RNA cleavage without hydrolysis. Splitting the catalytic activities of binase with Asn101 and Thr101 mutations


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Members of the microbial guanyl-specific ribonuclease family catalyse the endonucleolytic cleavage of single-stranded RNA in a two-step reaction involving transesterification to form a 2’,3’-cyclic phosphate and its subsequent hydrolysis to yield the respective 3’-phosphate. The extracellular ribonuclease from Bacillus intermedius (binase, RNase Bi) shares a common mechanism for RNA hydrolysis with mammalian RNases. Two catalytic residues in the active site of binase, Glu72 and His101, are thought to be involved in general-acid–general-base catalysis of RNA cleavage. Using site-directed mutagenesis, binase mutants were produced containing amino acid substitutions H101N and H101T and their catalytic properties towards RNA, poly(I), poly(A), GpC and guanosine 2’,3’-cyclic phosphate (cGMP) substrates were studied. The engineered mutant proteins are active in the transesterification step which produces the 2’,3’-cyclic phosphate species but they have lost the ability to catalyse hydrolysis of the cyclic phosphate to give the 3’ monophosphate product.

Keywords: active-site mutants/binase/ribonuclease/site-directed mutagenesis

Introduction

Microbial cyclizing ribonucleases have been studied over the past few years using a variety of different methods. The enzymes within this family have a preference for guanine bases on the 5’-side of the scissile phosphodiester bond. Structural studies identified a similar secondary structure for several microbial ribonucleases and a similar spatial organization of the active centres of these enzymes (Hill et al., 1983; Polyakov et al., 1987; Koepke et al., 1989; Pavlovsky et al., 1989; Sevcik et al., 1990; Bycroft et al., 1991; Sevcik et al., 1991).

Binase (RNase Bi) is a guanylic-specific extracellular ribonuclease produced by Bacillus intermedius. It is made up of a single polypeptide chain consisting of 109 amino acid residues (Schulga et al., 1992). Binase lacks sulphur-containing residues and has only a single histidine, which is located in the active site. It has been shown that the binding of a 3’-GMP nucleobase occurs at the ‘substrate recognition loop’ formed by amino acid residues 55–61 and that the phosphate group is fixed at the catalytic site nearby three other invariant residues among the microbial ribonucleases, Glu72, Arg86 and His101 (Sevcik et al., 1990) (Figure 1).

Binase and barnase (RNase Ba from Bacillus amyloliquefaciens), which have 85% identity (17 substitutions and 1 deletion) and almost identical three-dimensional structure share a common mechanism for RNA hydrolysis with mammalian RNases such as RNase A (Findlay et al., 1961; Hershlag, 1994). Each enzyme catalyses two reactions. The first of these is the endonucleolytic cleavage of single-stranded RNA in a transesterification reaction to form a 2’,3’-cyclic phosphate. The second reaction is the hydrolysis of the cyclic phosphate to yield the respective 3’-phosphate. For pancreatic RNase A, a general acid–general-base catalytic mechanism involving two histidines, His12 and His119, is widely accepted. In binase, Glu72 and His101 (Glu73, His102 in barnase) are thought to act as general base and general acid, respectively, with Arg86 playing a role in binding the substrate phosphate group (Arg87 in barnase).

It has been shown that the barnase mutant H102A is inactive (Paddon and Hartley, 1987; Mossakovska et al., 1989). The side chain of alanine can neither act as an acid/base nor does it have hydrogen bonding capacity. It cannot therefore act as a general base to activate water nor is it able to stabilize charged intermediates (transition state) formed in the reaction. In addition, the alanine substitution is likely to disturb the hydrogen bonding network and water structure at the active site of the enzyme. Moreover, its small size will not restrict the conformational freedom around the phosphate moiety in the substrate. For our study we chose to substitute histidine-101 by asparagine and threonine, whose side chains are closer in size to that of histidine and which can participate in hydrogen bonding. However, neither asparagine nor threonine can serve as a general base to activate the water molecule participating in the second step of the reaction. Consequently, one would expect these mutations to abolish the hydrolytic activity of binase without necessarily drastically reducing the rate of the transesterification reaction. To explore more precisely the role of His101 in binase catalysis, Asn101 and Thr101 substitutions were introduced into the enzyme by site-directed mutagenesis. The catalytic properties of these mutants, H101N and H101T, towards RNA, poly(I), poly(A), GpC and guanosine-2’3’-cyclic phosphate (cGMP) substrates were studied.

