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# Subsite Interactions of Ribonuclease $T_1$: Asn36 and Asn98 Accelerate GpN Transesterification through Interactions with the Leaving Nucleoside N†

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ABSTRACT: We previously presented evidence that ribonuclease $T_1$ (RNase $T_1$; EC 3.1.27.3) contains a subsite that, by interacting with the leaving nucleoside N of GpN dinucleoside phosphate substrates, contributes to catalysis. The $k_{cat}$ values for transphosphorylation follow the order GpC > GpA > GpU whereas the equilibrium dissociation constants for these substrates are very similar [Steyaert, J., Wyns, L., & Stanssens, P. (1991) *Biochemistry* (preceding paper in this issue)]. Consistent with this notion, we find that the rate of transesterification of the synthetic substrate GpMe, in which the leaving nucleoside is replaced by a methanol group, is at least 3 orders of magnitude lower than that of GpN substrates. The enzyme's affinity for GpMe is very similar to that for the various GpN substrates, indicating that the apparent contribution of the leaving nucleoside to ground-state binding is minimal. To identify the side chains that belong to the RNase $T_1$ subsite, we searched for amino acid substitutions that differentially affect the transesterification kinetics of GpNs versus GpMe. The Asn36Ala, Tyr38Phe, His92Gln, and Asn98Ala mutants have been analyzed. Of these, the Asn36Ala and Asn98Ala substitutions reduce the transphosphorylation rate of the different GpNs considerably whereas they have virtually no effect on the rate of GpMe transphosphorylation. This observation shows that the Asn36 and Asn98 amide functions are part of the RNase $T_1$ subsite. The sum of the contributions of the two residues accounts quite precisely for the differences in turnover rates among GpC, GpA, and GpU.

Early kinetic studies on the ribonuclease $T_1$ (RNase $T_1$; EC 3.1.27.3) catalyzed transesterification of dinucleoside phosphates suggested the existence of a subsite with a preference for cytidine as the leaving nucleoside. The preference for cytidine is barely significant with GpN[1] substrates because of compensatory changes in $k_{cat}$ and $K_m$ (Walz et al., 1979). We have shown that these compensatory changes result from a rate constant of product formation that exceeds the dissociation rate constant of the enzyme–substrate complex; i.e., the Michaelis constants ($K_m$) are higher than the corresponding equilibrium dissociation constants ($K_s$) if GpC or GpA is used as the substrate (Steyaert et al., 1991b). The equilibrium dissociation constants for GpA, GpC, and GpU appear to be very similar (~40 μM) whereas the $k_{cat}$ values follow the order GpC > GpA > GpU, indicating that the subsite primarily affects catalysis. The dependence of the transphosphorylation rate on the nature of the leaving nucleoside seems to be a general feature of RNases. For pancreatic ribonuclease, the transesterification rates of CpN and UpN were shown to vary more than 100-fold depending on the nature of N whereas the Michaelis constants remained virtually the same (Follmann et al., 1967). Similar observations have been made for other RNases (Imazawa et al., 1968; Yasuda & Inoue, 1982; Mossakowska et al., 1989).

The only structural information on the subsite derives from a crystallographic investigation of RNase $T_1$ complexed with the specific inhibitor 2′,5′-GpG (Koepke et al., 1989). The 3′-terminal guanine base forms no hydrogen bonds with the enzyme. The poor binding is reflected in the observation of two alternative positions for the 3′-terminal nucleoside. The data led them to propose intermolecular contacts between the 3′-terminal ribosome moiety and the Gly74 O, the Arg77 $N^{\eta 2}$, and the His92 $N^{\epsilon 2}$ for the major orientation and the Tyr38 $O^{\eta}$, the Asn98 $O^{\delta 1}$, and the Asn98 $N^{\delta 2}$ for the minor orientation.

In this study we present an experimental approach to identify amino acid side chains that belong to the RNase $T_1$ subsite. The method rests on the use of GpMe, a synthetic minimal substrate in which the leaving nucleoside is replaced by methanol. Two amino acid substitutions, Asn36Ala and Asn98Ala, have been found that differentially affect the transesterification kinetics of GpNs versus GpMe.

