Kinetic Characterization of the Recombinant Ribonuclease from *Bacillus amyloliquefaciens* (Barnase) and Investigation of Key Residues in Catalysis by Site-Directed Mutagenesis†

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ABSTRACT: Barnase, the ribonuclease from *Bacillus amyloliquefaciens*, has been cloned and expressed in *Escherichia coli* [Hartley, R. W. (1988) *J. Mol. Biol. 202*, 913–915], thus enabling the overproduction and site-directed mutagenesis of one of the smallest enzymes (M_r equals 12382). As barnase is also composed of just a single polypeptide chain with no disulfide bridges and has a reversible folding transition, it affords a fine system for studying protein folding and design. We show here that the recombinant enzyme has properties identical with those of the authentic enzyme, characterize the basic kinetics and specificity of the enzyme, and, using site-directed mutagenesis, identify key residues involved in catalysis to provide evidence that supports the classic ribonuclease mechanism. The wild-type enzyme catalyzes the hydrolysis of dinucleotides of structure GpN. There is a prime requirement for G and a preference for A > G > C > U for N. The pH–activity curve for the transesterification step of dinucleotides is bell shaped with an optimum for k_{cat}/K_M and k_{cat} at about pH 5. The enzyme is far more active toward long RNA molecules, and the pH optimum for k_{cat} is at 8.5. The activity of barnase toward dinucleotide substrates is about 0.5% of that of the highly homologous T_1 nuclease at pH 5.9, but barnase is twice as active as T_1 toward RNA at pH 8.5. There must be important subsite interactions that contribute to catalysis in barnase in addition to those immediately on either side of the scissile bond. The kinetics implicate a catalytic base of $pK_a \sim 4.4$ and an acid of $pK_a \sim 5.7$, the two values being too close to analyze with accuracy. ^1H NMR titration shows that His-102 at the active site has a pK_a of 6.3. Mutation of His-102 → Ala virtually abolishes activity as does mutation of Glu-73 at the active site. This supports the previously proposed mechanism whereby Glu-73 is the active site general-base and His-102 the general-acid catalyst. These results are in contrast to mutagenesis studies on T_1 nuclease which are in conflict with the classical mechanism and where it is proposed that a second histidine (His-40) is the general base. However, on mutation of the residue in barnase that is analogous to His-40 in T_1 (Asp-54 → Ala), there is still 9% activity against RNA.

Ribonucleases have played a central role in the development of enzyme chemistry. Their small size has facilitated rapid protein sequencing, X-ray crystallography, NMR, and other biophysical studies. Interest continues in them as model systems because of the advent of protein engineering. Their small size again makes them attractive targets for protein design: they are amenable to theoretical calculations that can be combined with structural studies on wild-type and engineered mutants. Barnase, the extracellular ribonuclease produced from *Bacillus amyloliquefaciens*, is especially suitable as a paradigm for protein design. The enzyme consists of a single polypeptide chain of only 110 amino acids with no disulfide bridges and M_r = 12382 (Hartley & Barker, 1972). The crystal structure has been solved at 2 Å (Mauguen et al., 1982) and diffracts to 1.4 Å (Dr. C. Hill, personal communication). The enzyme has a well-resolved ^1H NMR spectrum and is small enough for sequence-specific 2D NMR assignment (M. Bycroft, unpublished data).

Primary sequence homologies have shown that barnase is a member of the family of microbial ribonucleases which includes the ribonuclease T_1 from *Aspergillus oryzae* (Hartley, 1980). Detailed kinetic analysis has been performed on only one enzyme from the group of microbial ribonucleases, T_1, the eukaryotic ribonuclease from *A. oryzae*, because of low yields of enzyme from other organisms. The similarity between barnase and T_1 ribonuclease is emphasized by comparison of their tertiary structures; both proteins share common structural motifs that can be superimposed (Mauguen et al., 1982), although the T_1 enzyme contains two disulfide bridges. T_1 ribonuclease has been well characterized kinetically and shows a marked specificity for guanylyl linkages (Takahashi, 1965; Walz et al., 1979). The mechanism of action is similar to that of pancreatic ribonuclease in that a cyclic intermediate is formed in the first step (=transesterification) of the reaction followed by hydrolysis of this intermediate. The products of hydrolysis of RNA and various oligonucleotides by barnase are consistent with the formation of a cyclic intermediate and indicate the marked preference of barnase for -GpGp- and GpAp- linkages (Rushizky et al., 1963). Further characterization of barnase has been difficult owing to the low yields of protein obtained from *B. amyloliquefaciens*.

Attempts to clone the gene into a plasmid vector to increase yields have been reported (Paddon & Hartley, 1985, 1987), but it has proved impossible to clone the gene for active barnase on its own. This is in contrast to what has been found for other extracellular enzymes from *B. amyloliquefaciens* (Palva, 1982; Vasantha et al., 1984) and is most likely due to the lethal nature of the gene product. In *B. amyloliquefaciens*, the intracellular action of barnase is inhibited by a specific intracellular inhibitor, barstar (Hartley & Smeaton, 1973). The native gene for barnase has now been cloned together with the

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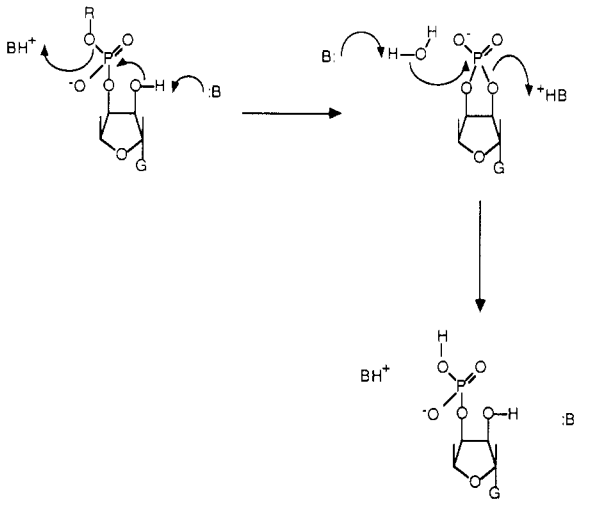

FIGURE 1: Classical mechanism of action proposed for ribonucleases. The first step is the transesterification (or cyclization) reaction where the general base is proposed to be His-12 in RNase A, Glu-58 in T_1, and Glu-73 in barnase and the general acid is His-119 in RNase A, His-92 in T_1, and His-102 in barnase. The role of the two residues is reversed in the subsequent hydrolysis step.

gene for barstar (Hartley, 1988). In this construction, the structural gene for barnase has been fused to the alkaline phosphatase promoter and the signal sequence from *Escherichia coli*, *phoA*; the barstar gene is located downstream of the barnase gene and is expressed from its own promoter. The availability of this construct has meant that sufficient quantities of barnase can be produced from small-scale cultures to perform detailed mechanistic and structural studies.