Materials and methods

Chemically pure reagents were obtained from Merck and Sigma. HPLC-grade acetonitrile (Fluka) was used in the preparation of buffers for reversed-phase high-performance liquid chromatography (RP-HPLC). Aqueous solutions were prepared using Milli-Q water (Millipore). The HPLC apparatus (Gilson) consisted of Model 305 pumps, a Model 803C manometric module, a Model 811B mixer with a 1.5 ml chamber, a UV112 detector (254–280 nm) and a Model 7125 injector (Rheodyne) with 20 µl, 100 µl and 1 ml loops. A
Fig. 1. Stereo diagram of wild-type binase in complex with 3'-GMP (coordinates provided by Dr K.M. Polyakov and also described in Pavlovsky et al., 1989). The protein backbone is shown with α-helical and β-strand regions highlighted. The side chains of amino residues Arg58, Glu59, Arg61, Glu72, Arg86 and His101 are represented in ball-and-stick form, as is 3'-GMP. Stereoview drawn in MOLSCRIPT (Kraulis, 1991).

Model 714 HPLC system controller (Gilson) was used to regulate chromatographic processes and record and analyse results.

All growth media were supplied by Difco Laboratories (Detroit, MI). Escherichia coli strains JM109 and XL-1 Blue MRF' (Stratagene) were used for DNA manipulations and for expression of binase and its mutants. RNase activity was assayed as described previously (Okorokov et al., 1994).

Mutagenesis and production of mutant proteins

The binase variants were obtained by site-directed mutagenesis of the binase coding sequence using the Altered Sites kit supplied from Promega and following the manufacturer’s specifications. The 900 bp BamHI–HindIII DNA fragment of plasmid pBIT36 (Schulga et al., 1994), containing the binase and barstar structural genes was subcloned into the pSELECT-1 vector. The following primers were used to introduce single amino acid mutations within the coding sequence for RNase Bi: 5'-C AAA ACA ACA GAC AA T TA T GCA ACT TTC-3' (H101N) and 5'-C AAA ACA ACA GAC ACT TA T GCA ACT TTC-3' (H101T). These oligonucleotides are complementary to the transcribed strand of the binase gene at sequences flanking codon 101 but they contain mismatches (underlined) at codon 101 that direct the mutations.

The introduction of the mutations within the RNase Bi gene was 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 (Schulga et al., 1994) was used to produce wild-type binase and its mutants in E.coli. Fusion to the phoA signal peptide directed the expression ribonuclease to the cell periplasm. The recombinant RNase Bi and its mutant derivatives were purified by RP-HPLC (Panov, 1994) to ~99% homogeneity. Protein yields ranged from 5–10 to 50–100 mg of pure protein per litre of cell culture for the mutant variants and wild-type binase, respectively.