## Experimental Procedures

*Materials.* Wild-type, Tyr38Phe, and His92Gln RNase $T_1$ have been previously discussed (Steyaert et al., 1990, 1991b). The Asn36Ala, Arg77Tyr, and Asn98Ala mutants were constructed by oligonucleotide-directed mutagenesis as described (Stanssens et al., 1989) and purified to homogeneity as described (Steyaert et al., 1990). The dinucleoside phosphate substrates GpA, GpC, and GpU and 3′-guanylic acid were from Sigma. Anhydrous pyridine (distilled over $CaH_2$), $NH_4OH$, and MSNT were purchased from Merck. Triethylamine was from Aldrich. HPLC-grade acetic acid and ammonium acetate were purchased from Baker Chemicals; methanol was from Romil Chemicals.

*Preparation of GpMe.* $N^2$,$O^{2'}$,$O^{5'}$-triacetyl-3′-guanylic acid was prepared essentially as described by Lohrmann and

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[1] Abbreviations: cGMP, 2′,3′-cyclic guanosine monophosphate; EDTA, ethylenediaminetetraacetic acid; 3′-GMP, 3′-guanylic acid; GpEt, guanosine 3′-(ethyl phosphate); 2′,5′-GpG, guanylyl(2′,5′)guanosine; GpMe, guanosine 3′-(methyl phosphate); MSNT, 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole; GpN, 3′,5′-linked dinucleoside monophosphate compounds (N represents any of the four common nucleosides); TEAB, triethylammonium bicarbonate.

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Khorana (1964). A total of 0.05 mmol of the pyridinium salt of $N^2,O^{2'},O^{5'}$-triacetyl-3'-guanylic acid was dissolved in 0.5 mL of anhydrous pyridine, 15 μL of triethylamine, and 1 mL of anhydrous methanol. After addition of 30 mg (0.1 mmol) of MSNT, this solution was stirred at room temperature for 30 min. Then, 1 mL of $H_2O$ was added and the total mix was left for another hour at room temperature. Following concentration under vacuum (30 °C), 2.5 mL of a 30% solution of $NH_4OH$ was added and the mixture was left for 3 h at 40 °C. This mixture was again concentrated under vacuum (30 °C) and treated with 1 mL of TEAB whereafter the material was coevaporated three times with 20 mL of $H_2O$. The product was then chromatographed on a DEAE-Sephadex A-25 column (1.5 × 50 cm) with use of a linear gradient from 0.05 to 0.15 M TEAB (600-mL total volume). GpMe eluted at 0.13 M TEAB to give 13 mg of the pure product.

*Kinetics.* All experiments were performed at 35 °C in a buffer containing 50 mM imidazole, 50 mM NaCl, and 2.5 mM EDTA at pH 6 (ionic strength 0.1 M). RNase $T_1$ concentrations are based on $A_{278}^{0.1\%} = 1.9$ for a 10-mm light path (Egami et al., 1964). The values and standard errors of $k_{cat}$ and $K_m$ were obtained from nonlinear least-squares analysis using the program ENZFITTER (Leatherbarrow, 1987).

*(A) Dinucleoside Phosphate Transesterification.* The kinetic parameters for the conversion of GpN to cGMP and N by Asn36Ala, His92Gln, and Asn98Ala RNase $T_1$ were determined from initial velocities by measuring the absorbance increase at 280 nm (Zabinski & Walz, 1976) as described by Steyaert et al. (1991a). The parameters of wild-type and Tyr38Phe RNase $T_1$ were taken from Steyaert et al. (1991b).

*(B) GpMe Transesterification.* The steady-state kinetic parameters for the transesterification of the synthetic substrate GpMe were determined from initial velocity measurements. The concentration of GpMe was determined with use of a molar extinction coefficient at 260 nm of 11 400 $M^{-1}$ $cm^{-1}$. After incubation for 20–1000 min, reaction mixtures were analyzed by reversed-phase HPLC on a Vydac C18 (218TP54) column with a linear gradient from 0 to 100% B (20-mL total volume; flow rate 0.5 mL/min). Solvent A was 50 mM ammonium acetate (pH 4.5) and solvent B was 50% A and 50% methanol (pH 4.9). Incubation times were chosen such that less than 10% of the substrate was converted to products at enzyme concentrations $\ll [S]_0$. Initial rates were derived from the peak areas ($A_{260}$) of the products (cGMP and 3'-GMP) and the remaining substrate (GpMe) with a Varian 4290 integrator. Whereas, in practice, the hydrolysis of cGMP need not be considered when measuring the transesterification rate of GpN substrates [$(k_{cat}/K_m)_{GpN}/(k_{cat}/K_m)_{cGMP} \approx 3000$ and $(K_m)_{cGMP} \gg (K_m)_{GpN}$; Takahashi & Moore, 1982], the cGMP product effectively competes with the GpMe substrate [$(k_{cat}/K_m)_{GpMe}/(k_{cat}/K_m)_{cGMP} \approx 1.5$]. The rate of breakdown of GpMe is therefore given by

