Contribution of arginine-82 and arginine-86 to catalysis of RNases from *Bacillus intermedius* (binase)

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Abstract To elucidate the functional role of Arg^{82} and Arg^{86} in the enzyme activity of binase, the extracellular ribonuclease of *Bacillus intermedius*, we used site-directed mutagenesis. On cleavage of various substrates the catalytic activity of binase mutant $Arg^{86}Ala$ is 2.7×10^3–7.7×10^3 times less than that of the native enzyme. The decrease in activity is determined preferentially by the decrease in the molecular rate constant k_{cat} with a relatively small change of enzyme-substrate affinity, characterized by K_m. This is the expected result if Arg^{86} acts to lower the energy of a transition state of the reaction. The replacement of Arg^{82} by Ala causes a 5–19-fold activity decrease, depending on the substrate. We propose that this residue does not have a direct catalytic function in the molecular mechanism of the binase action and that the activity decrease of binase on the replacement of Arg^{82} by alanine is mediated by the effect of Arg^{82} on the p*K* of catalytic residues.

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

1. Introduction

Binase is a extracellular bacterial ribonuclease produced by *Bacillus intermedius* (EC 3.1.4.23). It belongs to a large family of homologous microbial ribonucleases [1,2]. The binase gene has been cloned and expressed in *Escherichia coli* [3,4]. The enzyme consists of a single polypeptide chain of 109 amino acids with no disulfide bridges [5]. Binase catalyzse the cleavage of RNA to yield an RNA strand with a free 5′OH and another RNA strand with 2′,3′-cyclophosphate at the 3′ end. The enzyme then catalyzes the hydrolysis of this cyclophosphate. It has been shown to be guanyl-specific toward nucleotide-2′,3′-cyclophosphates and purine-preferential toward dinucleoside-phosphates and polynucleotides [6]. The chemical mechanism of the transesterification 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 an ionizing side chain at the catalytic site of the enzyme: Lys^{26}, Glu^{72}, Arg^{82}, Arg^{86} and His^{101} [2]. The effect on the enzyme properties of binase on substitution of glutamic acid for His^{101} indicates that His acts as a general acid, donating a proton to the leaving group in the course of enzymatic cleavage of an RNA phosphodiester bond [4]. This allows us to assume that the carboxyl group of Glu^{72} acts as a general base to deprotonate the nucleophilic ribose 2′OH group. It is likely that one or more of the other three positively charged residues acts to lower the energy of a transition state of the reaction. The replacement of Lys^{26} by Ala caused a relatively modest decrease in the enzyme activity [4]. This study reports the effects of substitution of alanine for Arg^{82} or Arg^{86} on the enzymic properties of binase.

2. Materials and methods

Wild-type binase and its mutants were purified by the procedure described in [7] from the culture medium of *E. coli* (JM107) carrying the appropriate expression plasmids. The plasmids for binase expression are identical to the barnase expression plasmid pMT 416 [8] with the exception of the structural gene encoding the ribonuclease. Mutants were prepared by the recombinant circle PCR method of Jones and Howard [9]. Codon changes were AGG to GCA for Arg^{82} and Arg^{86}. Dinucleoside phosphate GpA was purchased from Serva. Polynucleotides poly(I) and poly(A) were purchased from Sigma. The concentrations of binase and its mutant and substrates were determined spectrophotometrically. The following extinction coefficients were used: binase and its mutant $\varepsilon_{280} = 22\,500\ M^{-1}\ cm^{-1}$ [5]; GpA $\varepsilon_{280} = 9760\ M^{-1}\ cm^{-1}$ at pH 7 [10]; poly(I) $\varepsilon_{248} = 10\,000\ M^{-1}\ cm^{-1}$ at pH 7.8 [11]; poly(A) $\varepsilon_{257} = 10\,000\ M^{-1}\ cm^{-1}$ at pH 7.5 [12]. 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 $\Delta\varepsilon_{280} = 930\ M^{-1}\ cm^{-1}$ [13]; poly(I) $\Delta\varepsilon_{248} = 1330\ M^{-1}\ cm^{-1}$ [6]; poly(A) $\Delta\varepsilon_{260} = 5000\ M^{-1}\ cm^{-1}$ [6]. In kinetic measurements a buffer containing 0.1 M sodium citrate and 0.1 NaCl was used. pH was measured using pH meter pHM 64 (Radiometer, Denmark). The pH was adjusted by the addition of NaOH. Spectral and kinetic measurements were performed using spectrophotometer Specord M-40 (Carl Zeiss, Germany). The spectral width of the optical slit was 0.6–1.2 nm. Cells having an optical path length of 0.2–1 cm were used and were thermostatted at 25°C. The rate of hydrolysis for all substrates was changed hyperbolically with increase of substrate concentration. Kinetic parameters were determined from Lineweaver-Burk plots. These data were analyzed using a weighted least-square procedure.

