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Quantitative Analysis of the Contribution of Glu46 and Asn98 to the Guanosine Specificity of Ribonuclease T_1[†]

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ABSTRACT: In the crystal structure of the ribonuclease T_1 (RNase T_1; EC 3.1.27.3)–2′-GMP complex the hydrogen-bonding potential of the guanine base is saturated [Arni, R., Heinemann, U., Tokuoka, R., & Saenger, W. (1988) *J. Biol. Chem. 263*, 15358–15368]. The oxygens of the Glu46 carboxylate and the Asn98 main-chain carbonyl act as hydrogen-bond acceptors for the N(1)H–C(2)–N(2)H_2 part of the base. We measured the transesterification kinetics of wild-type and Glu46Ala RNase T_1 using the GpU, IpU, and XpU series of analogous substrates. We found that the N(1)H---Glu46 Oϵ_1, the N(2)H---Glu46 Oϵ_2, and the N(2)H---Asn98 O hydrogen bonds have an apparent contribution of 2.7, 1.1, and 1.2 kcal/mol to the interaction energy of the enzyme and the transition state of the substrate. Wild-type RNase T_1 discriminates guanine from nonionized xanthine (a guanine analogue in which the exocyclic amino group is replaced by an oxygen) by about 4.4 kcal/mol. Loss of the specific hydrogen bonds with the exocyclic amino group of the guanine base accounts for 2.4 kcal/mol of this discrimination energy; 2.0 kcal/mol is due to unfavorable non-H-bonded oxygen–oxygen contacts in the enzyme–xanthine complex. A pH dependence study shows that the deprotonated form of xanthine (i.e., the 6-keto-2-enolate anion; pK_a = 5.4) is far less preferred, if not excluded, as substrate by wild-type RNase T_1; this may be attributed to an electrostatic repulsion of the negatively charged xanthine by the Glu46 carboxylate group.

Ribonuclease T_1 (RNase T_1, EC 3.1.27.3) from the fungus *Aspergillus oryzae* is the best known representative of a family of homologous microbial ribonucleases (Hill et al., 1983). These enzymes cleave single-stranded RNA by a transesterification reaction, yielding a 2′,3′-cyclophosphate. In a second step this cyclic intermediate may be hydrolyzed to yield a terminal 3′-phosphate. RNase T_1 has a pronounced specificity for the base guanine; kinetic studies on the transesterification of dinucleoside phosphates revealed that the specificity constant (k_{cat}/K_m) for GpN[1] substrates is about 10^6-fold greater than that for corresponding ApNs and at least 10^8-fold greater than that for CpNs and UpNs (Walz et al., 1979).

The three-dimensional structure of RNase T_1, complexed with the competitive inhibitor 2′-GMP, has been determined to 1.9-Å resolution by Heinemann and Saenger (1982) [see also Arni et al. (1988) and Sugio et al. (1988) for more refined data]. The observed mode of 2′-GMP binding provides a structural basis for understanding the enzyme's specificity. In the crystal structure the hydrogen-bonding potential of the guanine base is completely saturated by complementary donor/acceptor sites on the enzyme as illustrated in Figure 1 (Arni et al., 1988). Glu46, a strictly conserved residue in the family of microbial RNases, forms a pair of H-bonds with the N(1)H-C(2)–N(2)H_2 part of the base: N(1)H---Glu46 Oϵ_1 and N(2)H---Glu46 Oϵ_2 (Figure 1). The exocyclic amino group of guanine forms an additional hydrogen bond with the Asn98 main-chain carbonyl. This pattern of nucleotide–protein interactions is supported by kinetic studies. Using the Glu46Ala mutant form of RNase T_1, Hakoshima et al. (1988) found that the carboxylate group contributes 3.3 kcal/mol to the catalytic efficiency (k_{cat}/K_m) of pGpC cleavage. Comparison of the transesterification kinetics of the substrates GpU and IpU revealed that the NH_2 group on C(2) contributes 2.6 kcal/mol to binding (Walz et al., 1979).

In this study we measured the kinetics of transesterification using both modified dinucleoside phosphate substrates and Glu46-substituted RNase T_1 mutants. The analysis of the contribution of particular groups to the binding energy of the enzyme–substrate complex affords a quantitative description of the roles of separate contacts in guanine recognition and its discrimination from other bases. The apparent contributions of the contacts with N(1)H and the C(2)-substituent of guanine, hypoxanthine, and xanthine are reported.