$$-\frac{d[GpMe]}{dt} = \frac{[GpMe]k_{cat}^{GpMe}[E]_{total}}{[GpMe] + K_m^{GpMe}(1 + [cGMP]/K_m^{cGMP})}$$

During our initial rate experiments the concentration of cGMP never exceeded 10 μM, i.e., 70 times lower than the Michaelis constant for cGMP. Under these experimental conditions ($[cGMP]/K_m^{cGMP} < 0.014$) the initial rates of GpMe breakdown reflect the steady-state parameters for the transesterification of this substrate.

## Results and Discussion

*Experimental Strategy.* The aim of this investigation is to identify amino acid side chains that belong to the RNase $T_1$ subsite, i.e., side chains that interact with the leaving nucleoside N of GpN substrates. In this study we made use of GpMe, a synthetic minimal substrate in which the leaving nucleoside N is replaced by methanol (Figure 1). The experimental approach consists of finding amino acid substitutions that differentially affect the transesterification kinetics of dinucleoside phosphate substrates (GpC, GpA, and GpU) on the one hand and GpMe on the other hand.

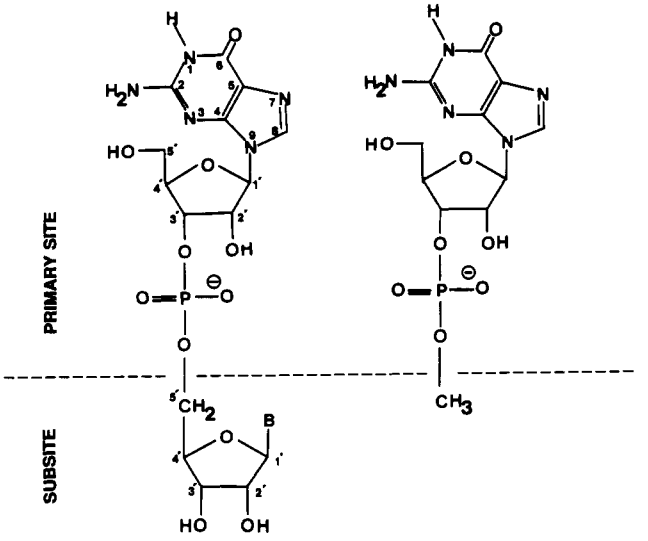
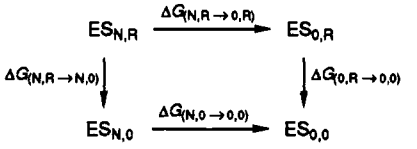



FIGURE 1: Chemical structure of the GpN (left) and GpMe (right) substrates. B represents the base of the leaving nucleoside of the GpN substrate. In the present study, we define the guanosine specific primary site as the collection of enzyme functional groups that interact with the substrate part shown above the dashed line; interactions with the lower part are referred to as subsite contacts. Note that contacts with the O5' atom of the leaving group are contained in the primary site.

The analysis of intermolecular interactions by means of mutant enzymes and modified substrates is essentially identical with the use of double mutants to study intramolecular interactions between two amino acid residues in a protein (Carter et al., 1984; Horovitz & Fersht, 1990). The energetics of the intermolecular interactions in an enzyme–substrate complex (ES) may be analyzed by constructing thermodynamic cycles of the type shown in Scheme I.

