

The role of Glu-60 in the specificity of the recombinant ribonuclease from *Bacillus amyloliquefaciens* (barnase) towards dinucleotides, poly(A) and RNA

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A computer model of the complex between G2'p5'G and barnase, the recombinant ribonuclease of *Bacillus amyloliquefaciens*, was constructed, based on the known structure of the complex RNAase T1·G2'p5'G. This model suggests that the conserved residue Glu-60 plays an important role in the specificity of barnase for guanosine. A barnase mutant was therefore made in which Glu-60 was replaced by Gln. This mutation increases the K_m for the dinucleotides GpC and GpA, by a factor of 10, but does not change the k_{cat} . For ApA, the k_{cat}/K_m decreases by a similar factor, but the individual parameters could not be determined. The mutation, however, has no influence on the k_{cat} and the K_m of barnase action towards RNA and poly(A). This demonstrates that the interactions between the substrate and the residue at position 60 must be different in the case of ApA and poly(A). For RNA, this conclusion is also likely, but not

absolutely certain, because barnase/RNA might be a Briggs–Haldane type enzyme/substrate pair. Therefore, if the effect of the mutation were limited to an increase of the dissociation rate constant of the substrate (k_{-1}), this would not be evident in K_m or k_{cat}/K_m . In view of the clear cut situation with poly(A), the pH profile for and the effect of salt concentration on the kinetic parameters of the mutant barnase were studied for this substrate. The influence of salt on the K_m can be interpreted via the linked function concept and shows a cooperative dissociation of 7–10 counterions upon poly(A) binding. The binding of the substrate is strongly reduced at high pH, and the pK_a involved decreases strongly at high salt concentrations. Poly(A) and RNA show a pH dependency of their absorbance spectrum, indicating a pH-dependent change of base stacking, which may influence the catalytic parameters.

INTRODUCTION

Barnase is a guanine-specific RNAase from *Bacillus amyloliquefaciens*. Like many enzymes that use polymeric substrates, barnase hydrolyses RNA much more efficiently than dinucleotides, indicating the presence of multiple subsites on the enzyme and demonstrating an influence of the interactions at these substrates on the conformation at the primary site.

A detailed study (Day et al., 1992) of barnase activity towards oligonucleotides of the type $Zp_0Gp_1Xp_2Y$, where X, Y and Z are any nucleoside and subscripts denote the position of a phosphoric diester, revealed that: (1) the minimal substrate is Gp_1X ($k_{cat}/K_m \sim 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$); (2) a considerable improvement is obtained when p_2 is present ($k_{cat}/K_m \sim 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$); (3) the limit of diffusion control is obtained when Y is also present ($k_{cat}/K_m \sim 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$); (4) Zp_0 does not influence the kinetic parameters. Furthermore, it is remarkable that the pH value of maximal k_{cat}/K_m is at a much higher pH for RNA hydrolysis than for the hydrolysis of dinucleotides (Mossakowska et al., 1989).

Here we report on the role of residue Glu-60. Our computer modelling studies suggest that Glu-60 determines in part the specificity of the enzyme, thanks to the H-bonds. $(G)H_1 \dots (Glu-60) \cdot OE_1$ and $(G)H_{21} \dots (Glu-60) \cdot OE_2$. Using the mutant E60Q, we show that Glu-60 contributes only to the specificity for the dinucleotides and not to that for poly(A) or RNA.

The effects of the ionic strength and pH on the catalytic parameters of poly(A) were studied in great detail. It is shown that binding of poly(A) is accompanied by the liberation of 7–10 counterions, and that poly(A) shows a pH dependency in its base stacking.

MATERIALS AND METHODS

Restriction enzymes were purchased from Boehringer (Mannheim, Germany). Dideoxy sequencing was done using the T₇ sequencing kit from Pharmacia (Uppsala, Sweden). SP-Trisacryl was purchased from IBF Villeneuve (La Garenne, France) and the Mono S and phenyl-Superose column were purchased from Pharmacia. The dialysis tubing (with cut-offs of 3500 and 13000 Da) was obtained from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). The purified samples were concentrated using Centricon microconcentrators (Amicon, Danvers, MA, U.S.A.). The substrates GpA, GpC, ApA and RNA were obtained from Sigma Chemical Co. Poly(A) was obtained from Pharmacia. Reagent-grade buffer materials, distilled and Millipore-filtered (pore diameter 0.45 μm) water were used in the preparation of buffers.