Experimental Procedures

Chemicals. The following ribonuclease T_1 substrates, purchased from Sigma, were used: the dinucleoside phosphates GpC, GpA, GpU, and IpU and Torula yeast RNA (type VI). $NaNO_2$ was from Aldrich. Common reagents were purchased at the highest purity available.

Preparation of XpU. XpU was prepared from GpU by deamination of the guanine base with nitrous acid under acidic conditions (Shapiro & Pohl, 1968); 10 mg of GpU and 90 mg of $NaNO_2$ were dissolved in 10 mL of 1 M formic acid (pH 3.0). After incubation for 2 h at 50 °C the XpU product was

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[1] Abbreviations: cGMP, 2′,3′-cyclic guanosine monophosphate; cXMP, 2′,3′-cyclic xanthosine monophosphate; EDTA, ethylenediaminetetraacetic acid; 2′-GMP, guanosine 2′-phosphate; GpN/ApN/CpN/UpN/GpA/GpC/GpU/IpU/XpU, 3′,5′-linked dinucleoside monophosphate compounds (N, any of the four common nucleosides; I, inosine or hypoxanthosine; X, xanthosine); IPTG, isopropyl β-D-thiogalactopyranoside; pGpC, 5′-phosphorylated GpC; Tris, tris(hydroxymethyl)-aminomethane.

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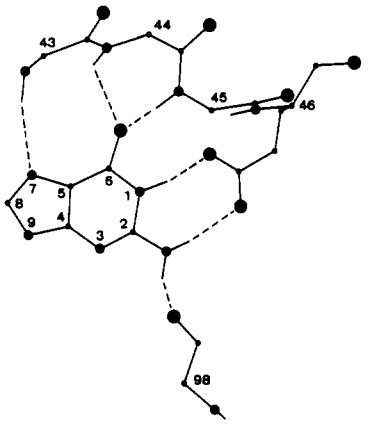

FIGURE 1: Pattern of the guanine–RNase T_1 hydrogen-bond interactions found in the crystalline structure of the RNase T_1–2′-GMP complex (Arni et al., 1988). The guanine base, the backbone atoms of Asn43, Asn44, Tyr45, Glu46, and Asn98, and the side chain of Glu46 are shown. The atoms O, N, and C are given in decreasing size. The residue number is shown near the Cα atom. The base numbering follows IUPAC conventions. Aliphatic protons are omitted for clarity. Dashed lines represent hydrogen bonds.

purified by reversed-phase HPLC on a Vydac C18 (218TP54) column. Fractions were eluted at 1 mL/min with a linear gradient from 20 to 60% B (20-mL total volume). Solvent A was 50 mM ammonium acetate (pH 4.5), and solvent B was 50 mM ammonium acetate/50% methanol (pH 4.9). Incubation of the purified dinucleoside phosphate with RNase T_1 yielded xanthosine monophosphate and uridine as shown by reversed-phase HPLC analysis and UV measurements on the hydrolysate (data not shown).

Overproduction and Oligonucleotide-Directed Mutagenesis. The overproduction of authentic RNase T_1 as a secretory protein in *Escherichia coli* has previously been described (Steyaert et al., 1990). Essentially, a chemically synthesized RNase T_1 coding region (Quaas et al., 1988) was fused to the *phoA* secretion signal and put under the control of the P_{tac} promoter. This expression cassette is present on a pMa/c type of phasmid (Stanssens et al., 1989), allowing expression and mutagenesis to be carried out from the same vector. The Glu46Ala, Glu46Gln, and Glu46Asp mutants were constructed as described by Stanssens et al. (1989). Oligonucleotides were synthesized by the phosphoramidite method (Beaucage & Caruthers, 1981) on an Applied Biosystems 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis (Wu et al., 1984). Mutants were identified by sequence determination (Sanger et al., 1977) of a few randomly picked clones; the entire sequence of each mutant gene was determined to check that no additional unwanted mutations had arisen during the mutagenesis procedure. The wild-type and mutant enzymes used in the present study are of the isoform which contains a Lys at position 25; they were purified to homogeneity as described (Steyaert et al., 1990).

Kinetics. All experiments were performed at 35 °C. The protein concentrations of wild-type RNase T_1 as well as the mutant forms of RNase T_1 were determined spectrophotometrically at 278 nm where $A_{0.1\%} = 1.9$ (Egami et al., 1964). In experiments requiring high substrate concentrations, 0.5- or 0.1-cm path-length cuvettes were used to diminish the background absorbance. Experimental data were analyzed with the program ENZFITTER (Leatherbarrow, 1987).

