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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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