Kinetic measurements

Experiments were performed with a Uvicon 1800 spectrophotometer (Contron Instruments). Thermostated cells (25°C) having an optical pathlength of 10 and 2 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 mutant, ε<sub>280</sub> = 22 500 M<sup>-1</sup> cm<sup>-1</sup> (Goldenberg et al., 1977); poly(I), ε<sub>248</sub> = 10 000 M<sup>-1</sup> cm<sup>-1</sup> at pH 7.8 (Chamberlin and Paterson, 1985); poly(A), ε<sub>257</sub> = 10 000 M<sup>-1</sup> cm<sup>-1</sup> at pH 7.5 (Blake and Fresko, 1966); and GpC, ε<sub>280</sub> = 12 600 M<sup>-1</sup> cm<sup>-1</sup> at pH 7.0 (Osterman and Walz, 1978). For the determination of the initial velocities of the substrate cleavage reactions, the following difference molar extinction coefficients were used: at pH 6.2 and 25°C, poly(I), Δε<sub>248</sub> = 1330 M<sup>-1</sup> cm<sup>-1</sup>; poly(A), Δε<sub>257</sub> = 5000 M<sup>-1</sup> cm<sup>-1</sup> (Blake and Fresko, 1966); and GpC, Δε<sub>280</sub> = 2050 M<sup>-1</sup> cm<sup>-1</sup> (Osterman and Walz, 1978). Each assay reaction contained substrate [poly(I) (3×10<sup>-5</sup>–1.2×10<sup>-3</sup> M; poly(A) (3×10<sup>-5</sup>–1.2×10<sup>-3</sup> M); GpC and barstar structural genes was subcloned into the pSELECT-1 vector. The following primers were used to introduce single mutations within the RNase Bi gene (ENZFITTER (Leatherbarrow, 1987). 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 (Schulga et al., 1994) was used to produce wild-type binase and its mutants in E.coli. Fusion to the phoA signal peptide directed the expression ribonuclease to the cell periplasm. The recombinant RNase Bi and its mutant derivatives were purified by RP-HPLC (Panov, 1994) to ~99% homogeneity. Protein yields ranged from 5–10 to 50–100 mg of pure protein per litre of cell culture for the mutant variants and wild-type binase, respectively.

**Analysis of products of hydrolysis of GpC, GpG and cGMP**

RP-HPLC was used for analysis of the cleavage products of GpC, GpG and cGMP substrates by binase and its mutants. The concentrations of binase, its mutants and the nucleic acid substrates were determined spectrophotometrically. The extinction coefficients used were as listed above together with those found for cGMP ε<sub>252</sub> = 13 700 M<sup>-1</sup> cm<sup>-1</sup> (Yakovlev et al., 1992). RP-HPLC was carried out using a 4×250 mm Nucleosil 100 C-18, 5 μm, column (Elsico, Russia). The following experimental procedures were used. For cGMP, the HPLC eluent was a 1 mM tetrabutylammonium (TBA) phosphate buffer, pH 3.0, containing 8% methanol which was pumped at a flow-rate of 1 ml/min. The reactions were performed variously in 50 mM sodium acetate, pH 5.0, 50 mM bis-Tris propane–HCl, pH 7.5, and 50 mM Tris–HCl, pH 8.0 (all buffers containing 0.2 M NaCl) at 25°C. The concentrations of cGMP and 3'-GMP in the reaction mixture were determined from the
integration of appropriate peaks according to previously obtained calibration curves. For GpG and GpC, the elution was performed with a 0–10% linear gradient of acetonitrile in 5 mM TBA formate buffer, pH 3.0, at a flow-rate of 1 ml/min. The reactions were performed at 25° C in 0.1 M sodium citrate buffer, pH 6.2, containing 0.1 M NaCl. The products of the reactions were identified from their retention times.

Binase H101N crystallization and determination of the structure

Crystals of binase H101N were grown in hanging drops by vapour diffusion. The hanging drop initially contained 12 mg/ml binase H101N in 40 mM glycine, pH 7.5, 8.75% polyethylene glycol (PEG) 10 000 and 2.5% saturated sodium citrate and the well contained 17.5% PEG 10 000, 60 mM glycine, pH 7.5, and 5% sodium citrate. Crystals grew within 2 days at 18°C. The largest crystals were ~1.1×0.1×0.08 mm³ and were in the space group P2₁2₁2₁, with unit cell dimensions a = 111.26, b = 69.15 and c = 33.37 Å. A single crystal was mounted in a 0.7 mm diameter glass capillary and diffraction data were collected to 2.2 Å spacing on a Rigaku R-axis II imaging plate detector using X-rays generated from a rotating copper anode. Data were processed using the program DENZO (Otwinowski, 1990) and the CCP4 suite of programs for protein crystallography (CCP4, 1994); 51 822 observed intensities were reduced to 13 288 unique reflections with an overall merging R-factor of 5.5% (Rmerge = Σ||Fobs| – |Fcalc||/Σ|Fobs|). The data set is 97.2% complete with 85% of the intensities greater than 3σ. The structure of the binase H101N mutant was refined using the restrained least-squares minimization method to a final Δ-factor of 5.5% (Otwinowski, 1990) and the CCP4 suite of programs for protein GpC 1.1 