(*A*) *Dinucleotide Phosphate Transesterification.* The kinetic parameters for the transesterification of GpN dinucleoside phosphates were determined from initial velocities by measuring the absorbance increase at 280 nm (Zabinski & Walz, 1976). The GpN dinucleoside phosphate concentrations were varied between 20 μM and 2 mM. Reactions were started by adding enzyme at a final concentration ranging from 5×10^{-10} to 1×10^{-7} M, depending on the enzyme used.

The second-order rate constants (k_{cat}/K_m) for the transesterification of IpU and XpU were derived from progress curves recorded at substrate concentrations much lower than K_m (refer to pH Dependence of k_{cat}/K_m). IpU or XpU was added to the buffer at a final concentration of 20 μM. Reactions were started by adding enzyme (final concentration between 1×10^{-7} and 2×10^{-5} M) and followed by monitoring the absorbance increase at 280 nm. All reactions were performed in 50 mM imidazole, 50 mM NaCl, and 2.5 mM EDTA at pH 6.0 (ionic strength 0.1 M).

(*B*) *RNA Cleavage.* The kinetic parameters for the cleavage of Torula yeast RNA were derived from initial rate experiments by following the absorbance decrease at 298.5 nm (Oshima et al., 1976). The RNA concentration was determined photometrically and is expressed in mononucleotide equivalents with a molar extinction coefficient at 260 nm of 8000 M^{-1} cm^{-1}. The RNA was extensively dialyzed against 50 mM Tris and 50 mM NaCl at pH 7.5 (ionic strength 0.1 M) to remove mono- and oligonucleotides. Kinetic measurements were performed in the same buffer at substrate concentrations varying between 0.5 and 30 mM. Reactions were started by adding enzyme at final concentrations ranging from 5×10^{-9} to 1×10^{-7} M.

(*C*) *pH Dependence of* k_{cat}/K_m. The pH dependence was determined for the dinucleoside phosphate substrates GpC and XpU in the pH range 4–7. The buffers had an ionic strength of 0.1 M and were sodium acetate/acetic acid at pH 4.0–6.0 and imidazole/HCl at pH 6.0–7.0. k_{cat}/K_m values were obtained by monitoring progress curves at substrate concentrations much lower than K_m. Under this condition the progress curve follows first-order kinetics with rate constant V_{max}/K_m (Fersht & Renard, 1974). The procedure allows the collection of many data and eliminates the need to consider the pH dependence of the difference molar extinction coefficients. Dinucleoside phosphates were added to the buffers at final concentrations of 10 μM. Reactions were started by adding enzyme (final concentration between 1×10^{-9} and 2×10^{-5} M) and followed by measuring the absorbance increase at 280 nm.

Crystal Structure. The atomic coordinates of the RNase T_1–2′-GMP complex were taken from the Protein Data Bank (1rnt; Bernstein et al., 1977). Structures were displayed on an Evans & Sutherland PS390 graphic terminal using the BRUGEL software package (Delhaize et al., 1984).

RESULTS

Effect of Glu46 Substitutions on the Transesterification of GpA and GpC. Table I lists the kinetic parameters for the transesterification of the dinucleoside phosphate substrates GpC and GpA by wild-type, Glu46Ala, Glu46Asp, and Glu46Gln RNase T_1 at pH 6.0 and an ionic strength of 0.1 M. The transesterification rates of Glu46Ala and Glu46Gln RNase T_1 were found to be linearly dependent on the concentration of GpA or GpC over the entire substrate concentration range which is experimentally accessible. It is not possible to follow the reaction at dinucleoside phosphate concentrations above 2 mM because of the high background absorbance at 280 nm. From these linear relationships we can only derive the apparent second-order rate constant for the transesterification reaction (k_{cat}/K_m) and define a minimum value for K_m. For Glu46Asp RNase T_1 there is no longer a linear increase in the reaction rate as the substrate concentration approximates a value of 2 mM; nonlinear analysis of

Table I: Kinetic Parameters of Wild-Type, Glu46Ala, Glu46Gln, and Glu46Asp RNase T_1 for GpC and GpA Transesterification[a]

	GpC			GpA		
	K_m (10^{-5} M)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (10^{-5} M)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
wild type	22 ± 3	429 ± 15	1950	7.1 ± 0.8	96 ± 3	1389
Glu46Ala[b]	>500		5.1	>500		3.5
Glu46Gln[b]	>500		12.2	>500		5.9
Glu46Asp	404 ± 154	452 ± 134	112	332 ± 115	143 ± 50	43.0

[a] Measurements were performed at pH 6.0, 35 °C, and 0.1 M ionic strength. [b] For these mutants the ratio k_{cat}/K_m is the only determinable parameter since the initial velocities are linearly proportional to $[S]_0$ (≤2 mM); a lower limit for K_m has been estimated. See text for more details.

