the Arg residues in the active site, since the GpA k_{cat} for binase is actually higher than that for barnase.

Table 1
Kinetic parameters of the cleavage reaction of polynucleotides and GpA by wild-type, $His^{101}Glu$ and $Lys^{26}Ala$ binase

Binase	Substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1} \cdot s^{-1}$)
Wild-type[a]	GpA	8.1	200	4.1×10^{-2}
	poly (I)	268	37	7.2
	poly (A)	9.4	40	2.4×10^{-1}
$His^{101}Glu$	GpA	0.2	180	1.1×10^{-3}
	poly (I)	4.7	32	1.5×10
	poly (A)	0.3	53	6.0×10^{-3}
$Lys^{26}Ala$	GpA	1.3	190	6.8×10^{-3}
	poly (I)	184	77	2.4
	poly (A)	2.2	70	3.1×10^{-2}

pH 6.2, I = 0.2 M and 25°C.
[a] The difference in activities of bacillar binase and recombinant binase was less than 7%.

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