The bacterial *Escherichia coli* strains used in this study were WK6 ($\Delta(lac-proAB) galE strA [F' lacI^a Z\Delta M15 proA^+ B^+]$) and WK6 *mutS* ($\Delta(lac-proAB) galE strA mutS125::Tn10 [F' lacI^a Z\Delta M15 proA^+ B^+]$). The WK6 strain was used for production of barnase, and the *mutS* derivative served for the mutagenesis experiment (Stanssens et al., 1989). For inducible expression, cells were grown in Hartley medium (Hartley and Rogerson, 1972), otherwise Luria broth (Lennox, 1955) was used. The construct barnase–barstar has been previously cloned in plasmid pMT416 by Hartley and Paddon (1987) and this recombinant plasmid was obtained from Dr. R. W. Hartley (National Institutes of Health, Bethesda, MD, U.S.A.). The plasmid pMa/c used for the mutagenesis and the expression of barnase was obtained from Dr. P. Stanssens (Stanssens et al., 1989).

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Table 1 Molar absorption coefficients (ϵ) used to calculate the concentrations of the substrates, and changes in molar absorption coefficients ($\Delta\epsilon$) used to calculate the activities

pH* denotes the pH at which $\Delta\epsilon$ was determined. Superscripts indicate external sources of data, as follows: † Zabinsky and Smith, 1983; ‡ Yakovlev et al., 1987; § Steyaert et al., 1991. The $\Delta\epsilon$ of RNA is expressed as formal mononucleotide molarity.

| Substrate | pH | λ (nm) | ϵ ($M^{-1}\cdot cm^{-1}$) | pH* | λ (nm) | $\Delta\epsilon$ ($M^{-1}\cdot cm^{-1}$) |
|-----------|-----|----------------|--------------------------------------|-----|----------------|--|
| GpC | 7 | 280 | 12.600† | 5 | 280 | 2.130† |
| GpA | 7 | 257 | 22.000† | 5 | 280 | 930† |
| ApA | 7 | 257 | 13.600† | 6.2 | 260 | 1.319 |
| Poly(A) | 6.2 | 260 | 10.000‡ | 6.2 | 260 | 5.000‡ |
| RNA | 7.5 | 260 | 8.000§ | 8 | 298 | -39 |

The expression cassette of barnase comprises the structural gene, the *tac* promoter and *phoA* signal sequence. Hence expression is induced with isopropyl β -D-thiogalactopyranoside and the product is secreted into the periplasmic space. An acid shock for separation of the periplasmic material and the first two chromatographic purification steps with SP-Trisacryl and a Mono S column were performed as described by Mossakowska et al. (1989). Further purification was achieved on a Pharmacia f.p.l.c. system with a prepacked phenyl-Superose column. Barnase was dialysed overnight against 50 mM imidazole/HCl, pH 6.6, containing 1.7 M $(NH_4)_2SO_4$. The protein was loaded onto a phenyl-Superose column pre-equilibrated with the same buffer. Barnase was eluted using a linear gradient of 50 mM imidazole/HCl, pH 6.6, and then dialysed against an appropriate buffer. After further concentration of the purified sample, the homogeneity of barnase was checked by SDS/PAGE (Midget system; Pharmacia).

The concentration of the enzyme and substrates were determined spectrophotometrically. The concentration of the enzyme was determined using $\epsilon_{280} = 27.411 M^{-1}\cdot cm^{-1}$, which was calculated from the amino acid composition (3 Trp and 7 Tyr) and the molar extinction coefficients for Trp and Tyr (Mach et al., 1992). The molar absorption coefficient corresponds to a specific-absorption-coefficient value of $2.18 cm^2\cdot mg^{-1}$.

The concentrations of dinucleotides, poly(A) and RNA were determined using the molar extinction coefficients given in Table 1. It should be noted that the concentration of poly(A) and RNA is thus expressed as the formal concentration of mononucleotides.