Table II: Apparent Kinetic Parameters for the Cleavage of RNA by Wild-Type, Glu46Ala, Glu46Gln, and Glu46Asp RNase T_1[a]

	K_m (mM)[b]	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
wild type	2.9 ± 0.4	1296 ± 57	452
Glu46Ala	57 ± 16	725 ± 148	12.7
Glu46Gln	40 ± 11	813 ± 155	20.6
Glu46Asp	16.3 ± 2.5	1881 ± 137	115.2

[a] Measurements were performed at pH 7.5, 35 °C, and 0.1 M ionic strength. [b] The RNA concentration is arbitrarily expressed in mononucleotide equivalents. See text for details.

these data yields an accurate value for the ratio k_{cat}/K_m and an approximate yet instructive value for the parameters K_m and k_{cat}. The Glu46 to Asp replacement results in a considerable increase in K_m concomitant with only small changes in k_{cat}, indicating that Glu46 is involved in substrate binding rather than in catalysis. The dramatic effects of the Glu46Ala and Glu46Gln mutations on the Michaelis constant for the transesterification of GpA and GpC (K_m must be above 5 mM; Table I) also indicate that Glu46 replacements interfere with substrate binding.

In contrast to our results, Hakoshima et al. (1988) claim that substitution of an Ala for Glu46 causes a 152-fold lowering in k_{cat} and only minor changes in K_m for the cleavage of pGpC. The reported decrease in k_{cat}/K_m is, however, similar to what we observe. The authors carried out initial rate experiments at substrate concentrations ranging from 0.1 to 0.2 mM, much below the K_m value (0.8 mM) they deduced from these measurements by a Lineweaver–Burk plot analysis. We assume that Hakoshima and co-workers also observed a linear relationship between the catalytic rate and the pGpC concentration.

Effect of Glu46 Substitutions on RNA Cleavage. The complete understanding of the RNase T_1 catalytic mechanism requires studies of its behavior with natural multimeric substrates. We therefore tried to extend our analysis based on dinucleoside phosphate model substrates by measuring the effect of Glu46 substitutions on RNA digestion. The cleavage of RNA can be followed by measuring the absorbance decrease at 298.5 nm (Oshima et al., 1976). The concentration of RNA (from Torula yeast) was arbitrarily expressed in mononucleotide equivalents, i.e., the concentration of ribonucleotides one would obtain after cleavage of all phosphodiester bonds (see Experimental Procedures). Initial rate experiments at RNA concentrations ranging from 0.5 to 30 mM result in a hyperbolic plot of catalytic rate versus substrate concentration, indicating that RNase T_1 follows saturation kinetics for the cleavage of RNA. Table II lists the apparent steady-state kinetic parameters for wild-type, Glu46Ala, Glu46Asp, and Glu46Gln RNase T_1 at pH 7.5 and an ionic strength of 0.1 M. It should be pointed out that the absolute values of k_{cat} and K_m are meaningless because of the arbitrary definition of the RNA concentration as well as expected differences in the reaction rates due to the heterogeneity of RNA isolated from biological sources. Nevertheless, the data in Table II are useful to evaluate the effect of mutations on the behavior of RNase T_1 toward its natural substrate. All three Glu46 mutations result in a substantial increase of the apparent K_m value concomitant with only minor changes in k_{cat}. Although the experiments on dinucleoside phosphates reveal a more pronounced effect on the k_{cat}/K_m ratio (see Table I)—a discrepancy which is not readily rationalized—the two complementary studies provide essentially identical results. It appears that Glu46 is engaged in the binding rather than in the chemical cleavage of dinucleoside phosphate model compounds as well as more complex polymeric substrates.