Chemical modification and primary amino acid homologies have provided evidence for the involvement of three residues in the activity of the microbial ribonucleases (Arata et al., 1979; Egami et al., 1980; Inagahi et al., 1981; Takahashi & Moore, 1982) which include His-92, Glu-58, and Arg-77 of T_1 ribonuclease. The residues considered to be catalytically important in barnase are the analogous residues His-102, Glu-73, and Arg-87. The catalytic mechanism of T_1 was originally thought to be the classic mechanism exhibited by RNase A (Findlay et al., 1962) with the Glu-58 acting as the catalytic base in the cyclization step (Figure 1). However, mutation of Glu-58 to Ala, Gln, and Asp gives enzyme retaining significant activity (Nishikawa et al., 1987). Barnase has been chosen by this laboratory as a system for intensive study for protein design (Kellis et al., 1988). This study reports a characterization of the kinetics of the recombinant enzyme produced by Hartley (1988). The relevance of residues His-102, Glu-73, and Arg-87 to catalysis is examined with site-directed mutagenesis as have the roles of Lys-27, Arg-83, and Asp-54. The pH dependence of k_{cat}/K_m and the specificity of barnase at the scissile bond have been investigated with dinucleotide substrates.

Materials and Methods

Bacterial Strains, Bacteriophages, and Plasmids. The *E. coli* strains used were TG2 [F′(*tra*D36 *pro*AB *laq*I^q *lac*Z ΔM15)/K12 Δ(*lac-pro*) *supE thi* recA Sr1::Tn10 c *hsd*5 (EcoKr$^-$m$^-$)] (Gibson, 1984) and for site-specific mutagenesis BMH71-18 *mut*L [F′(*pro*AB *laq*I^q *lac*Z ΔM15)/K12 Δ(*lac-pro*) *sup*E *thi mut*L::Tn10)] (Kramer et al., 1984). Bacteriophage M13mp8 (Messing & Vieira, 1982) was used for mutagenesis.

The barnase and barstar genes had previously been cloned into pUC19 by Hartley (1988). The recombinant plasmid, pMT410, was a gift from Dr. Hartley. After mutagenesis in M13, the fragment containing the barnase and barstar genes was cloned back into pUC9 (Messing & Vieira, 1982) as described below.

Enzymes and Chemicals. All enzymes were obtained from commercial suppliers and used according to the manufacturers' instructions. The radiochemicals were from Amersham International; RNA from yeast Torula, guanylyl-(3′–5′)-nucleosides (GpN), and T_1 ribonuclease were all purchased from Sigma. SP-Trisacryl was from IBF and dialysis tubing of M_r cutoff 3500 was obtained from Spectrum Medical Industries, Inc. All other reagents were purchased either from Sigma or from BDH.

Recombinant DNA Techniques. Plasmid DNA and replicative M13 DNA was isolated from *E. coli* by the method of Birnboim and Doly (1979). Transformation with plasmid DNA and transfection with M13 were carried out by standard procedures (Mandel & Higa, 1970; Messing, 1983). Before ligation, the inserts were purified from 0.8% agarose gels by running the DNA into NA45 membranes (Schleicher & Schuell) and eluting for 1 h at 70 °C into 1 M NaCl/50 mM arginine (E. Shephard, personal communication).

Recombinants of plasmid pUC9 were screened by an adaptation of the method of colony blotting described by Grunstein and Hogness (1975) in which a synthetic oligonucleotide phosphorylated with [γ-^{32}P]dATP was used as a probe. Recombinants of M13 were selected by standard methods (Messing, 1983) and screened by the method of Birnboim and Doly (1979) to verify that an insert of the correct size was present.

Site-Directed Mutagenesis. Single-stranded M13 template was prepared as described by Bankier and Barrell (1983). The mutagenic primers Lys-27 → Ala (TTCTGAT-C*G*TGTAAT), Asp-54 → Ala (CGGCGGAG-C*CATCTTCT), Glu-73 → Ala (ATCCGCT-G*CACGCCA), Arg-83 → Ala (TGAATTTG*C*-GAAGCC), Arg-87 → Ala (AAGAATCG*C*GTCTGA), and His-102 → Ala (TTGATAAG*C*GTCCGT) (asterisks follow the mismatches) were synthesized on an Applied Biosystems 380B DNA synthesizer. Phosphorylation of the primers, annealing, extension, and ligation were carried out as described by Zoller and Smith (1983). The mutant bacteriophages were identified by an oligonucleotide hybridization method described by Carter et al. (1984), using the mutagenic primer, phosphorylated with [γ-^{32}P]dATP, as a probe. The mutant phages were easily distinguished as hybridization-positive colonies after the filters had been washed twice for 2 min in 6× SSC (stock solution 20×: 175.3 g of NaCl and 88.2 g of sodium citrate per liter) at 3 °C below the calculated melting temperature (Suggs et al., 1981) for each primer.

Nucleotide Sequencing. The mutations were verified by dideoxy sequencing (Sanger et al., 1977) after plaque purification of phage from the hybridization-positive colonies. The sequencing primer used was either a universal primer for M13 or a specific primer with homology to a suitable stretch on the barnase gene. The primers were synthesized in this laboratory as described above.

