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Barnase Has Subsites That Give Rise to Large Rate Enhancements†

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ABSTRACT: Barnase is found to have a series of subsites for binding its substrates that confers large rate enhancements. Ribonucleotide substrates of the type $Zp_0Gp_1Xp_2Y$ have been synthesized, where p is phosphate, X, Y, and Z are nucleosides, and G is guanosine. G occupies the primary specificity site. The most important subsite is for p_2, followed by that for Y. There appears to be no subsite for the Z or p_0 positions. Occupation of the subsite for p_2 gives rise to a 1000-fold increase in k_{cat}/K_m, composed of a 100-fold increase in k_{cat} and a 10-fold decrease in K_m. The Y subsite gives rise to further 20-fold increase in k_{cat}/K_m. Rates approaching diffusion control for k_{cat}/K_m are observed. k_{cat} for the dinucleotide monophosphate GpU = 0.55 s^{-1}, and K_m = 240 μM; this compares with 53 s^{-1} and 20 μM for GpUp, and 3.3×10^3 s^{-1} and 17 μM for GpApA (the best substrate tested). Cleavage occurs at the 3′-phosphate of guanosine in all cases. There are differences in base specificity at the two subsites for X and Y downstream of the scissile bond. The binding energies of different substrates have been analyzed using thermodynamic cycles. These show that the contributions of the X and Y sites are nonadditive.

Barnase is a small extracellular ribonuclease from *Bacillus amyloliquefaciens* and is a member of a family of homologous microbial nucleases (Hill et al., 1983), of which the best characterized is RNase[1] T1. Barnase consists of a single polypeptide chain of 110 amino acids, M_r = 12 382, with no disulfide bridges (Hartley & Baker, 1972). The small size has facilitated structural and physical studies. The crystal structure has been solved at 2-Å resolution (Mauguen et al., 1982). More recently, a complex with the deoxydinucleotide d(GpC) at 1.9-Å resolution has also been solved (Baudet & Janin, 1991), but the binding of the dinucleotide is a nonproductive mode for a substrate. The sequence-specific 2D ^{1}H NMR spectrum of barnase has been assigned (Bycroft et al., 1990) and the solution structure determined (Bycroft et al., 1991). Barnase has also been extensively studied as a paradigm for protein folding and stability [e.g., Matouschek et al. (1990) and Serrano et al. (1991)].

The mechanism of action of barnase is similar to that of pancreatic ribonuclease in that a 2′,3′-cyclic nucleotide intermediate is formed in the first step (i.e., transesterification) of the reaction, followed by a much slower hydrolysis of this intermediate to give a 3′ nucleotide (Figure 1). On the basis of chemical modification, site-directed mutagenesis, and primary sequence homologies between members of the family of microbial ribonucleases, three important catalytic residues have been identified in barnase: His-102 (putative general acid), Glu-73 (putative general base), and Arg-87 (Arata et

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[1] Abbreviations: RNase, ribonuclease; d(GpC), 2′-deoxyguanylyl-(3′→5′)-2′-deoxycytidine; 2′,3′-cGMP, guanosine 2′,3′-cyclic monophosphate; 2′,3′-cAMP, adenosine 2′,3′-cyclic monophosphate; CpGc-2′,3′-p, cytosyl(3′→5′)guanosine 2′,3′-cyclic monophosphate; N, any nucleoside. For other nucleoside and nucleotide abbreviations, see IUPAC-IUB Commission on Biochemical Nomenclature (1970), for example, GpUp, guanylyl(3′→5′)uridine 3′-monophosphate.

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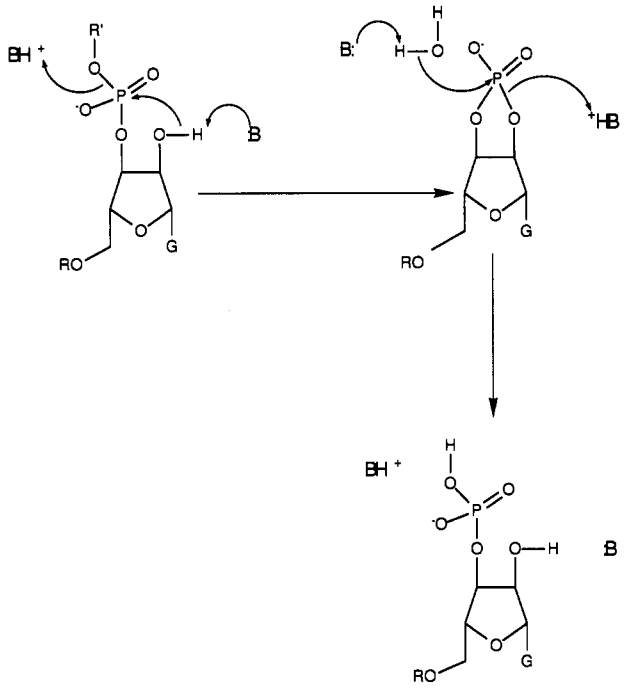