Specificity for GpU, IpU, and XpU. The use of substrate analogues represents a complementary approach to investigate enzyme–substrate interactions. IpU and XpU are two analogues of the dinucleoside phosphate GpU which have the guanine exocyclic amino group replaced by a hydrogen and an oxygen atom, respectively. Uridine was chosen as the 3′-nucleoside in this series of substrates because (1) IpU is commercially available and (2) uracil does not react with nitrous acid, permitting the preparation of XpU from GpU by deamination (see Experimental Procedures). We measured the specificity constants (k_{cat}/K_m) of wild-type and Glu46Ala RNase T_1 for the substrates GpU, IpU, and XpU. The data, listed in Table III, were obtained from initial rate experiments or from progress curves at substrate concentrations much lower than K_m (see Experimental Procedures). The kinetic data were used to determine the apparent contribution of a particular enzyme or substrate group to the interaction energy of the enzyme–substrate complex in the transition state. These group binding energies were calculated from the effect on the specificity constant (k_{cat}/K_m) upon removal of that group, i.e., replacement by a H atom, with the relation: $\Delta G = -RT \ln [(k_{cat}/K_m)_{group}/(k_{cat}/K_m)_H]$. Figure 2 shows two thermodynamic cycles that schematically illustrate the changes in interaction energy of the transition-state complex induced by removal of (1) the Glu46 side chain, (2) the guanosine exocyclic amino group, and (3) the xanthosine C(2) oxygen.

Table III: k_{cat}/K_m Values for the Transesterification of GpU, IpU, and XpU by Wild-Type and Glu46Ala RNase T_1[a]

	k_{cat}/K_m (mM^{-1} s^{-1})		
	GpU	IpU	XpU
wild type	1070 ± 158	23.3 ± 1.5	0.174 ± 0.020 (0.885 ± 0.060)[b]
Glu46Ala	2.05 ± 0.10	0.268 ± 0.016	0.073 ± 0.006

[a] Measurements were performed at pH 6.0, 35 °C, and 0.1 M ionic strength. [b] The value in parentheses gives the calculated specificity constant for nonionized XpU at pH 6.0 (the experimental value was divided by the fraction of nonionized XpU at pH 6.0). For this correction it is assumed that deprotonation of XpU (pK_a = 5.39 ± 0.05) prevents binding to wild-type enzyme (see text).

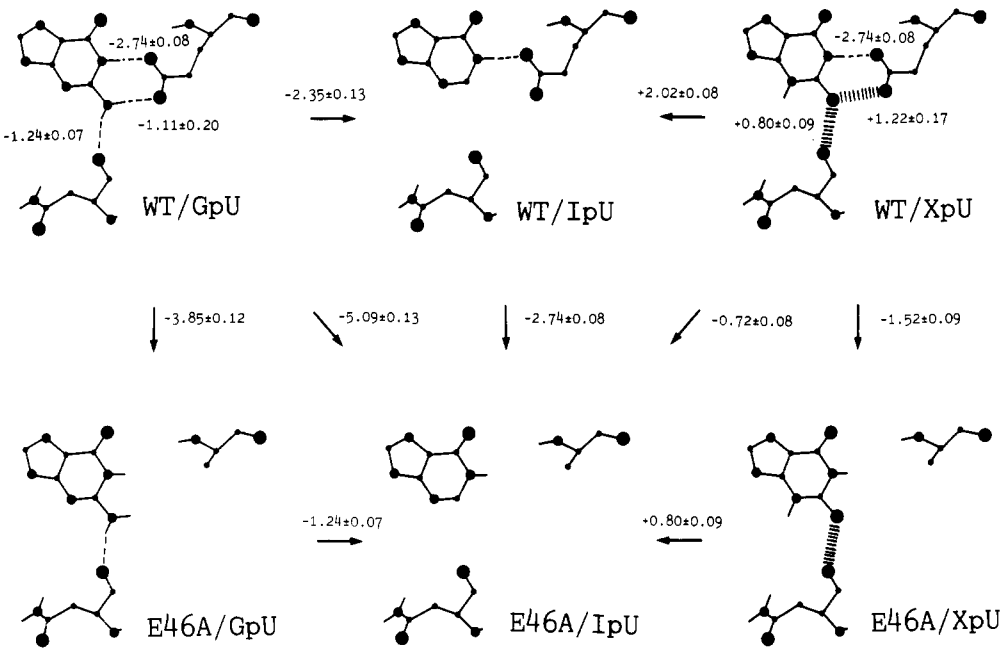

FIGURE 2: Thermodynamic cycles relating the changes in interaction free energy of the enzyme–substrate transition state induced by removal of (1) the Glu46 side chain, (2) the guanine C(2) exocyclic amino group, and (3) the xanthine C(2) oxygen. The base (guanine, hypoxanthine, or xanthine), Asn98, and the residue at position 46 (Glu or Ala) are shown for every enzyme–substrate couple. Atoms O, N, and C are drawn in decreasing size. Aliphatic protons are omitted for clarity. Dashed lines symbolize hydrogen bonds; hatched lines represent unfavorable contacts. The values near the arrows represent the loss (−) or gain (+) in enzyme–substrate interaction energy upon removal of a particular enzyme or/and substrate group. The values are expressed in kcal/mol and were calculated from the effect on the ratio k_{cat}/K_m (see Table III; for more details refer to text). For wild-type (WT) enzyme complexed with GpU or XpU the apparent contributions of individual enzyme–substrate contacts are given (see text for details).