3. Results and discussion

The transesterification reaction consists of an associative nucleophilic displacement at the phosphorus atom of the 5′ leaving group by the 2′ entering oxygen atom. The formation of the transition state with a pentacovalent phosphorus is accompanied by a large change in geometry about the phosphorus as it changes from tetrahedral in the ground state to trigonal bipyramidal in the transition state. In the pentacovalent trigonal bipyramidal state the second negative charge

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Abbreviations: 3′GMP, guanosine 3′-phosphate; GpA, guanylyl(3′-5′)adenosine; poly(I), polyinosinic acid; poly(A), polyadenylic acid

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Table 1
Kinetic parameters of the cleavage reaction of polynucleotides and GpA by wild-type, $Arg^{82}Ala$ and $Arg^{86}Ala$ binase

Binase	Substrate	k_{cat} (cm^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} cm^{-1})
Wild-type	GpA	8.1	20	4.1×10^4
	poly(I)	268	37	7.2×10^6
	poly(A)	9.4	40	2.4×10^4
$Arg^{82}Ala$	GpA	0.6	280	2.2×10^3
	poly(I)	355	240	1.5×10^6
	poly(A)	1.9	140	1.4×10^3
$Arg^{86}Ala$	GpA	–	–	7
	poly(I)	1	38	2.6×10^3
	poly(A)	0.0012	39	3.1

pH 6.2, I = 0.2 M and 25°C.

appears on one of the equatorial oxygens. The neutralizing of this second negative charge by a positive charge of ionogenic protein group decreases the free energy of transition state formation in a value of the energy of their electrostatic interaction. We may expect that the elimination of such a positive charge in binase active sites will cause a decrease of catalytic constant k_{cat} of as much as 10^3–10^4 times [14,15].

The steady-state 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 the replacement of Arg^{82} by Ala causes a 5–19-fold activity decrease, depending on the substrate. The activity decrease is due to the change in both k_{cat} and K_m for poly(A), and in k_{cat} alone for GpA and in K_m alone for poly(I). The catalytic activity of $Arg^{86}Ala$ binase is 2.7×10^3–7.7×10^3 times less than that of the native enzyme. The decrease in activity is determined preferentially by the decrease in the molecular rate constant k_{cat} with relatively small change of enzyme-substrate affinity, characterized by K_m. These results demonstrate the importance of Arg^{86} in accelerating the cleavage reaction of an internucleotide bond.

The modest decrease of activity on substitution of Ala for Arg^{82} enables us to rule out Arg^{82} as a residue stabilizing the substrate transition state. We propose that this residue does not have a direct catalytic function in the molecular mechanism of the binase action and that the activity decrease of binase on the replacement of Arg^{82} by alanine is mediated by the effect of Arg^{82} on the pK of catalytic residues. It is much more likely that the transition state of the binase-substrate complex is stabilized by Arg^{86} due to the interaction of this positively charged side chain with the phosphate group.

It has been reported that in barnase, the close homolog of binase from *Bacillus amiloliquefaciens*, the residue Lys^{27} is crucial to stabilizing the transition state [15]. Binase and barnase share about 85% sequence identity, with an almost superimposable structure. The corresponding lysine residue in binase, however, appears to be much less critical [4]. These results demonstrate that there must be subtle differences between the two close structures which affect the manner by which the transition state is stabilized.

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