FIGURE 1: Classical mechanism proposed for ribonucleases. The first step is the cyclization reaction where the general base is proposed to be Glu-73 and the general acid to be His-102 in barnase. The role of the residues is reversed in the subsequent hydrolysis step.

al., 1979; Egami et al., 1980; Inagahi et al., 1981; Takahashi & Moore, 1982; Mossakowska et al., 1989; Steyaert et al., 1990; Meiering et al., 1991).

There is a variation in the specificity of ribonucleases. Some, such as RNase T2, are not base-specific, whereas others such as RNases A, T1, and U2 show a strong specificity for pyrimidine, guanine, and purine, respectively (Uchida & Egami, 1971). Barnase lies between these two extremes and shows a more relaxed specificity. Barnase will cleave after only guanosine when catalyzing the hydrolysis of dinucleotide substrates GpN (Rushizky et al., 1963). With longer substrates, however, it preferentially cleaves after guanosine, but it will also cleave after other bases yielding a mixture of mono- and dinucleotides in a total RNA digest (Rushizsky et al., 1963). Barnase is also different from the homologous guanosine-specific RNase T1 in its preference for the nucleoside, N in GpN; RNase T1 shows a preference in the order C > A > G > U, whereas barnase shows the preference order A > G > C ≈ U (Osterman & Walz, 1978; Mossakowska et al., 1989). (The subsite nomenclature is shown in Figure 2.)

A further important difference between barnase and RNase T1 lies in their kinetic parameters for reaction with RNA and dinucleotide monophosphates. Barnase has a 1000-fold lower value of k_{cat}/K_m for transesterification of GpA than does RNase T1 but hydrolyzes RNA at twice the rate of RNase T1 (Walz et al., 1978; Mossakowska et al., 1989). The latter observation suggests that barnase has a very strong preference for substrates longer than dinucleotide monophosphates. We report here an investigation into this preference and also into the subsite specificity for groups 3′ to the N of GpN.

Materials and Methods

Chemicals. Tri- and tetranucleotides were synthesized as follows from RNA monomers supplied by Milligen Biosearch:

(*a*) *RNA Synthesis.* RNA synthesis was performed on an ABI 381A automated synthesizer (Usman et al., 1987). The synthesis cycles used were modified DNA coupling cycles (ABI 381A instruction manual): the coupling time was increased

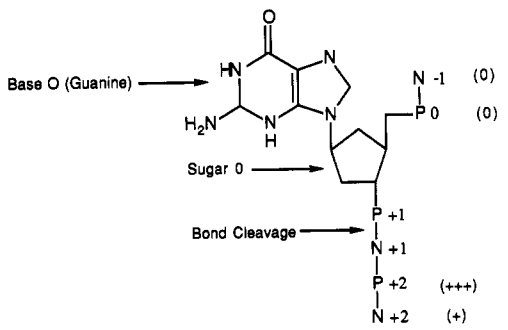

FIGURE 2: Schematic of ribonucleotide substrate of barnase. N is nucleoside; P is phosphate. The importance of subsites is indicated in parentheses; 0 indicates no binding observed.

to 10 min and iodine oxidation was followed by a second capping procedure. RNA cyanoethyl phosphoramidites were diluted with anhydrous acetonitrile to a concentration of 0.1 M. The syntheses were monitored by the spectrophotometric quantitation of released dimethoxytrityl cation at 498 nm. The functionalized solid support was synthesized by the method of Masada et al. (1991).