Table IV: Slopes from log (k_{cat}/K_m) vs pH Plots in the pH Range 5.5–6.5[a]

	wild type	Glu46Ala
GpC	−0.21 ± 0.03	−0.34 ± 0.02
XpU	−0.99 ± 0.08	−0.40 ± 0.04

[a] Calculated from the plots presented in Figure 3.

Effect of Xanthine Ionization on XpU Transesterification. Nonionized xanthosine has the 2,6-diketo form; on acid dissociation the proton is lost from N(3) to give the 6-keto-2-enolate anion (Roy & Miles, 1983). The pK_a of the xanthine moiety of XpU was determined by spectroscopic titration (280 nm, data not shown) and equals 5.39 ± 0.05 at 35 °C and an ionic strength of 0.1 M. We have analyzed whether or not this substrate ionization affects substrate binding and/or catalysis. Ionizations in both the free enzyme and the free substrate that are critical for substrate binding and/or catalysis are reflected in the pH dependence of k_{cat}/K_m. Figure 3 shows the profiles of k_{cat}/K_m in the pH range 4–7 for the transesterification of XpU by wild-type and Glu46Ala RNase T_1. The profiles for the transesterification of GpC have been included as references; it has been demonstrated that the protonation of the N(3) of cytosine, the only ionization of GpC in the pH range 4–7, is not critical for substrate binding and/or catalysis (Zabinski & Walz, 1976).

Inspection of Figure 3 reveals that the pH dependence for XpU transesterification by wild-type enzyme differs significantly in shape from the pH profiles characterizing the other enzyme/substrate couples. This is manifested in the slopes of the plots of log (k_{cat}/K_m) vs pH in the pH interval 5.5–6.5 (see Table IV). For wild-type enzyme, this slope changes from −0.21 to −0.99 when XpU instead of GpC is used as the substrate. From the graphical analysis of the plot of log (k_{cat}/K_m) vs pH for XpU transesterification, an apparent pK of about 5.4 can be estimated (see Figure 3). This value agrees

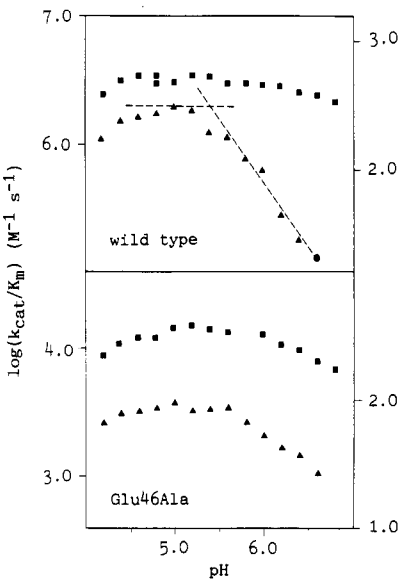

FIGURE 3: Plots of log (k_{cat}/K_m) versus pH for the transesterification of GpC (■; left ordinate) and XpU (▲; right ordinate) by wild-type and Glu46Ala RNase T_1. All experiments were carried out at 35 °C in 0.1 M standard buffers; other details are under Experimental Procedures. From the pH profile for XpU transesterification by wild-type enzyme, a pK of 5.4 was deduced with the graphical method of Dixon (1953). This pK value corresponds to the pH at which the straight-line portions with slopes of 0 and −1 intersect.

quite well with the acid dissociation constant of XpU determined spectroscopically under the same conditions. Taken together, the observations suggest that the deprotonation of the xanthine N(3), the only titratable group of XpU in the pH range 4–7, restricts substrate binding and/or catalysis. For Glu46Ala RNase T_1, the slopes in the pH range 5.5–6.5 are −0.34 and −0.40 when GpC and XpU are used, respectively. This result indicates that the ionization of the xanthine base

does not significantly affect the transesterification rate of XpU when Glu46 of RNase T_1 is replaced by an Ala residue.