Results and discussion

Binase catalyses the cleavage of phosphodiester bonds in ribonucleic substrates on the 3’-side of purine bases (Both et al., 1991). Like RNase A, binase is a distributive endonuclease. There are two distinct reactions catalysed at the same active site of the enzyme. The first is transphosphorylation of RNA proceeding via a nucleophilic attack on the phosphorus by the 2’-OH to form a 2’,3’-cyclic phosphodiester (cNMP); the second is hydrolysis of this cyclic intermediate to form a 3’-phosphomonooester (NMP). It is widely accepted that in the first reaction ionized Glu72 acts as general base accepting the proton from the 2’-OH while His101 acts in concert as a general acid donating a proton to the cleavage product. In the second reaction it is proposed that the roles of these catalytic residues are reversed.

We found that replacing the active site histidine with either an asparagine or a threonine residue does not completely abolish the ribonuclease activity of binase. Indeed, we were unable to obtain ampicillin-resistant transformants of several E.coli strains with plasmid constructs expressing mutant proteins without co-expression of the specific inhibitor, barstar. In order to overexpress and purify these mutants, we therefore co-expressed barstar using the plasmid pBIT36 (Schulga et al., 1994). A final yield of about 10 mg of each mutant protein was obtained per litre of culture. This amount is ~10-fold lower than that obtained for wild-type binase expressed and purified under the same conditions.

The His101 residue in binase corresponds to His102 in barnase, which has been shown to be involved in the protein’s interaction with the inhibitor barstar (Guilet et al., 1993). It was previously shown that the barnase–barstar complex has a very similar dissociation constant to the barnase–barstar complex (Yakovlev et al., 1995), consistent with the close sequence similarity of these two ribonucleases. Also, co-expression with barstar allows us to express wild-type binase and its active mutants in E.coli (Schulga et al., 1994; Okorokov et al., 1996). One may assume that any mutation of this conserved histidine in binase will affect the efficiency of inhibition by barstar and hence the ability of the host cell to withstand any ribonuclease activity which the mutated protein might still possess. It has been proposed that the barnase mutants Ala102, Gly102 and Leu102 are inactive because they can be efficiently expressed in E.coli in the absence of barstar, that is, they do not have deleterious effects on the growth of the host (Hartley, 1988; Jucovic and Hartley, 1995). Barnase H102A has no detectable activity towards RNA and less than 0.01% activity for GpA transesterification (Mossakovska et al., 1989).

Following expression and purification, the binase H101N and H101T mutants were analysed kinetically and their catalytic properties were compared with those of the wild-type protein (Tables I, II and III). The H101N and H101T mutants retain about 50 and 10%, respectively, of the activity of the wild-type enzyme.
type enzyme towards total yeast RNA substrates. The two mutants are able to catalyse the cleavage of poly(A) and poly(I) substrates but less efficiently than the wild-type enzyme. Again, the relative activity is lower for the H101T mutant than for the H101N mutant. The mutants are less effective catalysts of the cleavage of GpC and GpG relative to wild-type binase. In the absence of other substrates, cGMP is hydrolysed completely by wild-type binase to produce 3'-GMP. The mutants H101N and H101T are apparently unable to hydrolyse cGMP under Michaelis–Menten kinetic conditions, since no 3'-GMP was detected in the reaction mixtures even after a 60 min incubation when the substrate was in 100-fold molar excess over the enzyme.