(*b*) *Cleavage and Base Deprotection.* Cleavage of the assembled oligonucleotide from the support was carried out on the synthesizer using a mixture of ethanol/ammonia solution (1:3). The four 15-min wait times of the standard DNA end procedure were increased to 30 min to give a total cleavage time of 2 h; this was followed by heating to 55 °C for 6 h. Alternatively, cleavage was carried out by heating the resin in 3 mL of the ethanol/ammonia mixture at 55 °C for 6 h in a sealed glass vial and filtering off the glass beads. In either case, the solution was then lyophilized.

(*c*) *2′ Deprotection.* The *tert*-butyldimethylsilyl protecting groups on the 2′-hydroxyl groups of the ribose sugars were removed with an excess of 1 M tetrabutylammonium fluoride (50 equiv). After at least 8 h, an equal amount of 1 M ammonium acetate was added to quench the reaction. The solution was applied to a Dowex 50W X8 column (10 mL, Na^+ form) and eluted with 6 × 20 mL of distilled water. The combined eluates were evaporated to dryness, redissolved in a few milliliters of distilled water, and lyophilized. The lyophilization was repeated several times to remove the volatile buffer.

(*d*) *Purification.* The oligoribonucleotides were purified by reversed-phase HPLC [Brownlee Aquapore reverse-phase octyl (C8); 25 cm × 10 mm; buffer A = 0.1 M ammonium acetate, pH 7.0; buffer B = ammonium acetate, pH 7.0, with 20% acetonitrile]. The purified oligomers were desalted on a Sephadex G-10 column, eluted with distilled water, and lyophilized to dryness.

All other chemicals were purchased from Sigma Chemical Co. Ltd.

Recombinant Barnase. Barnase was expressed and purified from *Escherichia coli* strain TG2 harboring plasmid pMT410 (Kellis et al., 1989).

Assay of Barnase Transesterification Activity Using Oligonucleotides. All experiments were performed at 25 °C and pH 5.80 in 0.1 M ionic strength sodium acetate/acetic acid buffer containing 100 μg/mL molecular biology grade (acetylated) BSA. The total volume was 1 mL in 1-cm path-length cuvettes in a Perkin-Elmer Lambda 5 UV/visible spectrophotometer with a 4-nm band-pass. Background absorbance at high substrate concentrations necessitated the use of 0.2-cm path-length cuvettes, and the assay volume was reduced to 0.5 mL. The concentration of barnase was determined spectrophotometrically at 280 nm where $E_{0.1\%}$ = 2.2 (Loewenthal et

Table I: Molar Extinction Coefficients, Analytical Wavelengths, and Differences in Molar Extinction Coefficients between Oligonucleotide Substrates and Cleavage Products Obtained with Barnase[a]

substrate	ϵ (mM^{-1} cm^{-1}) at 260 nm	analytical wavelength for $\Delta\epsilon$ (nm)	$\Delta\epsilon$ (M^{-1} cm^{-1})
GpU		275	1280
GpUp	20.6[b]	275	1150
GpApC	30.6[b]	255	2690
CpGpApC	36.8[c]	255	3430
GpApA	37.2[d]	255	3640
GpUpC	28.5[d]	275	1720

[a] All measurements were made at pH 5.80 and 25 °C at 0.1 M ionic strength in sodium acetate buffer. [b] Taken from the measurements of Stanley and Bock (1965). [c] Calculated using the semiempirical nearest neighbor approach of Cantor and Tinoco (1965) using dinucleotide and trinucleotide molar extinction coefficients from Aoyagi and Inoue (1968) and Stanley and Bock (1965). [d] Taken from the calculated values of Cantor and Tinoco (1967).

al., 1991). The concentrations of stock oligonucleotide solutions were determined spectrophotometrically on the basis of either extinction coefficients at 280 nm (Zabinski & Walz, 1976; Osterman & Walz, 1978) or extinction coefficients at 260 nm allowing for hypochromicity (see Table I for values and references).

For GpA, GpC, and GpG, the values of K_m and V_{max} were determined from initial velocities by following the increase in absorbance at 280 nm (Zabinski & Walz, 1976). For other substrates, the wavelengths of maximal change in absorbance upon transesterification were determined by measuring difference spectra. Substrates containing the linkage GpU have nearly equal maximal changes at 257 and 275 nm; 275 nm was used to follow transesterification because of the lower background absorbance. Substrates not containing a GpU linkage have maximal changes at 255–257 nm, and transesterification was followed at 255 nm. The difference in molar extinction coefficients on total cleavage of the substrates by barnase was determined by observing the increase in absorbance at the appropriate wavelength (Table I).