Scheme I




In the present study N represents the leaving nucleoside of a GpN dinucleoside phosphate substrate (N = A, C, or U) while R stands for the side chain of a particular amino acid residue; 0 symbolizes the elimination of N or R in a manner considered to be nondisruptive (Fersht et al., 1987). $\Delta G_{(N,R\rightarrow 0,R)}$ and $\Delta G_{(N,0\rightarrow 0,0)}$ are the apparent free energy changes upon replacement of the leaving nucleoside of the substrate by methanol (i.e., using the synthetic molecule GpMe as the substrate) in the presence and absence of R, respectively. $\Delta G_{(N,R\rightarrow N,0)}$ and $\Delta G_{(0,R\rightarrow 0,0)}$ are similarly defined for a nondisruptive mutation of residue R. $\Delta G$ values equal the group's apparent contribution to ground-state binding if they are calculated from the effect on the equilibrium dissociation constant $K_s$ [$\Delta G^{bind} = +RT \ln (K_s/K_s^{group\rightarrow 0})$] and equal to the contribution to catalysis when derived from the effect on the turnover number [$\Delta G^{cat} = -RT \ln (k_{cat}/k_{cat}^{group\rightarrow 0})$].

Table I: Steady-State Kinetic Parameters of Asn36Ala, Tyr38Phe, His92Gln, Asn98Ala, and Wild-Type RNase $T_1$ for a Variety of Substrates[a]

| | GpC | GpA | GpU | GpMe |
|---|---|---|---|---|
| | | wild type | | |
| $K_s$[b] ($\mu$M) | 29.5 | 56 | 33 | |
| $K_m$ ($\mu$M) | 216 ± 29 | 78 ± 8 | 33 ± 3 | 52 ± 4 |
| $k_{cat}$ ($s^{-1}$) | 429 ± 15 | 100 ± 3 | 36 ± 1 | (37 ± 1) × $10^{-3}$ |
| $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | 1986 | 1282 | 1091 | 0.712 |
| | | Asn36Ala | | |
| $K_m$ ($\mu$M) | 60 ± 9 | 48 ± 7 | 55 ± 9 | 187 ± 28 |
| $k_{cat}$ ($s^{-1}$) | 39 ± 2 | 11 ± 1 | 6.8 ± 0.3 | (33 ± 2) × $10^{-3}$ |
| $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | 650 | 229 | 124 | 0.176 |
| | | Tyr38Phe | | |
| $K_m$ ($\mu$M) | 32 ± 3 | 33 ± 7 | 40 ± 7 | 48 ± 22 |
| $k_{cat}$ ($s^{-1}$) | 5.3 ± 0.1 | 1.3 ± 0.1 | 0.66 ± 0.03 | (54 ± 8) × $10^{-5}$ |
| $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | 166 | 39 | 16.5 | 113 × $10^{-4}$ |
| | | His92Gln | | |
| $K_m$ ($\mu$M) | 255 ± 32 | 130 ± 11 | 109 ± 7 | 188 ± 42 |
| $k_{cat}$ ($s^{-1}$) | 0.213 ± 0.01 | 0.046 ± 0.001 | 0.014 ± 0.001 | (20 ± 1) × $10^{-6}$ |
| $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | 0.835 | 0.354 | 0.128 | 106 × $10^{-6}$ |
| | | Asn98Ala | | |
| $K_m$ ($\mu$M) | 66 ± 8 | 33 ± 7 | 25 ± 6 | 63 ± 17 |
| $k_{cat}$ ($s^{-1}$) | 45 ± 2 | 38 ± 2 | 19 ± 1 | (39 ± 5) × $10^{-3}$ |
| $k_{cat}/K_m$ ($mM^{-1}$ $s^{-1}$) | 682 | 1151 | 760 | 619 × $10^{-3}$ |

[a] Measurements were performed in imidazole buffer (0.1 M ionic strength), pH 6.0, at 35 °C. [b] The wild-type–GpC and wild-type–GpA reactions obey Briggs–Haldane kinetics ($K_m > K_s$); the equilibrium dissociation constants have been taken from Steyaert et al. (1991b).

When the effects of the two substitutions (N → 0 and R → 0) are not independent of each other, then the coupling term $\Delta G_{(NR)} = \Delta G_{(N,R\rightarrow 0,R)} - \Delta G_{(N,0\rightarrow 0,0)} = \Delta G_{(N,R\rightarrow N,0)} - \Delta G_{(0,R\rightarrow 0,0)}$ will differ from zero. The coupling energy equals the apparent interaction energy between side chain R and leaving nucleoside N; whether the interaction energy results from a direct contact or is due to an indirect effect that is propagated via the substrate molecule or the enzyme cannot be decided from this analysis. $\Delta G_{(0,R\rightarrow 0,0)}$ measures the apparent contribution of R to primary site interactions.

