Subsite Interactions of Ribonuclease T_1: Binding Studies of Dimeric Substrate Analogues†

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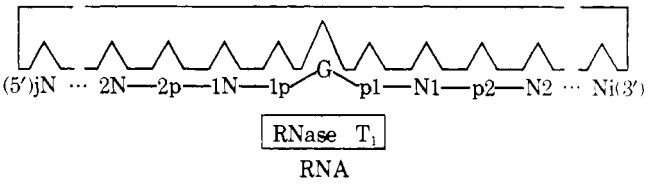
ABSTRACT: Ultraviolet difference spectral binding studies of ribonuclease T_1 with pGp, ApG, CpG, UpG, dGpdA, dGpdC, dGpdG, dGpdT, dTpdG, pdApdG, pdTpdG, pdGpdA, pdGpdG, pdGpdT, c(pdGpdA), and c(pdGpdG) were conducted at pH 5.0, 0.2 M ionic strength and 25 °C. Under these conditions, the characteristic difference spectrum and association constant for (1:1) ribonuclease T_1 binding were determined for each ligand. The binding of guanosine and deoxyguanosine containing ligands could be distinguished by the shapes of their difference spectra. The results indicated that the guanine moiety of each ligand was bound at the enzyme's primary recognition site. Evidence of a specific enzyme subsite for binding the adenine moiety of ApG and pdApdG is presented. The proposal of a specific enzyme subsite for binding the 5′-phosphate group of a complexed guanosine moiety (Sawada, F., Samejima, T., and Saneyoshi, M. (1973), *Biochim. Biophys. Acta 299,* 596) is not supported in the present work. Preliminary evidence for the existence of two additional enzyme subsites and the effect of oligomer conformation on enzyme binding are also discussed.

The existence and nature of subsite interactions for depolymerizing enzymes have been elucidated in studies on various proteolytic enzymes (Fruton, 1974), lysozyme (Dahlquist and Raftery, 1969), and *Bacillus amyloliquefaciens* amylase (Thoma et al., 1970, 1971). In the case of simple, low molecular weight polynucleotidases, electrostatic subsites for substrate phosphate groups have been evidenced with staphylococcal nuclease (Cuatrecasas et al., 1968) and recently with pancreatic RNase[1] A (Sawada and Irie, 1969; Li and Walz, 1974). However, subsites that interact with nucleotide base groups have not been systematically investigated for this class of enzymes.

The possibility of subsite interactions of polynucleotides with RNase T_1 was first suggested from RNA digestion studies (Pinder and Gratzer, 1970) and recently from binding studies of this enzyme with single-stranded DNA (Walz et al., 1975). Since RNase T_1 is a strongly acidic protein having a high proportion of aromatic amino acid residues (Takahashi et al., 1970), it was anticipated that possible subsites on this enzyme would interact in a more specific manner with base groups of RNA, rather than by nonspecific Coulombic interactions with the phosphate groups of this polynucleotide. On the basis of this preliminary evidence for subsites and their likelihood of exhibiting base group specificity, the present systematic investigation of RNase T_1 subsites was undertaken.

The system in Scheme I was devised to provide a subsite nomenclature as well as a basic experimental format for this study of RNase T_1 subsite interactions. In Scheme I, precedence is given to the binding of a G moiety of RNA at the enzyme's primary recognition site since endonucleolytic cleavage, catalyzed by RNase T_1 (i.e., between p1 and N1 in Scheme I), occurs exclusively at guanosine residues (Takahashi et al., 1970). The detailed specificity (Takahashi et al., 1970) and spectroscopic characteristics (Oshima and Imahori, 1972; Walz and Hooverman, 1973) of guanine residue binding at this site have been amply demonstrated. The possibility of enzyme subsite interactions with other nucleoside residues toward the 3′ terminus (e.g., with N1, N2, etc.) and/or 5′ terminus (e.g., with 1N, 2N, etc.) of an RNA strand are considered. The designations p1 and 1p represent phosphodiester groups attached to the 3′ and 5′ positions, respectively, of a guanosine residue occupying the primary recognition site. Subsite binding at these and other phosphate residues along the RNA chain is also considered. A RNase T_1 subsite interacting with a given nucleoside or phosphate group will be labeled with the designated position of this group according to Scheme I. Beyond illustrating possible subsite interactions and their nomenclature, Scheme I is not meant to offer any preconceived view regarding the nature of possible subsite interactions.