Reaction was initiated by the addition of 5–10 μL of barnase solution in the assay buffer at a concentration such that the initial rate could be measured in 1–2 min. The same batch of enzyme was used for all substrates with the exception of the GpN dinucleotides; kinetic constants determined for GpA and GpUp are identical within experimental error between different batches of enzyme. The data were analyzed by nonlinear regression analysis with the program ENZFITTER (Leatherbarrow, 1987) from which values of K_m and V_{max} were obtained.

Product Analysis. Reactions from all non-GpN substrates were allowed to continue for 30–60 min, and the products then frozen and lyophilized. The solid residue was dissolved in at least twice the initial volume of water and the pH adjusted to ≥7 with a minimum volume of 1 M triethylammonium hydrogen carbonate before application to a Mono Q 10/10 column on a FPLC system. The column was eluted with a 0.05–1.0 M triethylammonium hydrogen carbonate, pH 8.5, gradient. Fractions containing material which absorbed at 260 nm were collected and lyophilized repeatedly to remove the buffer. The residues were dissolved in D_2O and NMR spectra measured on a Bruker AMX 500 NMR spectrometer. The samples (2′,3′-cGMP, 3′-UMP, CpGc-2′,3′-p, UpC) were then lyophilized again and mass spectra measured on a Kratos Instruments MS 890 mass spectrometer.

A number of fragments (ApC and ApA) did not bind to the Mono Q column. The products were analyzed by HPLC as follows: known standards (ApA, ApC, 2′,3′-cGMP, and 2′,3′-cAMP from Sigma Chemical Co. Ltd.) were separated on a Gilson 715 HPLC system using an Anachem Spherisorb S5 SAX ion-exchange column developed isocratically with 37.5 mM KH_2PO_4, pH 3.5, for 25 min, followed by 100 mM KH_2PO_4, pH 3.5, for 25 min, at a flow rate of 1 mL/min. Substrates were digested with an excess of barnase in 7.5 mM sodium acetate, pH 5.8, adjusted to 25 mM KH_2PO_4, pH 3.5, and applied to the HPLC apparatus. As there were slight changes in retention times of the standards between runs, the identity of the barnase digestion products was confirmed by adding standards (corresponding to the expected products) to the digests and ensuring that no additional peaks were seen.

Table II: Product Analysis Determined after Reaction of Oligonucleotide Substrates with Barnase

substrate	products found[a]	products found[b]
GpUp	2′,3′-cGMP, 3′-UMP	
GpApC	2′,3′-cGMP	2′,3′-cGMP, ApC
CpGpApC	CpGc-2′,3′-p	ApC[c]
GpApA	2′,3′-cGMP	2′,3′-cGMP,ApA
GpUpC	2′,3′-cGMP, UpC	

[a] Products were separated by ion-exchange chromatography and analyzed by ^1H NMR and Mass spectrometry (see text). [b] Products were analyzed by HPLC (see text). [c] A peak consistent with CpGc-2′,3′-p was seen on the HPLC chromatogram (see text).

Results

Product Analysis. It has already been demonstrated for the GpN dinucleotides that transesterification and not hydrolysis is being measured (Mossakowska et al., 1989). The values of K_m and k_{cat} for hydrolysis of cyclic GMP are sufficiently poor relative to the rate of transesterification that there is no significant competition from overall hydrolysis when the initial rate (~5% of reaction) of GpN transesterification is followed.

The products of reaction of all the other substrates were analyzed by NMR and mass spectrometry after initial rate measurements. In all cases, NMR spectra and mass ions consistent with the products shown in Table II were seen. No other nucleotide-containing material was detected. For GpApA, GpApC, and CpGpApC, the expected ApA and ApC products were not detected. These products probably did not bind to the Mono Q column under the conditions used.