Growth Conditions. For small-scale isolation of barnase (1 L), an overnight 10-mL starter culture in Luria broth (Lennox, 1955) was used to inoculate two 2-L Erlenmeyer flasks containing 500 mL of a low-phosphate media (MOPS) described by Serpersu et al. (1986). Cells were routinely grown for 18 h at 37 °C with vigorous shaking before harvesting. Large-scale isolation of barnase was from 12-L fermentations grown in MOPS media with a 0.8% overnight inoculum grown in LB broth. Fermentations were routinely performed at 37 °C in

an MBR MCS10 Bioreactor with constant stirring at 700 rpm and aeration of 12 L/min. Barnase was purified from the media after approximately 20 h when the A_{600} of the cell suspension was above 2.

Purification of Barnase. Purification of barnase was adapted from the procedure outlined in Paddon and Hartley (1987). Culture medium was chilled to 0 °C before addition of glacial acetic acid at 55 mL/L of culture. Acidification releases residual barnase from the periplasm into the medium (R. W. Hartley, personal communication). The acidified media was centrifuged for 10 min at 4 °C and 10000*g* to remove the cells; SP-Trisacryl was added at 5 mL/L of supernatant. Barnase was allowed to adsorb to the SP-Trisacryl at 4 °C for 1.5–2 h with gentle mixing during which time >95% of the protein adsorbed to the column. The slurry was then allowed to settle before the supernatant was decanted and the SP-Trisacryl was washed in low-salt buffer (0.05 M sodium acetate/acetic acid, pH 5.0). Barnase was eluted from the SP-Trisacryl following packing of the material into a small column, washing extensively with the same buffer, and eluting with high-salt buffer (0.05 M sodium acetate/acetic acid, pH 5.0, containing 1.0 M NaCl). The barnase was dialyzed against low-salt buffer overnight with low M_r cutoff dialysis tubing; barnase at this stage is generally >90% pure. Further purification is achieved on the Pharmacia FPLC system. Barnase was loaded onto a MONO-S column preequilibrated with the low-salt buffer; elution was with a linear gradient of 0–40% with the high-salt buffer. The resulting protein is homogeneous as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis and isoelectric focusing.

Enzyme Assays. All experiments were performed at 25 °C. The concentration of enzyme was determined spectrophotometrically at 280 nm where $E_{0.1\%} = 2.01$ (Hartley & Rogerson, 1972). The values thus obtained are consistent with those obtained with the Bradford reagent (Bradford, 1976).

(*a*) *Rapid Assay of Barnase Activity.* Yeast Torula RNA was used to assay barnase activity as described by Rushizky et al. (1963). Alternatively, RNA hydrolysis was followed spectrophotometrically by diluting purified yeast RNA to 2 mg/mL in Tris-HCl, 0.1 M ionic strength, pH 8.5, and following the decrease in absorbance at 298.5 nm. The RNA solution (1 mL) was prewarmed before addition of 10 μL of appropriately diluted barnase. Estimations of enzyme activity were made by comparing the rates of RNA hydrolysis against those obtained by known quantities of barnase.

(*b*) *Assay of Barnase Activity Using GpN Dinucleotides.* The values of K_m and V_{max} for the transesterification step of GpN dinucleotides were determined from initial velocities by following the increase in absorbance at 280 nm (Zabinski & Walz, 1976). All reactions were performed in 0.1 M ionic strength sodium acetate/acetic acid buffer to give a total assay volume of 1 mL in a 1-cm path-length cuvette with a Gilford 2600 spectrophotometer. The changes in molar extinction coefficients on total hydrolysis of dinucleoside phosphate substrates were measured by observing the increase in absorbance at 280 nm in buffer; values so determined agreed with previously published values (Osterman & Walz, 1978). Values of $\Delta\epsilon_{280}$ for each of GpA, GpG, GpC, and GpU are 930, 1500, 2130, and 850 M^{-1} cm^{-1}, respectively. Initial velocities were followed over a substrate concentration of 2.0×10^{-5} to 1.2×10^{-3} M and enzyme concentrations of 5.0×10^{-7} to 2.0×10^{-6} M. Owing to the high background absorbance of these substrates at high concentrations, 0.2-cm path-length cuvettes were used, and the assay volume was reduced to 0.5 mL. A minimum of 15 initial velocities was recorded for a given substrate with low substrate concentrations repeated mostly in duplicate. The data were analyzed by nonlinear regression analysis with the program ENZFITTER (Leatherbarrow, 1987) from which values and standard errors of K_m and V_{max} were obtained.

(*c*) *Coupled Enzyme Assay for Transesterification Step of GpA.* The release of A from GpA in the transesterification reaction can be followed by measuring the release of adenosine with adenosine deaminase (Ipata & Felicoli, 1968); adenosine is deaminated to inosine with a concomitant change in absorbance at 265 nm. K_m and V_{max} were determined from initial velocities as described under (b) at the same pH except that the decrease in absorbance at 265 nm was followed. The total assay volume was 1 mL containing 2×10^{-7} M adenosine deaminase and 4×10^{-7} M barnase. Decreasing the amount of adenosine deaminase by half or doubling its concentration did not affect the rate of the transesterification step of GpA, indicating that the steady-state concentration of the products was being monitored and not the reaction of the coupling enzyme. Substrate concentration was varied from 5.0×10^{-5} to 5.5×10^{-4} M. At the higher substrate concentrations, the reaction was monitored in a 0.2-cm path-length cuvette. The $\Delta\epsilon_{265}$ for the change in absorbance in going from adenosine to inosine was taken as 8.2×10^3 M^{-1} cm^{-1} (Baer et al., 1968).

(*d*) *pH Dependence of K_m and k_{cat} for the Transesterification Step of GpA.* The variation of the Michaelis–Menten parameters with pH was determined by the direct spectrophotometric assay using initial rates of transesterification of GpA (0.02–1.0 mM). The buffers were all at 0.05 M ionic strength with KCl added to give a total ionic strength of 0.15 M. Sodium formate/formic acid was used at pH 3.5, sodium acetate/acetic acid at pH 4.0–5.5, and imidazole/HCl at pH 6.5–7.5.