The steady-state kinetic studies of cleavage of the substrates GpC, GpA, ApA, poly(A) and RNA were performed using a Kontron Uvicon 810P spectrophotometer. All the kinetic measurements were performed at 25 °C. The kinetic parameters were calculated using the changes in molar extinction coefficients, $\Delta\epsilon$, given in Table 1 at the pH of determination.

The poly(A) cleavage reaction was followed by an increase in A_{260} . The change in absorption upon complete digestion (A_{260}) was determined as a function of pH. The pH and ionic strength dependence on the poly(A) absorbance was experimentally determined and taken into account in the calculations wherever necessary.

The kinetics of RNA cleavage by barnase were studied by following the decrease in $A_{298.5}$ (Oshima et al., 1976). An amount of *Torula* yeast RNA was dissolved in distilled water. After filtration (pore diameter 0.22 μm), the solution was extensively dialysed (cut off 13000 Da) to remove short fragments. The RNA stock solution was then diluted into the appropriate buffer, just before the kinetic measurements were performed. The $\Delta A_{298.5}$ upon complete digestion was experimentally determined.

Where the pH dependence was determined, the following

buffer systems were used: formic acid/NaOH (pH 3.0–4.5), acetic acid/NaOH (pH 4.5–5.8), imidazole/HCl (pH 6.0–7.8) and Tris/HCl (pH 7.8–10.0), where I was always 0.1 M. No appreciable discontinuities were observed between buffer systems of comparable pH.

All curve fittings were done using Sigmaplot (Jandel Scientific, Erkrath, Germany).

RESULTS AND DISCUSSION

Computer modelling studies

The structure of the complex RNAase T1-G2'p5'G was determined previously (Köpke et al., 1989) and the coordinates of the complex were obtained from the Protein Data Bank (Entry 2RNT). The co-ordinates of barnase were obtained from Mauguen et al. (1982). The two structures were superimposed and the overlay of the backbones of the secondary structure elements was optimized, using the Brugel package. Hence it was found that a number of residues were involved in a network of hydrogen bonds with the guanosine ring in the active site (see Figure 1): (G)N-7...HO(Ser-57), (G)O-6...HO(Ser-57), (G)O-6...HN(Asn-58), (G)O-6...HN(Arg-59), (G)N-1H...OE₁(Glu-60) and (G)N-2H...OE₂(Glu-60). We therefore decided to construct the mutant E60Q. A recent comparison with the structure of binase (RNAase from *Bacillus intermedius*) led to the same suggestion for the role of Ser-57 and Glu-60 (Yakovlev et al., 1993). The deleterious effect of replacing Ser-57 by Glu has been demonstrated by the same authors. It should be noted however that Glu has a much larger side chain than Ser, such that the effect of

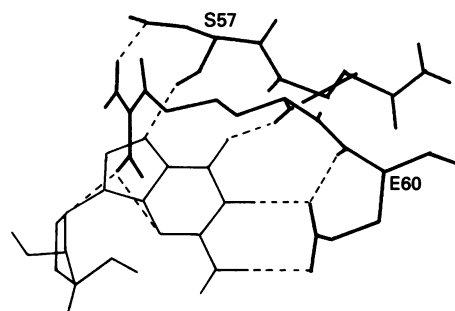


Figure 1 Structure of the guanosine-binding site of barnase, as obtained from the overlay of the structure of RNAase T1-G2'p5'G

The thick lines are parts of the protein (from S57 to E60) and the thin lines represent the guanosine part of GpA; the dotted lines represent potential H bonds.