Analysis by HPLC with an Anachem Spherisorb S5 SAX ion-exchange column gave baseline separation of the following substrates and commercial standards (average retention times in parentheses): 2′,3′-cGMP (17.1 min); 2′,3′-cAMP (13.3 min); ApA (8.5 min); ApC (5.5 min); GpApA (41.8 min); GpApC (36.7 min); CpGpApC (41.3 min). The reaction products of substrate digestion by barnase were identified by comparison with the retention times of the standards and confirmed by addition of standards to the digests as shown in Table II. This was not possible for the digest of CpGpApC as CpGc-2′,3′-p is not available commercially. However, the digest of CpGpApC gave rise to a peak corresponding to ApC and a peak with a retention time of 38.5 min which is consistent with a species having two negative charges, as expected for CpGc-2′,3′-p under the conditions used. CpGc-2′,3′-p was conclusively identified, however, in a digest of CpGpApC by mass spectrometry and NMR.

Thus, it has been shown for all of the substrates used in this study that the primary site of transesterification is immediately 3′ to the guanosine base.

Specificity of Barnase. The transesterification of oligonucleotides can be followed spectrophotometrically. The advantage of using defined substrates over RNA is that the kinetic constants, K_m and k_{cat}, may be measured for the transesterification of a specific bond. The optimal pH for

Table III: Kinetic Constants for Transesterification of Oligonucleotide Substrates by Barnase[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
GpA	62.7 (±4.8)	0.729 (±0.017)	1.16 (±0.09) × 10^4
GpG	40.0 (±3.5)	0.252 (±0.007)	6.30 (±0.58) × 10^3
GpC[b]	219 (±29)	0.338 (±0.020)	1.54 (±0.22) × 10^3
GpU[b]	236 (±28)	0.547 (±0.025)	2.32 (±0.29) × 10^3
GpUp	19.9 (±2.0)	53.1 (±1.7)	2.66 (±0.28) × 10^6
GpApC	43.5 (±5.2)	4.0 (±0.2) × 10^3	9.19 (±1.17) × 10^7
GpUpC	37.9 (±3.8)	1.68 (±0.06) × 10^3	4.44 (±0.47) × 10^7
GpApA	16.6 (±2.8)	3.26 (±0.23) × 10^3	1.97 (±0.35) × 10^8
CpGpApC	36.3 (±6.7)	3.61 (±0.21) × 10^3	9.94 (±1.93) × 10^7

[a] At pH 5.80, 25 °C, and 0.1 M ionic strength sodium acetate buffer. [b] It was not possible to go to high ($\gg K_m$) substrate concentrations with these substrates as the background absorbance is too high.

Table IV: Difference Free Energy Values Calculated from Steady-State Kinetic Constants[a]

substrate	$\Delta\Delta G^b$ K_m (kcal mol^{-1})	$\Delta\Delta G^c$ k_{cat} (kcal mol^{-1})	$\Delta\Delta G^d$ k_{cat}/K_m (kcal mol^{-1})
GpA	0.74 (±0.05)	0.46 (±0.01)	1.20 (±0.05)
GpG	1.01 (±0.06)	−0.17 (±0.02)	0.83 (±0.06)
GpC	0	0	0
GpU	−0.05 (±0.07)	0.28 (±0.03)	0.24 (±0.08)
GpUp	1.46 (±0.06)	3.01 (±0.02)	4.43 (±0.07)
GpApC	1.08 (±0.08)	5.58 (±0.03)	6.53 (±0.08)
GpUpC	1.05 (±0.06)	5.06 (±0.02)	6.10 (±0.07)
GpApA	1.65 (±0.11)	5.44 (±0.04)	6.99 (±0.11)
CpGpApC	1.20 (±0.12)	5.50 (±0.04)	6.58 (±0.13)

[a] All values are calculated relative to the "poorest" substrate, GpC. Positive values = better binding. [b] Apparent change in binding of substrate to enzyme. [c] Apparent change in difference in binding energy between transition state to enzyme and substrate to enzyme. [d] Apparent change in binding energy of transition state to enzyme

hydrolysis of RNA is 8.5 (Mossakowska et al., 1989); however, experiments were carried out at pH 5.8 as this has been shown to be the optimum pH for transesterification of dinucleotide monophosphates, GpN (Moskowska et al., 1989).

The values of K_m and k_{cat} for each of the substrates were determined from measurements of initial rates (Table III). There are differences in absolute values of K_m for GpN monophosphates between those found in this study and those found in the study of Moskowska et al. (1989); the values of K_m determined in this study are in reasonable agreement with those determined more recently by other workers in this laboratory.