(*e*) *pH Dependence of k_{cat}/K_m.* Both GpC and GpG were used as substrates. Stock buffers were chosen in the pH range 2.8–7.8 and at 0.1 or 1.0 M ionic strength; these were then diluted to give the required ionic strength. The buffers were sodium formate/formic acid at pH 2.8–4.0, sodium acetate/acetic acid at pH 3.8–6.0, and imidazole/HCl at pH 6.0–7.8. Substrate (20 μL of 0.5 mM stock made in water) was added to buffer in a total volume of 0.99 mL and preincubated at 25 °C in a quartz cuvette. The reaction was started by adding 10 μL of 0.5 mM enzyme and following the change in absorbance at 280 nm. Under these conditions, where the initial substrate concentration is at least 20 times lower than the K_m and there is no product inhibition, hydrolysis of substrate is found to obey first-order kinetics [cf. Fersht and Renard (1974)]. Since the substrate concentrations used are low, very small changes in absorbance are observed (typically 0.02 absorbance unit). To facilitate the measurement of such small changes, the digital output of the spectrophotometer was fed directly into a BBC microcomputer and was analyzed as described by Russell et al. (1987).

(*f*) *Hydrolysis of cGMP.* cGMP was prepared as a stock solution of 9 mM in a buffer containing 0.5 mM imidazole/HCl (pH 6.4) and 0.1 M KCl. This was diluted into the same buffer to give concentrations between 0.2 and 6 mM. Reaction was initiated by adding 5 μL of barnase (7 mg/mL stock) to 1.0 mL of substrate at 25 °C in a Metrohm pH stat. The initial rate of proton release at pH 6.4 was measured by the automatic addition of aliquots of 0.5 N NaOH under nitrogen.

RESULTS

Purification and Activity of Barnase from E. coli. Barnase was expressed from pMT410 to give routinely 25–30 mg of homogeneous protein per liter of cells after purification. The

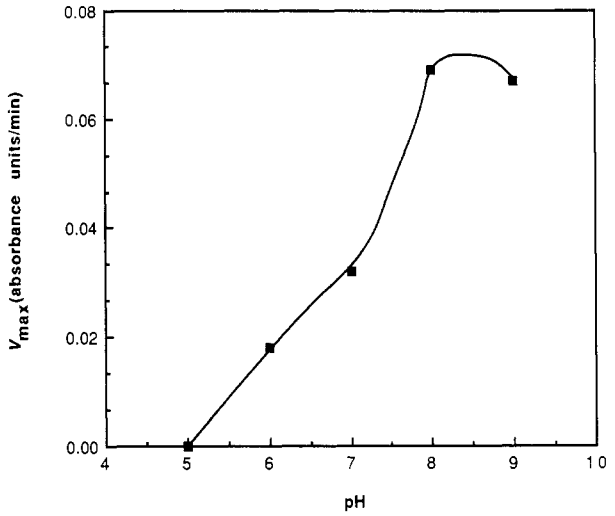

FIGURE 2: pH dependence of RNA hydrolysis. The hydrolysis was followed at different pHs in 0.1 M ionic strength buffer at 25 °C with the precipitation assay of Rushizky et al. (1963).

specific activity of this enzyme as measured by RNA hydrolysis (Rushizky et al., 1963) is $\sim 2 \times 10^6$ units/mg and corresponds to the activity obtained for enzyme purified from the original host (Hartley & Rogerson, 1972). The pH optimum for hydrolysis of RNA catalyzed by the recombinant enzyme is pH 8.5 (Figure 2) and is identical with that of the authentic enzyme from *B. amyloliquefaciens* (Rushizky et al., 1963). $NaDodSO_4$–PAGE of the purified recombinant barnase from *E. coli* using the method of Swank and Murkres (1971) for small molecular weight proteins gives a single band which corresponds to the M_r determined from the amino acid sequence.

Kinetics. (*a*) *Transesterification of Dinucleotides and Not Hydrolysis Is Being Monitored.* Preliminary experiments were performed to compare the rate of release of adenosine from GpA followed by the coupled assay using adenosine deaminase with that of the overall reaction by the pH stat (proton release occurs only on the hydrolysis of cGMP to GMP). The overall reaction showed nonlinear kinetics with a long lag phase followed by a proton release that was slower than the initial rate of release of adenosine. This was found to result from the barnase-catalyzed hydrolysis of cGMP having a relatively high value of K_m and a low value of k_{cat} (1.4 mM and 0.03 s^{-1} at pH 6.4 compared with 0.3 mM and 0.5 s^{-1} for release of adenosine). Thus, in terms of the classical mechanism of Figure 1, the cGMP formed from GpA rapidly diffuses from the enzyme, and the enzyme continues to cyclize more GpA until the concentration of cGMP is sufficiently high to compete with the binding and reaction of GpA. The scheme of Figure 1 is not an obligatory sequence of events as in, say, the acyl-enzyme pathway for a serine protease. The rates of hydrolysis and transesterification are equal when $[cGMP](k_{cat}/K_m)_{hydrolysis} = [GpA](k_{cat}/K_m)_{trans}$ [cf. Fersht (1985), p 112]. This occurs at pH 6.4 when [cGMP]/[GpA] = 28, i.e., after 96% reaction. In practice, therefore, the following of the reaction of GpA by initial rates ($\sim$5% of reaction) measures the transesterification step without any significant competition from overall hydrolysis. Some reactions described below examine the transesterification reaction by looking at a total progress curve for [GpA] < 10 μM. As the cGMP produced is so far below the value of K_m, it does not perturb the kinetics of transesterification as the enzyme is greater than 99% unligated.

(*b*) *Specificity of Barnase for Dinucleotides GpN.* The transesterification of GpN dinucleotides can be conveniently followed spectrophotometrically at 280 nm (Zabinski & Walz, 1976; Osterman & Walz, 1978). The advantage of using defined substrates over RNA is that a measure of both K_m and k_{cat} may be measured for the transesterification of a specific bond.