*Contribution of N to GpN Transesterification.* We previously presented evidence that the dinucleoside phosphate substrates GpC, GpA, and GpU bind to RNase $T_1$ with similar affinities; the equilibrium dissociation constants are 30, 56, and 33 $\mu$M, respectively (Steyaert et al., 1991b). We find that RNase $T_1$ binds the minimal substrate GpMe equally well (52 $\mu$M; Table I). Thus, the apparent contributions of the various leaving nucleosides to ground-state binding are minimal.

Substitution of methanol for the leaving nucleoside N in the GpN–wild-type RNase $T_1$ complex increases the activation energy for chemical turnover ($\Delta G^{cat}_{(N,R\rightarrow 0,R)}$) by 4.84, 5.73, and 4.21 kcal/mol for N being A, C, and U, respectively. If it is assumed that the synthetic and the natural substrates have similar intrinsic reactivities (see below), these experimental data clearly demonstrate that RNase $T_1$ possesses a subsite that substantially contributes to catalysis. It thus appears that RNase $T_1$, as many other depolymerizing enzymes, exhibits a greatly increased activity toward extended substrates as compared to minimal model compounds.

*Partial Mapping of the RNase $T_1$ Subsite.* Asn36, Tyr38, Arg77, His92, and Asn98 have been chosen as target residues for oligonucleotide-directed mutagenesis. Inspection of the X-ray structure of RNase $T_1$ complexed with 2′,5′-GpG (Koepke et al., 1989; see also introduction) reveals that the side chains of these residues constitute a number of potential hydrogen-bond donor/acceptor sites that are properly situated in the substrate binding cleft to interact with the leaving nucleoside.

The residues Asn36 and Asn98 were replaced by Ala while Tyr38 was mutated to Phe. His92 was substituted by a Gln residue. The crystal structure of His92Ala RNase $T_1$, an inactive enzyme (Nishikawa et al., 1987; Steyaert et al., 1990), reveals that the loop that contains residue 92 became flexible (Grunert et al., 1991). In contrast, the semiconservative His92Gln mutation gives an enzyme with significant residual activity (Steyaert et al., 1990; Table I). We have also constructed Arg77Tyr RNase $T_1$. The mutant gene was, however, found to be expressed very weakly, and it has thus far been impossible to isolate sufficient quantities of the mutant protein to perform kinetic studies.

From the kinetics of Asn36Ala, Tyr38Phe, His92Gln, Asn98Ala, and wild-type RNase $T_1$ for the substrates GpA, GpC, GpU, and GpMe (see Table I) a series of 12 thermo-

Table II: Energetic Contributions of Asn36, Tyr38, His92, and Asn98 to Chemical Conversion and to Ground-State Binding of GpN Substrates by RNase $T_1$

| | $\Delta G_{(N,R\rightarrow N,0)}$[a] | | $\Delta G_{(0,R\rightarrow 0,0)}$[a] | | $\Delta G_{(NR)}$[b] | |
|---|---|---|---|---|---|---|
| substrate | $\Delta G^{cat}$ | $\Delta G^{bind}$ | $\Delta G^{cat}$ | $\Delta G^{bind}$ | $\Delta G^{cat}$ | $\Delta G^{bind}$ |
| | | | Asn36Ala | | | |
| GpA | −1.35 | 0.09 | | | −1.28 | 0.87 |
| GpC | −1.47 | −0.43 | | | −1.40 | 0.35 |
| GpU | −1.02 | −0.31 | | | −0.95 | 0.47 |
| GpMe | | | −0.07 | −0.78 | | |
| | | | Tyr38Phe | | | |
| GpA | −2.69 | 0.32 | | | −0.10 | 0.27 |
| GpC | −2.66 | −0.05 | | | −0.07 | −0.10 |
| GpU | −2.45 | −0.12 | | | 0.14 | −0.17 |
| GpMe | | | −2.59 | 0.05 | | |
| | | | His92Gln | | | |
| GpA | −4.70 | −0.52 | | | −0.10 | 0.27 |
| GpC | −4.66 | −1.32 | | | −0.06 | −0.53 |
| GpU | −4.81 | −0.73 | | | −0.21 | 0.06 |
| GpMe | | | −4.60 | −0.79 | | |
| | | | Asn98Ala | | | |
| GpA | −0.59 | 0.32 | | | −0.62 | 0.44 |
| GpC | −1.38 | −0.49 | | | −1.41 | −0.37 |
| GpU | −0.39 | 0.17 | | | −0.42 | 0.29 |
| GpMe | | | 0.03 | −0.12 | | |