Free energy differences were calculated for each of the kinetic parameters (Fersht, 1985) (Table IV). All were calculated relative to GpC, which is the "poorest" substrate of those tested.

The greatest changes in activity are seen on going from the dinucleotide monophosphates GpN to GpUp and from GpUp to trinucleotide substrates; no change is seen on going from tri- to tetranucleotide substrates. We can analyze these effects in terms of their steady-state kinetic constants. For Michaelis–Menten kinetics, K_m gives a measure of the binding between enzyme and substrate. k_{cat}/K_m is related to the binding energy between enzyme and transition state and k_{cat} to the difference in binding energy of the enzyme with the transition state and substrate (Fersht, 1985).

Our analysis assumes that we have Michaelis–Menten kinetics, in which the on and off rate constants for formation of the ES complex are much faster than the rate constants for ES → EP, in which case $K_m \sim K_s$. This is a reasonable assumption for an enzymic reaction which has no intermediate. However, as the reaction becomes so fast that the limiting factor is diffusion of the reactants (diffusion control), the assumption breaks down and $K_m = (k_{-1} + k_2)/k_1$ for

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

This usually occurs at values of $k_{cat}/K_m \sim 10^9$ M^{-1} s^{-1} (Fersht, 1985). The substrate with the highest value of k_{cat}/K_m analyzed approaches this value (GpApA, 1.97 × 10^8 M s^{-1}). However, all other substrates analyzed have considerably lower values of k_{cat}/K_m, suggesting that diffusion control has not been reached in the reaction of these substrates.

The specificity constant, k_{cat}/K_m, is 1000-fold higher for GpUp than for GpU, corresponding to a total binding energy difference of 4.2 kcal mol^{-1}. The binding energy is divided between enzyme–substrate (1.5 kcal mol^{-1}) and enzyme–transition state (2.7 kcal mol^{-1}) interactions. When the length of the substrate is increased by an additional nucleoside to give GpUpC, k_{cat}/K_m increases by a further 20-fold, corresponding to an additional total binding energy of 1.7 kcal mol^{-1} (GpU to GpUpC, 5.9 kcal mol^{-1}). In this case, the binding energy is distributed more in favor of transition state binding; comparing GpU with GpUpC we now see that only 1.1 kcal mol^{-1} of the additional binding energy is associated with binding the substrate and 4.8 kcal mol^{-1} with binding the transition state.

We see no significant difference in the steady-state kinetic parameters when the trinucleotide substrate GpApC is extended in a 5′ direction to give the CpGpApC tetranucleotide.

Discussion

Barnase has very low activity toward dinucleotide monophosphates. We have shown that catalysis is greatly enhanced by occupation of subsites that primarily affect k_{cat}.

The Primary Base Site, B0. The primary site in barnase, B0, has a strong preference for G, the enzyme cleaving only after G with dinucleotide monophosphate substrates and cleaving preferentially after G with longer substrates (Rushizky et al., 1963).

The N +1 Site. Values of k_{cat}/K_m suggest that, for the dinucleotide monophosphate substrates GpN, barnase preferentially cleaves GpA and GpG linkages over GpC and GpU linkages in the order of specificity GpA > GpG > GpU ~ GpC. This order is essentially in agreement with previous work (Mossakowska et al., 1989). Here, the order of preference between GpU and GpC is reversed compared with the earlier studies. These differences are not very significant with regard to specificity; in both studies the difference in binding energy between GpU and GpC is small (~0.2 kcal mol^{-1}).

The difference in specificity between GpA and GpG is not large (~0.4 kcal mol^{-1}). Indeed, both k_{cat} and K_m are decreased in GpG relative to GpA, and it is possible that nonproductive binding may account for some of the observed differences in kinetic parameters (perhaps the 3′ guanosine binds in the 5′ site) although not, of course, for the difference in specificity constant. In any case, as the difference in binding energy between GpA and GpG is small, the second subsite specificity of barnase may more properly be thought of as one for purines over pyrimidines.

The P +2 Site. By far the largest difference in binding energy is seen on lengthening the substrate to occupy the P +2 site and not between different bases in particular subsites. The phosphate at the P +2 site gives rise to a 1000-fold rate enhancement worth 4.2 kcal mol^{-1} of total binding energy; there is a 10-fold decrease in K_m and a 100-fold increase in k_{cat}.