Table I: Specificity of Barnase for Dinucleotides GpN at pH 5.78[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
GpA	220	0.80	3640
GpG	103	0.22	2090
GpC	292	0.39	1320
GpU	375	0.36	950
GpA[b]	203	1.02	5024

[a] Transesterification of substrates was followed as described under Materials and Methods from the direc measurement of ΔA on hydrolysis. Substrate concentration was varied from 2.0×10^{-5} to 1.2×10^{-3} M; enzyme concentration was maintained at 1.8×10^{-6} M. All reactions were carried out at 25 °C in 0.1 M sodium acetate/acetic acid buffer at pH 5.78. [b] Determined from the coupled enzyme assay. The reaction contained 2.0×10^{-7} M adenosine deaminase and 4.0×10^{-7} M barnase. Otherwise, all reaction conditions were the same.

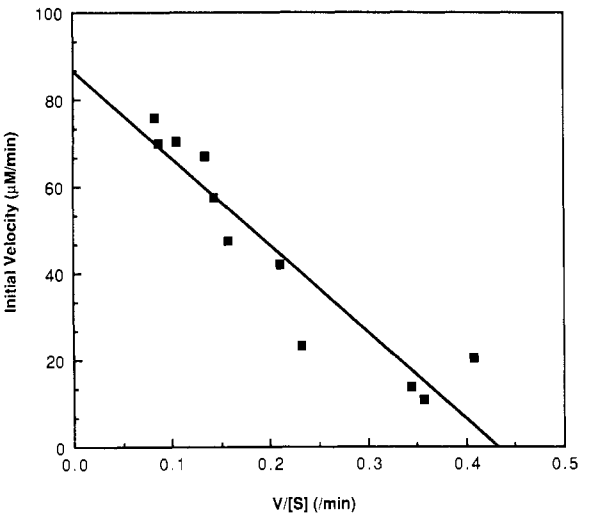

FIGURE 3: Eadie–Hofstee transformation of data from initial velocity measurements of GpU transesterification. Transesterification of GpU was followed at pH 5.78 in 0.1 M ionic strength sodium acetate/acetic acid buffer at 25 °C by measuring the increase in absorbance at 280 nm; other details are under Materials and Methods.

Initial experiments were performed at pH 8.5 since this is the optimal pH found for the hydrolysis of RNA. Minimal hydrolysis was observed at this pH although significant rates were observed between pH 5 and pH 6. From the measurement of initial velocities, the values for K_m and k_{cat} were determined for each of the substrates at pH 5.78 with the same batch of enzyme (Table I). Figure 3 shows an Eadie–Hofstee plot of the data for GpU. The background absorbance at high substrate concentrations is such that the highest concentrations used were at most $5K_m$. Values for k_{cat}/K_m indicate that the enzyme preferentially catalyzes the transesterification of GpA and GpG linkages over GpC and GpU with the order of specificity being GpA > GpG > GpC > GpU. This agrees with previous data (Rushizky et al., 1963) in which the products of RNA hydrolysis were analyzed. As a check of the assay system employed, the transesterification of GpA was observed with a different method. In this instance, a coupled enzyme assay system was used in which GpA transesterification was followed by measuring the release of adenosine with adenosine deaminase (Ipata & Felicoli, 1968). The values determined are within 25% of those measured by the direct assay (Table I), which is within the reproducibility of the procedures. Since there is no spectral change associated

Table II: Comparison of the Activities of T_1 Ribonuclease and Barnase[a]

enzyme	RNA hydrolysis (pH 8.5) specific activity [$\Delta A_{298.5}$ min^{-1} (nmol of enzyme)$^{-1}$]	GpA transesterification (pH 5.9) K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
barnase	0.00320	164	0.59	3.60×10^3
T_1	0.00165	56	110	1.95×10^6

[a] The activity of barnase and T_1 for RNA hydrolysis was determined in 0.1 M ionic strength Tris-HCl buffer at 25 °C. GpA hydrolysis was followed in 0.1 M ionic strength imidazole/HCl buffer at 25 °C; enzyme concentrations were 831 nM and 2.85 nM for barnase and T_1, respectively. Substrate was varied from 5×10^{-6} M to 1.2×10^{-3} M.

with the transesterification of any other dinucleotides NpN, the coupled enzyme assay was used to detect transesterification of ApA. No transesterification was observed under the conditions of the experiment even when the enzyme concentration was raised 10-fold. However, this does not preclude that a very low level of transesterification may be occurring and may be detectable if prolonged incubation times as well as more sensitive detection methods were employed. Walz et al. (1979) have found that T_1 ribonuclease does hydrolyze ApA, albeit at very low levels.

(*c*) *pH Dependence of K_m and k_{cat} of Barnase.* Previous study of the specificity of T_1 ribonuclease for the transesterification of GpN nucleotides has shown that there is a broad pH optimum for all the substrates covering a pH range of 5–7 (Osterman & Walz, 1978). This is unlike barnase which shows a very slow transesterification of GpN nucleotides at pHs above 6. Further, T_1 has values of k_{cat}/K_m of $\sim 10^3$ times greater than those for barnase at pH 5.9 (Walz et al., 1979). To ensure that this variation was not a result of the assay system employed, activities of barnase and T_1 were compared in parallel for GpA transesterification at pH 5.9 and RNA hydrolysis at pH 8.5 (Table II). No discrepancy was found between those values presented here and those previously reported for GpA transesterification. Further, the specific activity of barnase for RNA hydrolysis at pH 8.5 is twice that of T_1 ribonuclease.

The pH dependence of k_{cat}, K_m, and k_{cat}/K_m for the transesterification of GpA is shown in Figure 4: k_{cat}/K_m is a sharp bell-shaped curve; K_m increases above pH 6; and k_{cat} is a broader bell-shaped curve, not decreasing so rapidly at higher pH.