Although H101N and H101T fail to catalyse hydrolysis of cGMP under Michaelis–Menten conditions, we found that incubation of substrate for 30 h in the presence of high concentrations of binase H101N (enzyme to substrate concentration ratio 1:5:1) does lead to partial degradation of cyclic GMP. As a similar percentage of cGMP degradation was observed following incubation of cyclic GMP under identical conditions with lysozyme (data not shown), cGMP may be breaking down as a result of some non-specific interaction with the enzyme, e.g. at binase surface subsites which bind the phosphate backbone of RNA.

The steady-state kinetic parameters for wild-type binase and the two mutants are presented in Table II for the various substrates. The value of $K_M$ for H101N binase is the same as for wild-type binase on a poly(A) substrate, but $K_{cat}$ is reduced about 10-fold, so that $K_{cat}/K_M$ at $1.5 \times 10^{-4}$ M is also 10-fold lower for the mutant binase. Similar but smaller changes are observed with the poly(I) substrate. The value of $K_M$ is about the same but $K_{cat}$ is threefold lower, leading to a fourfold decrease in $K_{cat}/K_M$. The efficiency of catalysis is more dramatically decreased towards the GpC substrate: the value of $K_{cat}$ is reduced 40-fold while $K_M$ decreases about twofold, giving a 20-fold lower $K_{cat}/K_M$. Similar results were obtained for H101T, except that the decreases in $K_{cat}$ were even more pronounced. Neither H101N nor H101T catalyses hydrolysis of guanosine 2',3'-cyclic phosphate (cGMP). Indeed, the final products of the GpC and GpG cleavage are 3'-GMP and cytosine or guanosine for wild-type binase, but cGMP and cytosine or guanosine for the mutant variants (Table III). This implies that the products of cleavage of larger substrates by H101N and H101T will also have a cyclic phosphate group at their 3'-termini.

It has recently been suggested by several groups that the primary reaction catalysed by mammalian and microbial RNases is transphosphorylation leading to RNA cleavage and that this is not necessarily followed by hydrolysis of the cyclic phosphate formed in this reaction (Cuchillo et al., 1993; Thompson et al., 1994). This is in contrast to the previously held idea that the cyclic phosphate is an enzyme-bound intermediate which is subsequently converted into a 3'-monophosphate before being released into solution. It has been shown in a study of the barnase-catalysed cleavage of GpA that the cGMP intermediate is hydrolysed only when its concentration is sufficiently high to compete effectively with the GpA (Mossakovska et al., 1989). It has also been shown that barnase yields cyclic phosphates as the initial products of its cleavage of various di-, tri- and tetranucleotide substrates (Day et al., 1992). Another group demonstrated that when cytidylyl-3',5'-cytidine (CpC) is cleaved by pancreatic RNase A, no 3'-CMP is produced until all of the CpC has been converted into cCMP (Cuchillo et al., 1993). This demonstrates again that the hydrolysis step is slower than the cyclization reaction. In contrast, when RNA is hydrolysed non-enzymatically either in imidazole buffer or at high pH, the rate of transphosphorylation is limiting and cyclic phosphates do not accumulate (Thompson et al., 1994).

To draw reliable conclusions from site-specific mutagenesis
experiments, it is recognized that one has to establish the extent to which a mutation perturbs the structure of the protein. The recombinant H101N binase has been crystallized and its X-ray structure determined (Offen, 1995). The structures of the mutant and the wild-type enzyme are very similar. A least-squares fit of the main chain atoms of the mutant and wild-type enzymes gives an r.m.s. deviation in atomic positions of 0.32 and 0.30 Å for the A and B molecules of the asymmetric unit, respectively. The only noticeable difference between the two structures is the shortening of the position-101 side chain where asparagine replaces histidine (Figure 2). Neither His101 in the wild-type enzyme nor Asn101 in the mutant are hydrogen bonded to any other protein groups. Indeed, the pattern of interactions with well-defined water molecules is altered. The kinetic effects of the mutation can therefore be attributed to the substitution of the histidine and rearrangement of the water molecules located at the catalytic site of enzyme and possibly involved in catalysis.