The N +2 Site. The presence of a cytidine at the N +2 subsite leads to a further increase in total binding energy of 1.7 kcal mol^{-1} (GpUp → GpUpC). In this case, the binding energy is manifested primarily in k_{cat}; binding in the transition state has increased by 2.1 kcal mol^{-1}, and substrate binding

has decreased by 0.4 kcal mol^{-1}. One interpretation of this observation is as follows. There is a large binding energy contribution from the terminal phosphate of GpUp, but the substrate is not ideally oriented for catalysis, and too much of the binding energy is manifested in the ground state. When a nucleoside binds in the N +2 site, however, some of the binding energy due to this moiety is used to orient the substrate in a conformation that shifts the binding energy (due to P +2 and N +2) from substrate to transition state and thus to increase the catalytic rate. Another interpretation is that some of the binding energy is used to distort the enzyme to align it. This is to be expected for an enzyme whose primary function is probably the cleavage of RNA into small oligomers rather than the cleavage of dinucleotides; subsites have evolved to increase the catalytic efficiency of the enzyme with the natural RNA substrate. A third interpretation is that subsite binding energy is used to overcome nonproductive binding.

Comparison of the binding energies of GpApC and GpApA shows that there is also a subsite specificity for the third base position; GpApA has 0.6 kcal mol^{-1} more binding energy than GpApC with barnase, and this binding energy is manifested mostly in K_m. One could imagine that both C and A bind in the same subsite and optimally orient the substrate for bond cleavage. Thus, any difference in binding between C and A in the N +2 subsite could manifest itself only in K_m. The value of k_{cat}/K_m for GpApA is so high as to be approaching diffusion control. If this is so, it is possible that the actual difference in binding energy between A and C in the N +2 subsite is higher than that observed. It is not possible from these data to tell if the discrimination is only between purines and pyrimidines or if it is more specific.

It may also be seen that the increase in total binding energy on going from U to A in the +1 subsite is half as much when the +2 site is occupied as when it is not and the binding is distributed differently in the two cases; there is no increase in substrate binding on going from U to A when the +2 site is occupied, but there is an increase in transition state binding. Clearly, A in the N +1 position binds better than U in both the ground and the transition state. When there is a C in the N +2 subsite, however, catalysis, and not substrate binding, is maximized. The presence of C in the N +2 position may decrease the total interaction energy at the +1 subsite such that the difference in binding energy between A and U in this position is also decreased. Further evidence that the binding energy is better utilized for catalysis in the trinucleotides than the dinucleotides comes from the observation that all of the binding energy difference between U and A in the +1 subsite of the trinucleotide is in k_{cat}, whereas most is in K_m for the dinucleotides.

The N −1 and P 0 Subsites. There is no difference, within experimental error, between the steady-state kinetic parameters of GpApC and CpGpApC. This suggests either that any binding energy due to N −1 and P 0 is less than experimental error or that there are not subsites at the N −1 or P 0 subsites. One could also argue that as the rates observed with these substrates are beginning to approach the values associated with diffusion control (10^9 M^{-1} s^{-1}; Fersht, 1985), any difference in binding energy between the two substrates would not be seen. However, we see a doubling of k_{cat}/K_m on going from GpApC to GpApA, which indicates that diffusion control has not been reached in the former substrate.

Interaction between the +1 and +2 Sites. It is possible to measure the interaction between successive groups on the substrate (i.e., between the B +1 and +2 positions of the substrate) by means of thermodynamic cycles [cf. Carter et al. (1984) and Horowitz (1987)]. These interactions may be direct or mediated via the enzyme.