Table 2 Steady-state parameters for a variety of substrates of wild-type (WT) and E60Q barnase

All measurements were performed at $I = 0.1$ M and at the optimal pH values (indicated) for the particular substrate reaction. For all substrates (except ApA), a full Michaelis–Menten curve was obtained using 15 points well spread over the concentration range 0.01–1.2 mM (for RNA, the concentration range was 0.01–10 mM). The errors for k_{cat} and K_m were calculated using Sigmaplot, and for k_{cat}/K_m were calculated by the rules for the propagation of errors. In the case of ApA, only k_{cat}/K_m could be determined because a linear relationship between v and the substrate concentration was obtained. For this substrate, the errors for k_{cat}/K_m were obtained from linear least-squares fitting to the 15 data points. Values are means \pm S.D.

| Substrate | Enzyme | pH | k_{cat} (s^{-1}) | K_m (μM) | k_{cat}/K_m ($\text{s}^{-1} \cdot \text{M}^{-1}$) |
|-----------|--------|-----|--------------------------------------|-------------------------|--|
| GpA | E60Q | 5.0 | 0.77 ± 0.17 | 2530 ± 700 | 304 ± 150 |
| GpA | WT | 5.0 | 0.81 ± 0.12 | 220 ± 35 | 3640 ± 1100 |
| GpC | E60Q | 5.0 | 0.41 ± 0.12 | 3934 ± 1300 | 104 ± 60 |
| GpC | WT | 5.0 | 0.50 ± 0.02 | 235 ± 19 | 2119 ± 250 |
| ApA | E60Q | 6.2 | — | — | 1.2 ± 0.1 |
| ApA | WT | 6.2 | — | — | 17 ± 1 |
| RNA | E60Q | 8.0 | 5727 ± 551 | 4873 ± 714 | $1175 \times 10^3 \pm 300 \times 10^3$ |
| RNA | WT | 8.0 | 3506 ± 551 | 2103 ± 360 | $1667 \times 10^3 \pm 500 \times 10^3$ |
| Poly(A) | E60Q | 6.2 | 32.4 ± 1.3 | 106 ± 13 | $301 \times 10^3 \pm 50 \times 10^3$ |
| Poly(A) | WT | 6.2 | 23.0 ± 1.1 | 130 ± 30 | $180 \times 10^3 \pm 50 \times 10^3$ |

this mutation is not necessarily limited to the absence of an H-bond with Ser-57.

Kinetic studies of the E60Q mutant

To investigate the consequences of the mutation E60Q, a steady-state comparative kinetic study was made using a variety of substrates. The results are shown in Table 2.

A comparison of the kinetic parameters of the wild-type and the mutant E60Q for the substrates GpA and GpC shows that this mutation does not alter the k_{cat} but increases K_m by a factor of 12–20. This proves that the E60Q mutation destabilizes the ground state of the enzyme–substrate complex. Because the influence on k_{cat} is minimal, we must conclude that the mutation destabilizes the transition state to the same extent, so that the free-energy difference between the two remains the same. This suggests that the enzyme–substrate complex does not change its conformation as a consequence of the mutation; the other interactions with the substrate are sufficiently strong to keep the substrate in the correct conformation.

It can also be concluded that in this part of the enzyme–substrate complex no conformational change (change of interactions) occurs upon going from the ground state to the transition state, otherwise it would be very unlikely that the mutation would influence both states to the same extent.

The low value of k_{cat}/K_m and the fact that k_{cat} and K_m can be changed independently suggests that the enzyme–substrate pair shows a Michaelis–Menten behaviour and that K_m equals K_s , the true dissociation constant of the substrate.

For the substrate ApA only the parameter k_{cat}/K_m could be determined. This parameter is decreased by a factor of ~ 14 in the mutant. This indicates that the E60Q mutation does not render the enzyme more specific for adenosine. This is a rather surprising result, as it indicates that Glu-60 stabilizes adenosine binding as well as guanosine binding, which was not expected from the results of our modelling studies. It seems that some flexibility of the side chains is involved.

The study of poly(A) yielded a different result. First of all, it should be noted that for both enzymes, the k_{cat} value for this substrate is ~ 40 -fold greater than for the dinucleotides, probably indicating a slightly different orientation of the substrate in the main active site, resulting from the influence of the interactions of additional subsites (Day et al., 1992). The K_m of the wild-type enzyme for poly(A) is similar to that for the dinucleotides [when

the poly(A) concentration is expressed in mononucleotide equivalents]. This suggests that the enzyme still behaves as a Michaelis–Menten enzyme and that $K_m = K_s$. When the wild-type is compared with the mutant enzyme using poly(A) as substrate, the various parameters are not changed by more than a factor of two. The strong destabilizing effect of the mutation on the binding of the dinucleotides is not present. We therefore must come to the conclusion that the interactions between poly(A) and the residue at position 60 are different from those involving the dinucleotide.