(*d*) *pH Dependence of k_{cat}/K_m.* The pH dependence of k_{cat}/K_m generally follows the ionization constants of the free enzyme and substrate, although there can be artifacts (Fersht, 1985). As the pH dependence of k_{cat}/K_m is a bell-shaped curve, many data points are required to fit theoretical curves. These were obtained by the method of Fersht and Renard (1974), which allows a large number of points to be obtained on the same day with the same batch of enzyme under identical conditions. At substrate concentrations much lower than K_m and when there is no product inhibition, the rate of decrease of substrate, $d[S]/dt$, is equal to $[E]_0(k_{cat}/K_m)[S]$ or $V_{max}/K_m[S]$. That is, there is a first-order progress curve with rate constant V_{max}/K_m. At $[S]_0 \ll K_m$, the amount of products formed is low, and product inhibition is negligible. The complete progress curve for transesterification is measured in a single experiment to give V_{max}/K_m. This is a far more accurate procedure than measuring initial rates since first-order kinetics with a change in signal of close to 100% are easier to monitor than the 5% change of signal in an initial rate experiment. Further, although the first-order reaction is monitored over a manyfold change in [S], the first-order constant is independent of [S], thus removing errors of dilution on preparing substrate solutions.

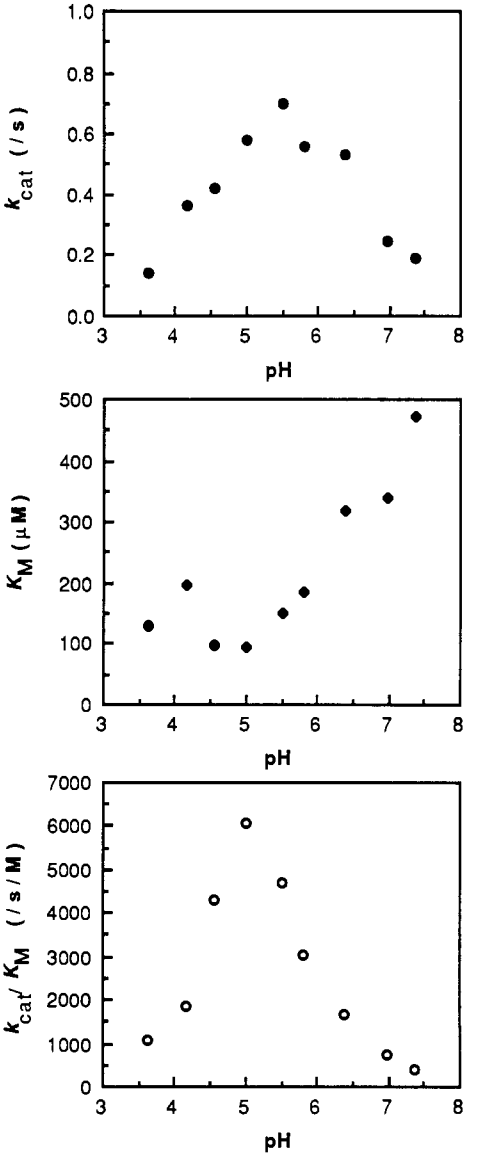

FIGURE 4: pH dependence of k_{cat}, K_M, and k_{cat}/K_M for the transesterification of GpA at 25 °C and ionic strength 0.15 determined from initial rate experiments.

Determinations were made for two dinucleotide substrates, GpN and GpC. Since the change in absorbance on transesterification of GpN dinucleotides is very small, even on complete hydrolysis (typically 10%), GpC was used as the preferred substrate since it gives the largest change in absorbance and $\Delta\epsilon_{280}$ does not change in the pH range 3–8 (Zabinski & Walz, 1976). Substrate concentration was chosen at 10 μM, which is <5% of the K_m at all values of pH studied. k_{cat}/K_m was determined for a minimum of 20 different values of pH. The data so obtained were fitted to the theoretical bell-shaped curves of the ionization of an acid and a base (Figure 5).

The pH dependence of k_{cat}/K_m in buffers of ionic strength 0.1 M fits to a bell-shaped curve generated by two groups with pK_a's estimated at 4.5 and 5.6 by use of ENZFITTER (Leatherbarrow, 1987). Slopes of the plots of log (k_{cat}/K_m) vs pH are useful for determining the number of protons involved in the ionizations. This is difficult to apply here because of the narrow bell shape and having to use data at the extreme limits of detection. The slopes tend to approximately 1 for the ascending limb and −0.8 for the descending limb (not shown),

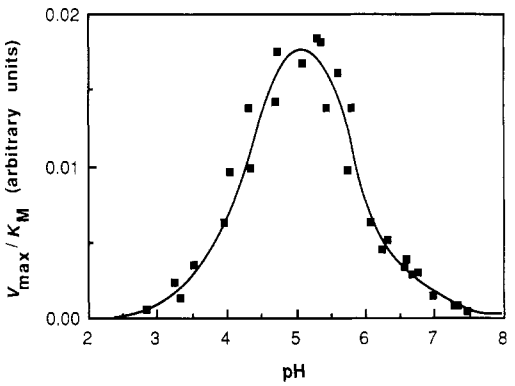

FIGURE 5: pH dependence of V_{max}/K_M for GpC transesterification by barnase derived from first-order determinations. Progress curves were followed at 25 °C at 280 nm with 1×10^{-5} M GpC and 5×10^{-6} M barnase in 0.1 M standard buffers.