[a] $\Delta G$ values (in kilocalories per mole) were calculated from Table I according to Scheme I. Data under $\Delta G^{cat}$ are the apparent free energy changes calculated from the effect on $k_{cat}$; data under $\Delta G^{bind}$ refer to free energy changes as calculated from the effect on $K_s$. [b] $\Delta G_{(NR)}$ equals the coupling energy as defined in the text.

dynamic cycles can be constructed according to Scheme I. The free energy changes with respect to binding and catalysis have been ordered in Table II. The data afford a quantitative description of the apparent contributions of Asn36, Tyr38, His92, and Asn98 to primary site and subsite interactions with the substrates GpA, GpC, and GpU.

The Asn36Ala mutation reduces the transphosphorylation rate of the different GpN dinucleoside phosphate substrates considerably whereas the rate of GpMe transphosphorylation is identical with that of wild-type enzyme (see Table I). This result indicates that the Asn36 $-CONH_2$ function contributes to catalysis exclusively through contacts with the leaving nucleoside N. According to Scheme I, the catalytic coupling energy ($\Delta G^{cat}_{(NR)}$) equals −1.28, −1.40, and −0.95 kcal/mol for N being A, C, and U, respectively whereas $\Delta G^{cat}_{(0,R\rightarrow 0,0)}$ = −0.07 kcal/mol (see Table II). The overall contribution of the Asn36 amide group to ground-state binding of the GpN dinucleoside phosphates ($\Delta G^{bind}_{(N,R\rightarrow N,0)}$) is significantly smaller than for GpMe ($\Delta G^{bind}_{(0,R\rightarrow 0,0)}$; see Table II). Formally, this shows up as there being repulsive ground-state binding contacts with the leaving nucleoside. A reasonable explanation is that the presence of the leaving nucleoside N prevents or weakens the contribution of Asn36 to ground-state binding at the primary site. In any event, it appears that the Asn36 side chain is involved in ground-state binding of GpMe while it primarily accelerates the turnover of GpNs.

The interpretation of the data on the Tyr38Phe mutant form of RNase $T_1$ is straightforward. The mutation has essentially identical effects on the transesterification kinetics of all four substrates (GpA, GpC, GpU, and GpMe; see Table I). Removal of the phenolic hydroxyl group does not affect ground-state binding but reduces $k_{cat}$ about 70-fold. Tyr38 thus appears to be an example of a pure catalytic residue. The observation that the contribution to catalysis is almost independent of the nature of the leaving group ($\Delta G^{cat}_{(N,R\rightarrow N,0)}$ = $\Delta G^{cat}_{(0,R\rightarrow 0,0)}$ = 2.6 kcal/mol) indicates that Tyr38 belongs to the primary site; i.e., the phenolic hydroxyl makes no contact with the leaving nucleosides A, C, or U ($\Delta G^{cat}_{(NR)} \cong 0$). This is consistent with crystallographic data (Arni et al., 1988; Koepke et al., 1989), which indicate that the Tyr38 hydroxyl function could form an H bond with one of the phosphate oxygens. Clearly, this interaction must occur in the transition-state conformation since rate and not binding is affected by the Tyr38Phe substitution.

The general acid His92 protonates the 5′ oxygen of the leaving group during its expulsion (Heinemann & Saenger, 1982; Steyaert et al., 1990). The crystal structure of the RNase $T_1$–2′,5′-GpG complex (Koepke et al., 1989) suggests that the His92 side chain may, in addition, be engaged in a stacking interaction with the guanine base of the 3′-terminal nucleoside. If this type of interaction occurs at all with A, C, or U, it is not used for rate enhancement. The 4.6 kcal/mol contribution of His92 to catalysis is entirely realized in the primary site ($\Delta G^{cat}_{(NR)} \approx 0$ for N being A, C, or U; Table II). His92 is apparently involved in ground-state binding as well. However, it cannot be ruled out that intermolecular interactions between the His92 imidazole and the GpN substrates are compensated for by interactions with the Gln92 amide of the mutant. For this reason we are reluctant to interpret the data on ground-state binding.