Discussion

On the basis of the three-dimensional structure of the RNase T_1–2′-GMP complex, it has been proposed that the guanidinium part of the guanine base is specifically recognized through three hydrogen bonds: N(1)H---Glu46 $O\epsilon_1$, N(2)H---Glu46 $O\epsilon_2$, and N(2)H---Asn98 O (Arni et al., 1988; see Figure 1). Substitution of Glu46 by an Asp, Gln, or Ala residue results in a decrease in k_{cat}/K_m for the transesterification of GpN model substrates as well as for the cleavage of RNA (see Tables I and II). This reduced specificity is mainly due to an increase in K_m. Likewise, removal of the guanine exocyclic amino group of the dinucleoside phosphate GpU lowers k_{cat}/K_m by increasing the Michaelis constant (Walz et al., 1979). Both observations suggest that the enzyme–substrate interactions involving the Glu46 carboxyl group and the guanine exocyclic amino group contribute to binding and do not accelerate chemical cleavage.

We have used mutant enzymes and modified substrates to investigate the contributions of the N(1)H---Glu46 $O\epsilon_1$, N(2)H---Glu46 $O\epsilon_2$, and N(2)H---Asn98 O hydrogen bonds to substrate specificity. We determined how the loss of the Glu46 side chain (Glu46 to Ala) and/or the guanine exocyclic amino group (GpU to IpU) affects the binding energy of the RNase T_1–GpU transition-state complex. The contribution of a particular group to the interaction energy, i.e., the group binding energy, was calculated from the change in k_{cat}/K_m upon removal of that group (Table III). The results have been arranged in a thermodynamic cycle (see left part of Figure 2). It appears that the group binding energy of the Glu46 carboxylate depends on the presence of the $N(2)H_2$ group of guanine; ΔG equals -3.85 ± 0.12 kcal/mol when GpU is used as the substrate and -2.74 ± 0.08 kcal/mol in the case of IpU. Similarly, the incremental energy for the removal of the exocyclic amino group depends on the presence of the Glu46 carboxylate (-2.35 ± 0.13 and -1.24 ± 0.07 kcal/mol, respectively). Thus, the interaction energy associated with one group clearly depends on the presence of the other group. This observation can adequately be explained in terms of a direct contact between the two groups; i.e., the result is consistent with the existence of the N(2)H---Glu46 $O\epsilon_2$ hydrogen bond inferred from the X-ray data. If it is assumed that the above enzyme and/or substrate lesions have no further structural consequences superimposed on the binding energetics, the apparent contributions of particular enzyme–substrate contacts to the overall binding energy may be deduced. The free energy difference between the Glu46Ala RNase T_1–GpU and the Glu46Ala RNase T_1–IpU complex correpsonds to the apparent contribution of the N(2)H---Asn98 O hydrogen bond. The group binding energy of the Glu46 carboxylate, with IpU as the substrate, equals the contribution of the N(1)H---Glu46 $O\epsilon_1$ hydrogen bond. The dependence of the Glu46 group binding energy on the presence of the exocyclic NH_2 group (and vice versa) measures the apparent contribution of the N(2)H---Glu46 $O\epsilon_2$ hydrogen bond to guanine binding [(2.74–3.85) kcal/mol = (1.24 − 2.35) kcal/mol = −1.11 kcal/mol]. The apparent contributions of the individual hydrogen bonds to the binding energetics of the enzyme–substrate transition state are included in Figure 2.

The cavities in the complex that result from the removal of the Glu46 side chain and the substrate $N(2)H_2$ group are probably sufficiently large to allow access of water. If so, the apparent contributions of the individual hydrogen bonds are genuine incremental binding energies, i.e., the net free energy contribution of each bond to transfer the substrate from aqueous solution to the enzyme–substrate complex (Fersht, 1987). The total energetic contribution of the three bonds (5.1 kcal/mol) is within the range expected for one neutral–charged and two neutral–neutral hydrogen bonds (Fersht et al., 1985; Street et al., 1986; Bass & Cech, 1984; Freier et al., 1986). From our analysis it would appear that the N(1)H---Glu46 $O\epsilon_1$ hydrogen bond contributes more to binding than the N(2)H---Glu46 $O\epsilon_2$ bond. This seems consistent with the crystallographic data (Kostrewa et al., 1989); the N(1) to Glu46 $O\epsilon_1$ distance (2.7 Å) is shorter than the N(2) to Glu46 $O\epsilon_2$ distance (3 Å) for comparable donor–proton–acceptor angles (161° and 168°, respectively). The three contacts with the N(1)H-C(2)–$N(2)H_2$ hydrogen-bond donor region of guanine (a chemical configuration unique for this base) represent a factor of about 4000 in specificity and must therefore be considered important for the discrimination of guanine from other bases.