Table III: pK_a's Derived from pH Dependence of k_{cat}/K_m for Dinucleotide Substrates and NMR[a]

ionic strength (M)	substrate	$(pK_a)_1$	$(pK_a)_2$	pK_a of His-102 from NMR
0.10	GpC	~4.5	~5.6	
0.10	GpG	~4.0	~5.6	
0.02	GpC	~4.4	~5.7	
0.02				6.3 ± 0.05

[a] Reactions were followed at 280 nm at 25 °C in the presence of 10.0×10^{-6} M substrate and 5.0×10^{-6} M enzyme. pK_a's were derived from fitting the bell-shaped curve to two ionization constants with the program ENZFITTER (Leatherbarrow, 1987). The two pK_a's are too close for accurate determination.

which suggest that a single ionizing group on the free enzyme or substrate is necessary in its acidic form and one group in its basic form. Repetition of the kinetics using GpG as the substrate gave two pK_a's of 4.0 and 5.6. The different value of the lower pK_a may indicate that substrate ionization is contributing to this pK_a; slopes from a plot of log (k_{cat}/K_m) gives values closer to 1.6 and −1.2 for the ascending and descending slopes, respectively, suggesting that the lower pK_a may represent more than a single ionization. The pH dependence of k_{cat}/K_m for GpC was repeated at a lower ionic strength of 0.02 M so that a direct comparison could be made with NMR data (see below). The data so obtained gave two pK_a's of 4.4 and 5.7, which are not significantly different from those at higher ionic strength. The pK_a's determined for the different substrates and ionic strengths are listed in Table III. From previous descriptions of microbial ribonuclease activity, the values of the two pK_a's may represent the active site His-102 and Glu-73. It must be emphasized that the proximity of the two pK_a's makes accurate estimates of their values difficult—at least two units of separation is desirable for accurate analysis. At best, it is realistic to state that there is one pK_a in the range 4–5 and the other in the range 5.5–6.5.

^{1}H NMR has now been performed on barnase (Sali et al., 1988) and shows that the active site His-102 titrates with $pK_a = 6.3 \pm 0.05$ at low ionic strength (0.02 M). This value is not inconsistent with the kinetic data given the uncertainties in the measurements.

Mutagenesis. Several amino acids have been implicated as functionally important in catalysis by barnase (Hill et al., 1983). To investigate the role of these amino acids in both structure and function, site-directed mutagenesis was employed. The genes for barnase and barstar are located on a 1.4-kb fragment between *Eco*RI and *Hin*dIII sites in pUC19. For mutagenesis, this fragment was excised with the appropriate restriction enzymes and ligated to M13mp8. The ligation mixture was transformed into *E. coli* TG2, and 24 colorless plaques were selected for further identification. This was carried out by agarose gel electrophoresis of *Eco*RI–*Hin*dIII digested from small-scale RF preparations.

Table IV: Activity of Mutant Barnase Enzymes

mutation	RNA hydrolysis (%)[a]	GpA transesterification[b] K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
H102 → A	no activity	no activity (<0.01%)		
E73 → A	0.2	no activity (<0.01%)		
K27 → A	1.3	352	0.000216	6
D54 → A	9	75	0.059	787

[a] Percent activity of wild-type enzyme against yeast Torula RNA at pH 8.5 and 25 °C. [b] At pH 5.78 and ionic strength 0.1 M, as in Table I.

The mutant bacteriophages were identified by colony hybridization and nucleotide sequencing. The frequency of formation of mutants was always greater than 1%.

Expression and Activity of Mutant Enzymes. For the purpose of expressing barnase in the absence of its intracellular inhibitor in *E. coli* (Paddon & Hartley, 1987), His-102 was altered to Asp with the concomitant loss of enzyme activity. Here we have replaced His-102 by alanine. The mutant protein is expressed at similar levels to wild-type barnase giving 20–24 mg of pure protein per liter of culture; no activity was observed for the mutant either against RNA or GpA, which confirms the essential role of this residue in catalysis.

The second active site residue to be altered was Glu-73 → Ala. Measurement of activity against RNA revealed that only 0.2% of wild-type activity remains and that no transesterification of GpA was observed under the experimental conditions. These results again confirm the essential role of this residue in catalysis.

The third amino acid to be altered was Lys-27 → Ala. It was suggested that this residue may play a role in catalysis by binding the 3′-phosphate and hence have a significant function (M. Prevost and S. Wodak, personal communication). Measurement of RNA and GpA hydrolysis has confirmed the importance; only 1.3% of wild-type activity remains against RNA whereas k_{cat} for GpA transesterification decreases by 3500-fold (Table IV).

Mutation of Asp-54 → Ala results in a decrease of 91% in activity against RNA. This residue is in an analogous position to His-40 of T_1 ribonuclease, which has been shown to be essential in catalysis by this enzyme. The decrease in activity for Asp-54 → Ala is not substantial enough to speculate that this residue is essential in catalysis.

Arg-87 and Arg-83 were both altered to Ala. As yet, it has not been possible to isolate significant quantities of the mutant proteins to perform a kinetic analysis. From the crystal structure both Arg-87 and Arg-83 are in position to form a number of contacts with other amino acids and water molecules; e.g., Arg-83 forms a salt bridge with the buried Asp-75. Consequently, altering these residue contacts could change the stability of these mutant proteins. It is found that destabilizing the structure of barnase greatly reduces the yield of enzyme (Kellis et al., 1988), as has been found for these mutants.

Discussion

Properties of the Recombinant Enzyme. The expression of recombinant barnase from *E. coli* gives enzyme that has the same specific activity, M_r, and pH profile for RNA as does the authentic enzyme from *B. amyloliquefaciens*. These results confirm that there has been no observable change in this

ribonuclease expressed from *E. coli* and that large quantities of enzyme can be purified from small-scale fermentations.

Catalytic Mechanism of Barnase. Site-directed mutagenesis has been used to identify catalytically important residues in barnase. Modification of Lys-27 → Ala, of which there is no analogous residue in T_1 ribonuclease, does not alter K_m significantly for GpA and so is not involved in binding of the substrate. However, k_{cat} for the reaction decreases to 0.03% of wild-type activity, demonstrating the importance of this residue in catalysis. A possible function has been proposed in the binding of the 3′-phosphate. This must occur in the transition state since rate, and not ground-state binding, is affected.