Asn98 has been implicated in guanine binding at the primary site through a hydrogen bond between the Asn98 main-chain carbonyl and the exocyclic amino group of the guanine base [see Steyaert et al. (1991a) and references cited therein]. In contrast, the Asn98 side chain does not belong to the primary site; $\Delta G^{cat}_{(0,R\rightarrow 0,0)}$ and $\Delta G^{bind}_{(0,R\rightarrow 0,0)}$ are essentially zero (Table II). The magnitude of the values of $\Delta G^{cat}_{(NR)}$ indicates that the Asn98 side chain contributes to catalysis via subsite interactions. The observation that replacement of the Asn98 side chain by an Ala residue does not influence ground-state binding at the primary site indicates that the ground-state contact between the Asn98 side chain and the leaving nucleoside ($\Delta G^{bind}_{(NR)}$) does not affect the strength of the Asn98 O···guanine $N^2H$ hydrogen bond at the primary site.

The above data show that the Asn36 and the Asn98 amide functions are part of the RNase $T_1$ subsite, which interacts with the leaving nucleoside of GpN substrates. If the effects of Asn36 and Asn98 are independent of each other, these residues together contribute 2.81, 1.90, and 1.37 kcal/mol to the binding of the transition-state conformation of the leaving nucleosides C, A, and U, respectively.

*GpN vs GpMe Transesterification Rate.* From the comparison of the transesterification rates of GpNs and GpMe it would appear that the leaving nucleosides C, A, and U contribute 5.73, 4.84, and 4.21 kcal/mol to catalysis, respectively. Subtraction of the apparent contributions provided by the Asn36 and Asn98 side chains (2.78, 1.91, and 1.37 kcal/mol for C, A, and U, respectively) shows that the two residues quite precisely account for the difference in turnover rate between GpC, GpA, and GpU. Independent of the nature of the leaving nucleoside, about 2.9 kcal/mol of the difference in activation energy between GpNs and GpMe remains unexplained. This suggests that several other functional groups on the protein (e.g., the Gly74 O and the Arg77 $N^{\eta 2}$; see introduction) interact favorably with the transition-state conformation of the leaving nucleoside. The remaining activation energy could also be explained in terms of methanol being a worse leaving group than a nucleoside. The Brønsted dependence of $k_{cat}$ for the RNase $T_1$ catalyzed cyclization of guanosine 3′-(benzyl phosphate) esters versus the $pK_a$ of the leaving groups yields a regression line with slope −0.34 (Satoh & Inoue, 1975; Davis et al., 1988). This is consistent with our observation that the $k_{cat}$ value for the wild-type-catalyzed transphosphorylation of GpEt (a synthetic substrate in which ethanol is the leaving group; $k_{cat}$ = 0.028 $s^{-1}$ and $K_m$ = 60 μM, unpublished results) is somewhat lower than that of GpMe (see Table I). The $pK_a$ values of ethanol and methanol are 15.9 and 15.5, respectively (Ballinger & Long, 1960).

*Use of Subsite Binding Energy for Chemical Activation.* Interactions at the subsite contribute considerably to catalysis, indicating that binding energy is converted into chemical activation energy (Fersht, 1985). The catalyzed transesterification reaction consists of an in-line nucleophilic attack of the 2′ oxygen on the 3′ phosphate (Eckstein et al., 1972) requiring that the O2′, P, and O5′ atoms are linearly arranged (Westheimer, 1968). In addition, theoretical studies on the phosphodiester electronic structure have suggested that the strength of the P–O5′ scissile bond depends considerably on the torsion angles α and ζ (see Figure 2; Gorenstein et al., 1977, 1979). For α and ζ angles that are consistent with the minimal energy of the P–O5′ bond, the RO–P–OR bond angle is smaller than 104°, and therefore distortion in the direction of the trigonal bipyramid transition-state geometry (104° → 90°) is achieved. These stereochemical considerations could in part explain how interactions with the leaving nucleoside decrease the chemical activation energy of transphosphorylation.

Until more structural information becomes available, any hypothesis on the interactions between the Asn36 and Asn98

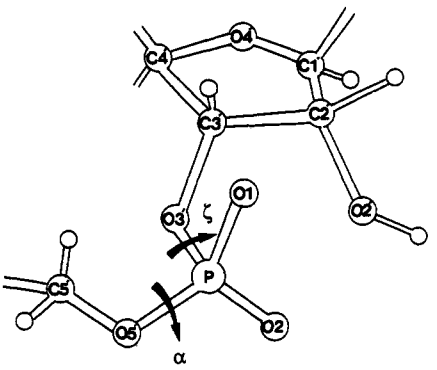



FIGURE 2: Ball-and-stick representation of the phosphodiester moiety of a dinucleoside phosphate substrate. The torsion angles $\alpha$ and $\zeta$ are indicated.

side chains and the various leaving nucleosides remains speculative. At present, we are synthesizing GpRib, a synthetic substrate in which the leaving nucleoside is replaced by D-ribose; comparison of the transesterification rate of this substrate with that of GpMe and GpNs will yield further information on the RNase $T_1$ subsite.