Scheme I



Considering Scheme I, possible subsites on RNase T_1 at positions N1, 1N, p1, 1p, and 2p have been examined in the present study using the following ligands: pGp, ApG, CpG, UpG, dGpdA, dGpdC, dGpdG, dGpdT, dTpdG, pdGpdA, pdGpdG, pdGpdT, pdApdG, pdTpdG, c(pdGpdA),[2] and c(pdGpdG) (the last two compounds are cyclic 3′:5′ and 3″:5″ dinucleotides; e.g., c(pdGpdA) could also be written c(pdApdG)). The uv difference spectral technique was employed to evaluate binding constants for these ligands with RNase T_1 under the same set of experimental conditions which were previously used in similar binding studies of the enzyme with Guo, dGuo, and their mononucleotides (Walz and

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[1] Abbreviations used: RNase, ribonuclease; uv, ultraviolet; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl; nucleotide and oligonucleotide abbreviations follow the recommendation of the IUPAC-IUB Commission as reported in *Biochemistry 9,* 4025 (1970).

[2] Because of compositional difficulties, the expression $\widehat{\text{pdGpdA}}$ (and other comparable structures) is designated by the form cyclic (pdGpdA), c(pdGpdA).

Hooverman, 1973). Evidence of the characteristic difference spectrum observed with monomeric guanine nucleosides and nucleotides (Oshima and Imahori, 1972; Walz and Hooverman, 1973) in experiments using the dimeric, guanine containing ligands listed above was used as a criterion to certify whether the guanine moiety of a particular dinucleotide or dinucleoside phosphate was occupying the enzyme's primary recognition site. On the basis of comparing the difference spectra and binding affinities of the 23 dimeric and monomeric ligands tested so far, as well as the results of recent steady-state kinetic studies of the enzyme using GpC and GpU as substrates (Zabinski and Walz, submitted), the following tentative conclusions regarding RNase T_1 subsites have been made: (1) there is a 1N subsite that is specific at least for purine bases and possibly for adenine; (2) there is no specific 1p binding site and the single phosphate group binding locus at the active site preferentially binds at the p1 vis a vis the 1p positions; and (3) there is an N1 subsite, of currently unknown specificity, that is apparently reflected in interactions with guanosine dinucleoside phosphate substrates but not with deoxyguanosine dinucleoside phosphate substrate analogues. Furthermore, preliminary evidence is discussed which indicates the possibility of a 2p subsite and the effect of ligand conformation on RNase T_1 binding.

Materials and Methods

Ribonuclease T_1 was prepared and its concentration was determined as described previously (Walz and Hooverman, 1973). P-5′-Guo-3′-P was obtained from Boehringer-Mannheim Corp.; this material was shown to be homogeneous and free of P-5′-Guo and Guo-3′-P when co-chromatographed on cellulose TLC plates (Eastman) using three solvent systems (isobutyric acid–concentrated NH_4OH–water (57:4:39); 1 M sodium acetate (pH 6.6)–saturated $(NH_4)_2SO_4$–isopropyl alcohol (18:80:2); ethanol–1 M ammonium acetate (75:30)). The concentration of P-5′-Guo-3′-P was determined using an extinction coefficient at 253 nm of 1.37×10^4 M^{-1} cm^{-1} at pH 7.0. The ribose dinucleoside phosphates ApG, CpG, and UpG were products of Sigma Chemical Co. and their molar extinction coefficients were determined according to Warshaw and Tinoco (1966). The deoxyguanosine ligands including dGpdA, dGpdC, dGpdG, dGpdT, dTpdG, pdTpdG, pdApdG, pdGpdA, pdGpdG, pdGpdT, c(pdGpdA), and c(pdGpdG) were obtained from Collaborative Research, Inc. The concentrations of the deoxyribose dinucleoside phosphates were determined using the molar extinction coefficients at 260 nm and pH 7.2 from Cantor et al. (1970). The same molar extinction coefficients were also used for the corresponding, homologous dinucleotides. All other chemicals were reagent grade. Distilled, deionized water with a specific resistance of at least 5 MΩ was used in all solutions.