Mutation of His-102 → Ala abolishes all detectable catalytic activity (>99.99%), consistent with an essential role of this residue in catalysis. Further, modification of Glu-73 → Ala reduces catalytic activity against RNA to 0.2% of wild type and to <0.01% for GpA. This is in contrast to T_1 ribonuclease in which the same mutation on the analogous Glu-58 gives rise to 5% residual activity for dinucleotide substrates (Nishikawa et al., 1987). Since there is significant activity remaining in the Glu-58 → Ala mutation, Nishikawa et al. (1987) have proposed that His-40 and not Glu-58 is the true catalytic base in RNase T_1 as mutation of His-40 to Ala results in a complete loss of activity. This hypothesis appears to be inconsistent with NMR and kinetic data on T_1. NMR shows that His-40 and His-92 titrate with pK_a's of 7.9 and 7.8, respectively (Arata et al., 1979; Inagaki et al., 1981), whereas kinetics indicate that binding and/or catalysis depends on two unprotonated groups on the free enzyme of apparent pK_a's of 3.4 and 4.3 and two protonated groups having apparent pK_a's of 7.5 and 8.1 (Osterman & Walz, 1878). However, the lower pK_a's may have complications from substrate ionization. The groups with pK_a values of 4.3, 7.5, and 8.1 appear to correspond to Glu-58, His-40, and His-92, respectively. Since both His-40 and His-92 are in their protonated forms, His-40 cannot act as the catalytic base and as yet has an undefined role in catalysis of T_1 ribonuclease. His-40 is conserved only among the eukaryotic group of microbial ribonucleases whereas in barnase and the other prokaryotic enzymes the analogous residue is aspartate or threonine. Modification of this aspartate in barnase, Asp-54 → Ala, does not reduce catalytic activity substantially (Table IV). The two pK_a's deduced from the pH dependence of k_{cat}/K_m are consistent with His-102 and Glu-73 being the catalytic acid and base, respectively. These results are consistent with the classical mechanism of action of RNase A holding for barnase.

Specificity of Barnase. The results presented here have reaffirmed the original observation of Rushizky et al. (1963) that barnase hydrolyzes GpA and GpG linkages. The differences in the specificities for GpN dinucleotides suggest that barnase has a second subsite specificity. There is evidence that T_1 ribonuclease has subsite specificity for the leaving nucleoside but unlike barnase expresses a preference for cytidine (Osterman & Walz, 1978). From the original crystallographic data it seemed that barnase and T_1 are very similar. However, detailed kinetic study of barnase has served to highlight the differences between these two enzymes. The value of k_{cat} for the transesterification of GpA by barnase is 190 times lower that by T_1 ribonuclease (Table II) even though barnase catalyzes RNA hydrolysis twice as effectively. The slow transesterification of GpN nucleotides by barnase is not a consequence of the primary specificity of the enzyme: there is no detectable hydrolysis of ApA, and Rushizky et al. (1963) found no evidence that any other linkages are hydrolyzed more rapidly in RNA than GpA or GpG.

Importance of Subsite Interactions in Barnase. The slow hydrolysis of dinucleotide substrates by barnase is a direct contrast to its efficient hydrolysis of RNA. This may indicate that these minimal substrates are binding nonproductively and that further subsite interactions may need to be utilized to produce efficient hydrolysis. Additional subsite interactions may also explain the large difference in the pH optimum (~3 pH units) between RNA and dinucleotide hydrolysis. Some evidence exists that T_1 ribonuclease may possess additional binding sites for longer substrates (Osterman & Walz, 1979).

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DNA Minor Groove Binding Agents Interfere with Topoisomerase II Mediated Lesions Induced by Epipodophyllotoxin Derivative VM-26 and Acridine Derivative *m*-AMSA in Nuclei from L1210 Cells†

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ABSTRACT: This study demonstrated that agents capable of interacting with the minor groove in nuclear DNA interfere with topoisomerase II mediated effects of antitumor drugs such as VM-26 and *m*-AMSA. Distamycin, Hoechst 33258, and DAPI were used as agents capable of AT-specific binding in the minor groove of DNA while producing no profound long-range distortion of DNA structure. In intact nuclei from L1210 cells, these minor groove binders inhibited the induction of topoisomerase II mediated DNA damage (DNA–protein cross-links and DNA double-strand breaks) by VM-26 and *m*-AMSA. The inhibitory effects of distamycin reflected prevention of formation of new lesions but not reversal of preexisting damage. The minor groove binders did not differentiate between lesions induced by an intercalator, *m*-AMSA, or by a DNA-nonbinding drug, VM-26. All three groove binders inhibited DNA breaks more strongly than DNA–protein cross-links. The inhibitory potency correlated with the size of minor groove binders and the size of their DNA-binding sites: distamycin (5 bp) > Hoechst 33258 (4 bp) > DAPI (3 bp). The results showed that DNA minor groove binders are a new type of modulators of the action of topoisomerase II targeted drugs.

DNA topoisomerase II has attracted considerable attention recently as a new target for antitumor drugs [for review see Ross (1985) and Wang (1987)]. This important enzyme controls topology of cellular DNA and plays a role in vital cellular processes such as replication, transcription, and mitosis (Brill et al., 1987; Liu & Wang, 1987; Snapka et al., 1988; Uemura et al., 1987). In the course of its reaction, topoisomerase II introduces a transient double-strand break in the DNA and binds covalently to 5′ ends of the broken strands (Ross, 1985; Wang, 1987; Tewey et al., 1984; Chen et al., 1984). This reaction intermediate is referred to as the "cleavable complex". A number of antitumor drugs trap the enzyme at this stage, preventing the restoration of intact DNA structure (Ross, 1985; Wang, 1987). Drug-induced stabilization of the cleavable complexes can be monitored in whole cells or nuclei as induction of DNA–protein cross-links accompanied by DNA double-strand breaks. The formation of these lesions correlates with antiproliferative effects of topoisomerase II targeted antitumor drugs (Ross, 1985; Wang, 1987).

Drugs stabilizing cleavable complexes of topoisomerase II belong essentially to two groups. The first group consist of drugs capable of binding to DNA by intercalation. A representative agent of this group is the acridine derivative *m*-AMSA (Ross, 1985; Tewey et al., 1984). However, interca-

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