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## REFERENCES

Arni, R., Heinemann, U., Tokuoka, R., & Saenger, W. (1988) *J. Biol. Chem. 263*, 15358–15368.

Ballinger, P., & Long, A. (1960) *J. Am. Chem. Soc. 82*, 795–798.

Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835–840.

Davis, A. M., Regan, A. C., & Williams, A. (1988) *Biochemistry 27*, 9042–9047.

Eckstein, F., Schulz, H. H., Rüterjans, H., Haar, W., & Maurer, W. (1972) *Biochemistry 11*, 3507–3512.

Egami, F., Takahashi, K., & Uchida, T. (1964) *Prog. Nucleic Acid Res. Mol. Biol. 3*, 59–101.

Fersht, A. R. (1985) *Enzyme Structure and Function*, 2nd ed., pp 331–343, W. H. Freeman, New York.

Fersht, A. R., Leatherbarrow, R. J., & Wells, T. N. C. (1987) *Biochemistry 26*, 6030–6038.

Follmann, H., Wieker, H.-J., & Witzel, H. (1967) *Eur. J. Biochem. 1*, 243–250.

Gorenstein, D. G., Findlay, J. B., Luxon, B. A., & Kar, D. (1977) *J. Am. Chem. Soc. 99*, 3473–3479.

Gorenstein, D. G., Luxon, B. A., & Findlay, J. B. (1979) *J. Am. Chem. Soc. 101*, 5869–5875.

Grunert, H.-P., Zouni, A., Beineke, M., Quaas, R., Georgalis, Y., Saenger, W., & Hahn, U. (1991) *Eur. J. Biochem. 197*, 203–207.

Heinemann, U., & Saenger, W. (1982) *Nature (London) 299*, 27–31.

Horovitz, A., & Fersht, A. R. (1990) *J. Mol. Biol. 214*, 613–617.

Imazawa, M., Irie, M., & Ukita, T. (1968) *J. Biochem. 64*, 595–602.

Koepke, J., Maslowska, M., Heinemann, U., & Saenger, W. (1989) *J. Mol. Biol. 206*, 475–488.

Leatherbarrow, R. (1987) ENZFITTER, Biosoft Hills Road, Cambridge.

Lohrmann, I. R., & Khorana, H. G. (1964) *J. Am. Chem. Soc. 86*, 4188–4194.

Mossakowska, D. E., Nyberg, K., & Fersht, A. R. (1989) *Biochemistry 28*, 3843–3850.

Nishikawa, S., Morioka, H., Kim, H. J., Fuchimura, K., Tanaka, T., Uesugi, S., Hakoshima, T., Tomita, K.-I., Ohtsuka, E., & Ikehara, M. (1987) *Biochemistry 26*, 8620–8624.

Satoh, K., & Inoue, Y. (1975) *Chem. Lett.*, 551–556.

Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., & Fritz, H.-J. (1989) *Nucleic Acids Res. 17*, 4441–4454.

Steyaert, J., Hallenga, K., Wyns, L., & Stanssens, P. (1990) *Biochemistry 29*, 9064–9072.

Steyaert, J., Opsomer, C., Wyns, L., & Stanssens, P. (1991a) *Biochemistry 30*, 494–499.

Steyaert, J., Wyns, L., & Stanssens P. (1991b) *Biochemistry* (preceding paper in this issue).

Takahashi, K. & Moore, S. (1982) *Enzymes (3rd Ed.) 15*, 435–467.

Walz, F. G., Osterman, H. L., & Libertin, C. (1979) *Arch. Biochem. Biophys. 195*, 95–102.

Westheimer, F. H. (1968) *Acc. Chem. Res. 1*, 70–78.

Yasuda, T., & Inoue, Y. (1982) *Biochemistry 21*, 364–369.

Zabinski, M., & Walz, F. G. (1976) *Arch. Biochem. Biophys. 175*, 558–564.