A similar trend is visible in the studies with RNA. Compared with poly(A), the value of k_{cat} is further increased by a factor of 130–180. The mutation does not change the k_{cat} and K_m significantly. It should be remembered that the concentration of RNA was expressed as mononucleotides. If it were expressed as the concentration of specific (G) sites, K_m would be decreased and k_{cat}/K_m would be increased by a factor of ~ 4 . This assumes that all of the guanines are equally accessible.

The interpretation of this result is likely to be the same as in the case of poly(A). However, if the barnase/RNA pair is treated as a Briggs–Haldane pair, the following alternative explanation can not be excluded: the deleterious effect of the mutation is present in the binding of RNA but it is not visible in the parameters. This situation would occur when two conditions are met: (1) the barnase/RNA pair is a real Briggs–Haldane pair; and (2) the decreased stability of the substrate binding in the mutant results from an increased dissociation rate constant of the substrate. This dissociation rate constant does not contribute to the value of K_m in the case of the a true Briggs–Haldane substrate, where the dissociation rate constant can be neglected relative to k_{cat} . In such a case, the destabilizing effect of the mutation would not be visible in K_m and in k_{cat}/K_m .

The results of Day et al. (1992) show that substrates as long as tetranucleotides exhibit Briggs–Haldane behaviour, with $k_{\text{cat}}/K_m = 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$. On the basis of these data, one would expect the barnase/RNA pair also to be a Briggs–Haldane pair.

In the case of RNA, however, we observed a value for k_{cat}/K_m of only $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. It should be noted that the parameters that we obtained for the wild-type with the dinucleotides are in full agreement with the data of Day et al. (1992) and of Mossakowska et al. (1989). Also, the k_{cat} values that we obtained for RNA are very similar to those observed for the tetranucleotides. The K_m value we obtain for RNA is, however, much higher than the K_m values for the tetranucleotides. Our high values for K_m are very

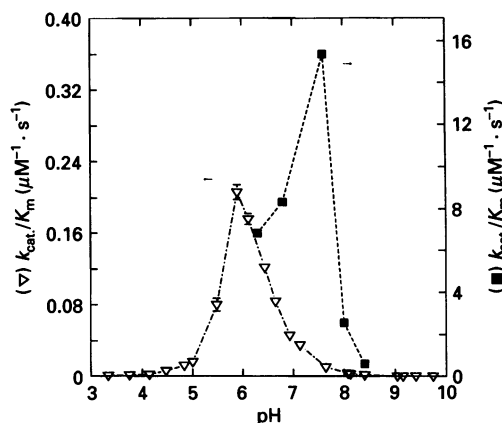


Figure 2 pH dependence of k_{cat}/K_m of barnase for the hydrolysis of poly(A)

The experiments were done at two different I values, 0.1 (▽) and 0.01 M (■).

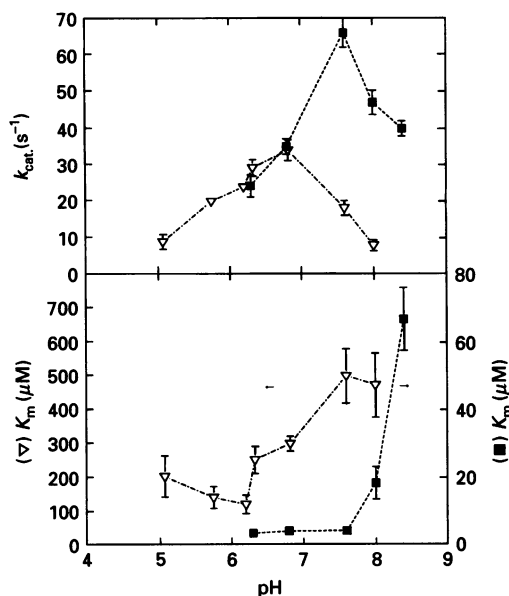


Figure 3 pH dependence of k_{cat} and K_m of barnase for the hydrolysis of poly(A)