The ultraviolet difference spectra resulting from RNase T_1–ligand binding were obtained in the same manner and under the same experimental conditions as those previously described (Walz and Hooverman, 1973), except that a Cary 118C scanning spectrophotometer was employed in the present study. Difference spectra were determined by scanning in the range, 240–310 nm, for each experiment. All experiments were conducted at 25 °C in a standard buffer solution containing 0.1 M KCl, 0.05 M Tris, 0.05 M sodium acetate adjusted to pH 5.0 with acetic acid. In general, the concentration of RNase T_1 was $\sim 4.3 \times 10^{-5}$ M and the concentration of the ligands varied in the range $\sim 5 \times 10^{-5}$ to $\sim 4 \times 10^{-4}$ M.

All pH measurements were conducted at 25 °C using a Radiometer pHM-26 pH meter.

Results and Treatment of the Data

On the average about 10 difference spectra were obtained for RNase T_1 binding of each ligand. These difference spectra were characterized as follows: (1) the wavelengths where the difference absorbance, ΔA, reach a maximum (λ_{max}) and a minimum (λ_{min}) were averaged and standard deviations were computed; and (2) the difference spectra were normalized at λ_{max} for each ligand and the mean and standard deviations of the normalized difference absorbance values were calculated at 5-nm intervals in the range 240 to 310 nm. The values of λ_{max} and λ_{min} for the 16 ligands tested are listed in Table I. The normalized difference spectrum for P-5′-Guo-3′-P was identical with those previously obtained for Guo-3′-P and P-5′-Guo (Walz and Hooverman, 1973) and is not shown. The normalized difference spectra for the remaining 15 ligands are presented in Figure 1. The deviations of the normalized difference spectrum for a particular ligand were apparently random and not correlated to the concentrations of the reactants.

In order to calculate the association constant, K, and the difference molar extinction coefficient, $\Delta\epsilon_{290}$, for RNase T_1 binding of a given ligand, ΔA was measured at 290 nm at a constant total enzyme concentration, $[E]_0$, while varying the total ligand concentration, $[L]_0$. The resulting binding isotherms could be described by the equilibrium expression

$$K = \frac{[EL]}{[E][L]} = \frac{\Delta A_\lambda/\Delta\epsilon_\lambda}{([E]_0 - (\Delta A_\lambda/\Delta\epsilon_\lambda))([L]_0 - (\Delta A_\lambda/\Delta\epsilon_\lambda))} \quad (1)$$

where λ is 290 nm, and [EL], [E], and [L] are the concentrations of the enzyme–ligand complex, the free enzyme, and the free ligand, respectively. The best fit values of K and $\Delta\epsilon_{290}$ were determined by finding the value of $\Delta\epsilon_{290}$ that minimized the coefficient of variation for K (Walz and Hooverman, 1973) and are listed in Table I. The data for dGpdG and dTpdG were too ambiguous to yield best fit values for K and $\Delta\epsilon_{290}$. The values of $\Delta\epsilon_{290}$ in Table I for these dinucleoside phosphates were calculated to be $0.92 \times \Delta\epsilon_{290}$ for their corresponding, homologous dinucleotides (i.e., for pdGpdG and pdTpdG, respectively). The justification for this assumption was based on the observed relationships of $\Delta\epsilon_{290}$ with other dinucleoside phosphate–dinucleotide pairs that did yield best fit values of K and $\Delta\epsilon_{290}$: i.e., $\Delta\epsilon_{290}$ for dGpdA was $0.87 \times \Delta\epsilon_{290}$ for pdGpdA, and $\Delta\epsilon_{290}$ for dGpdT was $0.97 \times \Delta\epsilon_{290}$ for pdGpdT. Considering the uncertainty of this assumption, the values of $\Delta\epsilon_{290}$ and their corresponding values of K which are listed in Table I for dGpdG and dTpdG should only be considered as representative approximations. The values of K and $\Delta\epsilon_{290}$ listed in Table I were used with eq 1 to generate the theoretical curves in Figure 2. In all cases there was a good fit between the experimental and theoretical binding isotherms. The number of binding sites per mole of RNase T_1, n, was also estimated from these data (Walz and Hooverman, 1973) and in all cases was consistent with $n = 1$ which was previously found for guanine mononucleotides (Walz and Hooverman, 1973). The standard free energies for enzyme–ligand binding were calculated for each ligand tested ($\Delta G^\circ = -RT \ln K$) and are also listed in Table I.