The second case this paper deals with is the substitution of a hydrogen-bond acceptor for a hydrogen-bond donor on the substrate. This single substitution introduces "mispairing" between donor and acceptor sites on enzyme and substrate. Xanthine differs from guanine essentially in the C(2) exocyclic substituent, which is an oxygen for the former and an amino group for the latter. We made the assumption that the N(3) proton of xanthine which is not present on guanine does not influence the enzyme–subtrate interaction. A hydrogen bond from the 5′-OH of the ribose to the guanine N(3) has been observed in the RNase T_1–2′-GMP crystal structure (Arni et al., 1988). The possibility that an equivalent intramolecular hydrogen bond of opposite polarity forms in the case of xanthosine seems to justify the above assumption.

Nonionized xanthosine has the 2,6-diketo form. On acid dissociation ($pK_a = 5.39 \pm 0.05$; see Results) the N(3) proton is lost to give the 6-keto-2-enolate anion (Roy & Miles, 1983). A pH dependence study shows that the specificity constant k_{cat}/K_m of wild-type enzyme with XpU as the substrate depends on the ionization of the xanthine base (Figure 3 and Table IV); it would appear that only the nonionized form is accepted as a substrate. A similar observation was made by Irie et al. (1970) for the hydrolysis of cXMP by wild-type RNase T_1. In contrast, the plot of log (k_{cat}/K_m) vs pH for Glu46Ala RNase T_1 indicates that this mutant transesterificates the ionized and the nonionized form of XpU with similar rates. We believe that the dramatic effect of the xanthine ionization on the specificity constant of wild-type enzyme results primarily from an electrostatic repulsion between the negatively charged xanthine and the Glu46 carboxylate. Although this type of repulsive interaction is eliminated in Glu46Ala RNase T_1, this mutant as well is expected to exhibit a lower specificity for ionized xanthine because of the greater energetic toll associated with an unpaired charged acceptor at the enzyme–substrate interface. Why this is not the case is not well understood. It may in part be explained if it is assumed that the Glu46Ala mutation allows entry of water to the binding site; this would neutralize the difference in desolvation energy of the charged xanthine compared to that of the neutral species.

The specificity constants for XpU transesterification have been used to extend the thermodynamic analysis (see Figure 2). The k_{cat}/K_m value for XpU transesterification by wild-type enzyme, measured at pH 6.0, was corrected for the ionization of the substrate (see Table III). Comparison with the data for IpU transesterification allows the deduction of the apparent contribution of particular enzyme–substrate contacts to the

overall interaction energy between wild-type RNase T_1 and nonionized XpU. We propose that the Glu46 side chain and the Asn98 main-chain carbonyl form one attractive and two repulsive contacts with the N(1)H–C(2)–O(2) part of xanthine. Assuming that the O(2) substituent does not distort the transition-state conformation, it is likely that the hydrogen bond between the Glu46 $O\epsilon_1$ and the N(1)H group (apparent $\Delta G = -2.74 \pm 0.08$ kcal/mol) also forms in the T_1–XpU complex. The contacts between the substrate O(2) and the enzyme oxygens Glu46 $O\epsilon_2$ and Asn98 O apparently reduce the interaction energy by $+1.22 \pm 0.17$ and $+0.80 \pm 0.09$ kcal/mol, respectively. This can be due to steric repulsion; in the crystalline RNase T_1–2′-GMP complex the distances from N(2) to Asn98 O and to Glu46 $O\epsilon_2$ are 2.9 and 3.0 Å, respectively (Kostrewa et al., 1989). In water, the minimum separation between non-H-bonded oxygen atoms varies between 3.1 and 3.6 Å depending on the relative orientations of the "lone pair" regions of the water oxygens (Savage & Finney, 1986). Even in the absence of steric repulsion the xanthine O(2) substituent should result in a considerable specificity decrease because the O(2) can still hydrogen bond as acceptor with bulk water whereas it remains unpaired upon association with the enzyme; i.e., water should more effectively compete with the enzyme for the XpU than for the IpU substrate (Fersht, 1987; Street et al., 1987).

In total, the present experiments show that RNase T_1 discriminates nonionized xanthine from guanine (corresponding to the substitution of an oxygen for NH_2 in an O---HNH---O complex) by 4.4 kcal/mol. The two hydrogen bonds between the Glu46 and Asn98 residues of RNase T_1 and the exocyclic NH_2 group of guanine account for 2.4 kcal/mol of the discrimination energy. The non-H-bonded oxygen–oxygen contacts appear to disfavor the xanthine–RNase T_1 complex formation by 2.0 kcal/mol. The ionized form of xanthine is rejected by the wild-type RNase T_1 through electrostatic repulsion involving the Glu46 carboxylate group.

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