The experiments were done at two different I values, 0.1 (▽) and 0.01 M (■).

similar to the ones obtained for RNAse T1 and RNA (Steyaert et al., 1991). This suggests that the value of k_{cat}/K_m can not be extrapolated from tetranucleotides to RNA. Despite the relatively low value of k_{cat}/K_m , it is possible that RNA is a Briggs-Haldane substrate. Also, the fact that both k_{cat} and K_m increase upon going from poly(A) to RNA suggests this. It is thus likely that the interpretation of the results with poly(A) can be extrapolated to RNA, but the alternative explanation can not strictly be excluded.

In view of the pure Michaelis-Menten behaviour of barnase with poly(A), we decided to study this substrate in further detail. Synthetic homopolymers such as poly(A) have the advantage that their structure is known (Saenger, 1984) and that all of the nucleotide bases are the same. The homopolymer poly(G) can

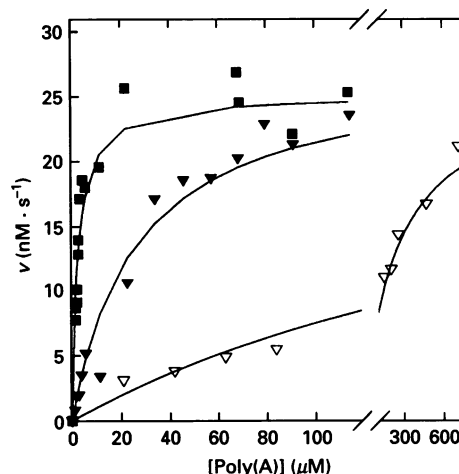


Figure 4 The Michaelis-Menten curves for the hydrolysis of poly(A) by barnase at $I = 0.0125$ (■), 0.0625 (▼) and 0.125 M (▽)

The pH was 6.2 for the three sets of experiments.

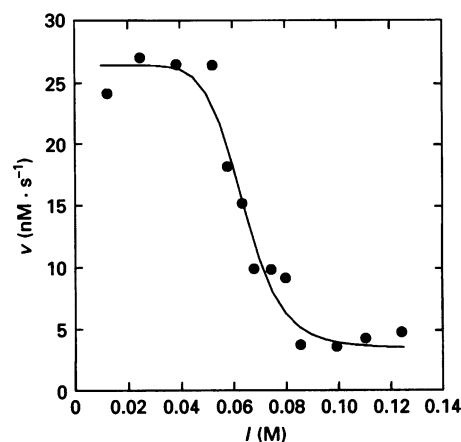


Figure 5 The dependence on I ([NaCl]) of the rate of poly(A) hydrolysis (v).

The experiment was done with a substrate concentration of $20 \mu\text{M}$ in a Tris/acetate buffer, pH 6.2.

not be used because of its strong tendency to form aggregates (Saenger, 1984).

The pH dependence of the k_{cat}/K_m value of the poly(A) reaction is shown in Figure 2. The experiments were done at two different ionic strengths (0.1 and 0.01 M). At $I = 0.1$ M and a substrate concentration of 5% of the K_m value at pH 6.2 [$15 \mu\text{M}$ poly(A)] the k_{cat}/K_m value was measured directly from the exponential time dependence of the absorbance change. Similar experiments were impossible to execute at low I (0.01 M) because the K_m value is far too small. Consequently, the full kinetic saturation curves were measured at different pH values. At high I , the optimum value is obtained at pH = 6, whereas at low I the optimum shifts 2.5 units towards a higher pH. The optimal rate constant k_{cat}/K_m is 100-fold larger at low I . It should be noted that the pH profile of the dinucleotide GpC is independent of I : experiments at $I = 0.02$ and 0.1 M gave no detectable differences (Mossakowska et al., 1989).