Conclusions

It has recently been suggested by several groups that the primary reaction catalysed by mammalian and microbial RNases is transphosphorylation, which results in RNA cleavage that this is not necessarily followed immediately by hydrolysis of the cyclic phosphate so formed (Mossakovska et al., 1989; Day et al., 1992; Thompson et al., 1994; Thompson and Raines, 1995). Data obtained here provide further experimental evidence that the cyclic phosphate is not an intermediate in a two-step reaction mechanism, but instead is the product of the first and faster primary transphosphorylation reaction, which is sufficient to cleave RNA substrates. The cyclic phosphate products are hydrolysed by the enzyme only when all poly- or oligoribonucleotides have been cleaved.

Catalysis of cyclic phosphate formation by ribonucleases requires that the 2′-OH group on the substrate be activated for nucleophilic attack at the phosphorus centre by groups on the enzyme. Glu72 performs this function by acting as a base which abstracts a proton from the 2′-OH group, so initiating the transphosphorylation step. In the classical mechanism of RNase action, His101 also facilitates the cyclization reaction by acting as a general acid which protonates the leaving group. The side chain amide of Asn101 and the hydroxyl group of Thr101, however, are poor general acids since their pK_a values are similar to that of a water molecule. In RNase A the analogous general acid, His119, contributes a 10^3-fold rate enhancement relative to its absence in the Ala119 mutant (Thompson et al., 1994). The magnitude of this rate enhancement is consistent with His119 acting as a general acid in catalysis taking into account the pK_a value of His119 (6.2), the pK_a of water (15.7) and an estimated Brønsted α-value of 0.5 for proton transfers between nitrogen and oxygen (Thompson et al., 1994). The pK_a of His101 in binase is 6.5 (Karpeisky et al., 1981) and the pK_a value of the amide group (proton dissociation from the neutral amide) of Asn should be close to 15 (Hine and Hine, 1952; Bordwell 1988). Consequently, one would expect His101Asn/Thr mutations to cause severe decreases in K_cat, if the major contribution of His101 to catalysis is protonation of the leaving group (general acid catalysis). However, the His101Asn/Thr mutations decrease K_cat for the transesterification reaction by only ~20 fold. The data suggest, therefore, that His101 has a function in binase catalysis which is unconnected with protonation of a leaving group. This function is preserved in His101Asn/Thr binase mutants but not in the His102Ala mutant of barnase (Mossakovska et al., 1989). Asn101 and Thr101 are able either to act as direct hydrogen bond donors or to promote hydrogen bond donation by adjacent water molecules. Thus, asparagine and threonine can stabilize the developing charge on the leaving group RO^- even though they are unable to act as proton donors. The implication of the present findings, therefore, is that leaving group protonation by His101, an integral component of the generally accepted mechanism, is not critical for RNase catalysis.

We expect that any amino acid residue which has a hydrogen bond donor group could stabilize the leaving group and therefore promote the transesterification reaction. It has recently been reported that an H102K mutant of barnase retains residual activity though as yet detailed measurements of its kinetics have not been published (Jucovic and Hartley, 1995). We expect that as in the case of binase H101N and H101T, the barnase H102K mutant will also catalyse only the first cleavage step of the RNase reaction to form cyclic phosphate products. Perhaps surprisingly, the binase mutant H101E also has been reported to have 2% of the activity of the wild-type enzyme (Yakovlev et al., 1994); however there are no data concerning cGMP hydrolysis by this mutant.

Finally, by mutating the catalytic histidine of binase to asparagine and threonine, we have engineered catalysts which are functionally distinct from the wild-type enzyme. The mutant proteins retain enzymatic activity in the transesterification step of RNA cleavage but lack catalytic activity towards guanosine 2′,3′-cyclic phosphate. In an active site that catalyses two similar reactions with subtly different chemistry, these binase mutants impose, in some sense, a step-selectivity. We hope that more of the hidden details of the ribonuclease catalytic mechanism will be revealed by analysis of crystals of the H101N and H101T mutants complexed with cGMP.

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A.L. Okorokov et al.


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