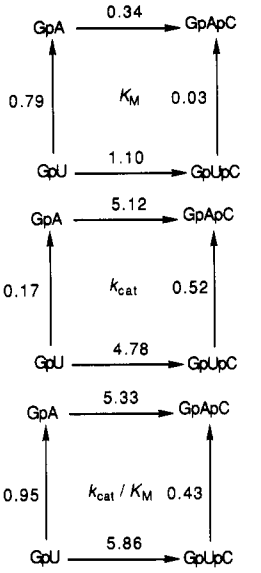

FIGURE 3: Thermodynamic cycles for changes in binding energy of substrate to enzyme on permutation of subsite occupancy: (top) apparent substrate binding (calculated from K_m); (middle) relative binding of transition state and substrate (calculated from k_{cat}); (bottom) binding of transition state to enzyme (calculated from k_{cat}/K_m). The energy changes in the direction of the arrows are in kilocalories per mole. A, $\Delta G_{int} = -0.82$; B, $\Delta G_{int} = 0.28$; C, $\Delta G_{int} = -0.53$. ΔG_{int} values are calculated by subtraction of the right-hand side from the left-hand side of the cycle and thus are a measure of the influence of the P +1/N +2 positions on the N +1 position. Values in kilocalories per mole are taken from Table III.

In Figure 3 are three cycles related to free energies calculated from K_m, k_{cat}, and k_{cat}/K_m for the substrates GpU, GpA, GpApC, and GpUpC. An interaction energy, ΔG_{int}, may be calculated: ΔG_{int} = rhs of cycle − lhs of cycle (Carter et al., 1984). If the binding due to a group at a particular position (e.g., N +1) is not influenced by a group at another position (e.g., P +2, N +2), then $\Delta G_{int} = 0$ and the binding due to the two groups is additive. If $\Delta G_{int} \neq 0$, then the binding is nonadditive and one group is affecting the way the other binds.

In all cases looked at for K_m, k_{cat}, and k_{cat}/K_m, binding is nonadditive. In addition to interaction between the groups bound to the enzyme, nonadditivity in binding energies calculated from K_m and k_{cat}/K_m could result from different conformations of the substrate in solution. We also see nonadditivity in k_{cat}, however, suggesting that site–site interactions do occur when the substrate is bound. By comparing ΔG_{int} values calculated from K_m and k_{cat}, it is possible to see how this interaction varies between the Michaelis complex and the transition state. The value of ΔG_{int} changes sign on going from the Michaelis complex (−0.76 kcal mol^{-1}) to the transition state (0.35 kcal mol^{-1}). This implies that a favorable interaction in the Michaelis complex becomes unfavorable in the transition state. It is also clear from these cycles that most of the enhancement of catalysis on going from dinucleotide monophosphates to longer substrates is manifested as an increase in k_{cat}.

Comparison with Other Ribonucleases. Crystallographic data suggest that barnase and RNase T1 are members of the same structural family (Mauguen et al., 1982), and the primary sequence of RNase U2 suggests that it is also a member of this family (Yasuda & Inoue, 1982). However, there are structural differences between these enzymes. For example, RNase T1 is acidic and barnase is basic. Barnase has a catalytically important lysine (27) on the second α-helix.

Mutation of this residue leads to a 3500-fold decrease in k_{cat} for GpA transesterification (Mossakowska et al., 1989); this residue is at the N-terminal end of a helix that is not present in RNase T1 or RNase U2 (Hill et al., 1983). These differences are reflected in kinetic differences in both subsite specificity and also activity toward substrates of varying length. RNase T1 is less discriminatory between dinucleotide monophosphate substrates and RNA than is barnase. The value of k_{cat} for the transesterification of GpA catalyzed by barnase is 190-fold lower than that of RNase T1; but barnase is twice as effective at RNA hydrolysis (Walz et al., 1978; Mossakowska et al., 1989). In addition, there is evidence for a subsite in the N −1 position in RNase T1 (Osterman & Walz, 1979). RNase U2 shows a completely different specificity to barnase, cleaving after A. There is a similar propensity for increase in k_{cat}/K_m as the length of the substrates increases, albeit less pronounced: about 20-fold on adding a terminal phosphate to ApN and a further 10-fold on adding a cytidine to give ApNpC. However, RNase U2 appears to have a "negative" N −1 subsite; addition of a nucleotide 5′ to ApGp to give NpApGp leads to between a 2- and 20-fold decrease in k_{cat}/K_m (Yasuda & Inoue, 1982).

In summary, barnase has a specificity for G in the B0 subsite, which is very high in small substrates but is relaxed in RNA. There is a preference for purines over pyrimidines in the +1 subsite. There is also discrimination between C and A in the B +2 site. There is a very large gain in catalytic efficiency on increasing substrate length from dinucleotide monophosphates to dinucleotide diphosphates and a further significant gain on addition of another nucleoside at the 3′-position.

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