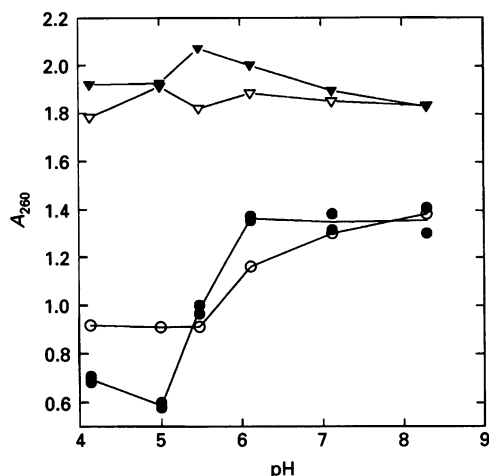


Figure 6 The A_{260} of poly(A) as a function of the pH at two different I values, 0.0125 M (●) and 0.125 M (○).

After the hydrolysis of poly(A) by barnase, the A_{260} increased as a result of unstacking [$I = 0.0125$ (▼) and 0.125 M (▽)].

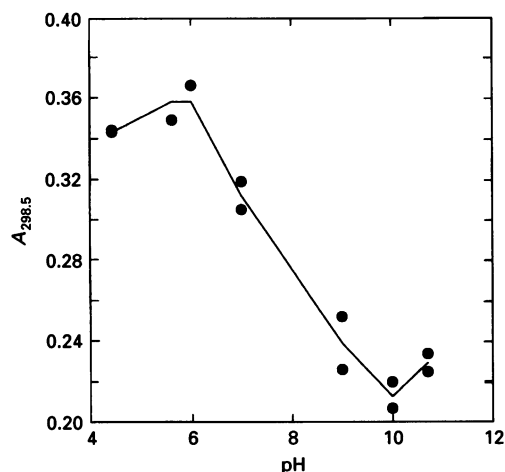


Figure 7 The $A_{298.5}$ of RNA as a function of the pH at $I = 0.1$ M.

The pH dependence of the kinetic constants $k_{\text{cat.}}$ and K_m at $I = 0.01$ and 0.1 M are shown in Figure 3.

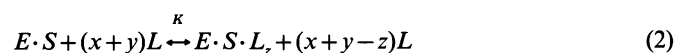
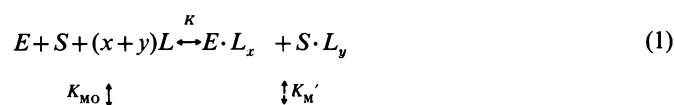
For $k_{\text{cat.}}$, the optimum pH is 7.5 at low I and 6.8 at high I . The optimal $k_{\text{cat.}}$ values at both I values do not differ by more than a factor of 2. At pH values lower than 6.8, the $k_{\text{cat.}}$ s become very similar for the two salt concentrations.

The K_m value is significantly lower at low I , and this is true for the whole pH region investigated. At low I , the K_m value increases sharply above pH 7.6. At high I , the K_m is much higher over the whole pH region, and the sharp rise of K_m starts when the pH surpasses 6.2.

A further illustration of the salt dependence is shown in Figure 4. Here, the full hyperbolic rate curves are shown at $I = 0.0125$, 0.0625 and 0.125 M. The pH is 6.2 for the three sets of experiments. Figure 4 and the parameters obtained by curve fitting show clearly that a 10-fold increase in I induces a 100-fold

increase of the K_m of barnase for poly(A). The catalytic rate constant ($k_{\text{cat.}}$) does not change at pH 6.2 (in agreement with Figure 3).

The I dependence of the poly(A) reaction rate was measured at constant substrate concentration (see Figure 5). The buffer solution was Tris/acetate, pH 6.2, and the experiment was done with a substrate concentration of 20 μM . It is clear from Figures 2 and 3 that, at pH 6.2, only the K_m value changes as a function of I and this phenomenon is reflected by the transition. The experimental values can be fitted to an equation that describes the linkage between a conformational change and the preferential binding of ligands (this application of the law of mass action can safely be made because K_m is a true dissociation constant). It describes the following equilibria:



$$K_M = K_{M0}(1 + KL)^m \quad (3)$$

$$K_{M0} = \frac{[E][S]}{[ES]} \quad (4)$$

$$m = x + y - z \quad (5)$$

where E is barnase, L is the salt providing the counterions and S is poly(A), and K is the binding constant of the salt, which is assumed to be the same for E , S and $E \cdot S$, in order to reduce the number of parameters. K_{M0} is the salt-independent Michaelis constant, and K_M is salt-dependent. z , x and y are the number of ion pairs formed between the counterions and the substrate-enzyme complex $E \cdot S$, and the free enzyme E and substrate S respectively, and m is the differential binding coefficient of the salt and a measure of the steepness of the transition. The data presented in Figure 5 could be fitted to eqn. (6). Because the rate does not decrease to zero at high I , $v_{\text{min.}}$ had to be introduced as the lower limit of v , the reaction rate. Furthermore, in order to obtain a good fit, the salt binding function [eqn. (3)] had to be changed into its full cooperative equivalent of eqn. (7):

$$v = V_{\text{max.}} \frac{[S]}{K_M + [S]} + V_{\text{min.}} \quad (6)$$

$$K_M = K_{M0}(1 + (KL)^m) \quad (7)$$

With the data of Figure 5, $[S] = 20 \mu\text{M}$, $V_{\text{max.}} = 28.5 \text{ nM/s}$, $V_{\text{min.}} = 3.5 \text{ nM/s}$, the following fitting parameters were obtained: $K_{M0} = 4.9 \pm 1.2 \mu\text{M}$, $K = 18.8 \pm 1.2 \text{ M}^{-1}$ and $m = 8.8 \pm 1.7$. This suggests that 7–10 ions are co-operatively released upon the binding of poly(A) to barnase.

Conformational changes of polymer as a function of pH and ionic strength

The possibility of pH-dependent conformational changes in poly(A) or RNA may also have to be considered. The u.v. absorbance of poly(A) as a function of the pH at two different I values (0.0125 and 0.125 M) is shown in Figure 6. It is clear from the shape of the curves that there is a transition of the structure leading to a change in base stacking. After full digestion of poly(A), the absorbance should be independent of the pH (see Figure 6). Hence, ΔA (and $\Delta \epsilon$) should be pH dependent and this

is taken into account in the calculations of activity/pH profile. It should be noted that in its absorbance spectrum RNA shows similar changes as a function of pH (Figure 7). These spectral changes, which reflect alterations in the extent of stacking of the bases, might influence the catalytic parameters for this substrate.

Conclusion

The E60Q mutation causes a strong destabilization of the binding of dinucleotides yet k_{cat} is not changed. Therefore, the destabilization is the same for the transition state and the ground state. This suggests strongly that in this part of the enzyme-substrate complex the conformation of the ground state and the transition state are the same.

This destabilization of the binding is not observed for poly(A) and for RNA. For poly(A), which clearly is a Michaelis-Menten substrate, the conclusion must therefore be that the conformation of the substrate at the position of residue 60 must be different from that of the dinucleotides. This can be explained by the effect of the additional subsites on the orientation of the specific base in the primary active site.

For RNA, similar results are obtained. Because the k_{cat} is so much higher for RNA than for dinucleotides, it is likely that the orientation of the oligonucleotide in the active site is different from that of, for example, GpC. This is probably also true at the position of Glu-60. However, because RNA might be a Briggs-Haldane substrate, an alternative explanation can not be excluded.

The activity of barnase towards poly(A) is very strongly dependent on the pH and on I . At low I , the binding of poly(A) is very strong but decreases rather sharply when the pH is raised above 8. When I is raised to 0.1 M, the binding is weakened and the pK of the groups involved seems to be reduced. The k_{cat} shows a more or less bell-shaped behaviour, again with a strong shift of the right pK towards lower values at high I . It seems likely that electrostatic interactions in the additional sites are responsible for the salt concentration and pH dependency at the right side of the bell-shaped curves.

Both poly(A) and RNA show pH-dependent absorbance

changes which reflect changes in stacking and might influence the catalytic parameters.

Note added in proof (received 18 April 1994)

While this paper was being proofed-out, a paper by Buckle and Fersht (1994) appeared which describes the interaction of the guanine base with barnase and demonstrates that our modelling is correct.

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