Discussion

The work presented here is a continuation of a project aimed at the systematic elucidation of the detailed binding topology of RNase T_1 with native, polymeric substrates. This project was initiated in binding studies of the enzyme with Guo, dGuo,

TABLE I: Values of the Binding Parameters from Difference Spectral Measurements of RNase T_1 with Substrate Analogues.[a]

Substrate Analogues	λ_{max} (nm)	λ_{min} (nm)	$10^{-3}\Delta\epsilon_{290\,nm}$ (M^{-1} cm^{-1})	10^{-3} K (M^{-1})	$-\Delta G°$ (kcal/mol)
pGp	290.5 ± 0.2	246.0 ± 0.4	4.50	20.6 ± 0.9	5.88
ApG	291.8 ± 0.3	247.4 ± 0.6	3.70	10.0 ± 0.8	5.45
CpG	291.4 ± 0.6	245.0 ± 1.0	2.85	2.06 ± 0.19	4.52
UpG	290.5 ± 0.4	246.3 ± 0.7	5.75	1.30 ± 0.06	4.25
dGpdC	289.3 ± 0.3	245.2 ± 0.3	5.30	1.99 ± 0.19	4.50
dGpdA	288.7 ± 0.5	246.0 ± 0.2	2.85	1.93 ± 0.09	4.48
dGpdT	288.3 ± 0.5	245.9 ± 0.7	4.50	1.60 ± 0.11	4.37
dGpdG[b]	289.0 ± 0.4	245.8 ± 0.8	5.65	0.80 ± 0.08	3.96
dTpdG[b]	288.3 ± 1.0	246.3 ± 1.0	6.70	0.34 ± 0.05	3.44
pdGpdG	289.2 ± 0.3	246.7 ± 0.4	6.15	6.76 ± 0.72	5.24
pdGpdT	288.9 ± 0.3	246.9 ± 0.6	4.65	5.73 ± 0.96	5.12
pdGpdA	289.2 ± 0.2	247.0 ± 0.6	3.30	4.74 ± 0.23	5.01
pdApdG	289.4 ± 0.4	248.1 ± 1.1	5.65	2.83 ± 0.12	4.71
pdTpdG	289.3 ± 0.4	247.8 ± 0.4	7.30	1.11 ± 0.07	4.15
c(pdGpdG)	289.6 ± 0.2	246.3 ± 0.5	3.25	8.07 ± 0.35	5.43
c(pdGpdA)	289.6 ± 0.3	248.0 ± 0.5	4.00	5.87 ± 0.57	5.14

[a] All experiments were conducted at pH 5.0, 0.2 M ionic strength and 25 °C. [b] The value of $\Delta\epsilon_{290}$ was assumed to equal $0.92\Delta\epsilon_{290}$ of the corresponding homologous dinucleotides; see text for details.

and their corresponding mononucleotides using uv difference spectroscopy. The shape of the difference spectra resulting from RNase T_1 binding with these simple, monomeric ligands was very similar in all cases and evidenced only minor differences between ribose and deoxyribose compounds (Walz and Hooverman, 1973). In the present study the normalized difference spectra for the 15 dinucleotides and dinucleoside phosphates examined (see Figure 1) were remarkably similar to those observed with guanosine mononucleotides especially regarding λ_{max} and λ_{min} values (see also Table I). *This finding strongly suggests that the guanine moieties of all the ligands tested are bound at the enzymes' primary recognition site.* A feature of the difference spectra in the present study that distinguishes the binding of deoxyribose and ribose compounds was that λ_{max} of the former were at 289 ± 0.5 nm; whereas, those of the latter were at 291 ± 0.5 nm (see Table I).

The significant variations in both the normalized difference spectra presented in Figure 1 and the values of $\Delta\epsilon_{290}$ in Table I are currently not understood. Nevertheless, these variations can be correlated with ligand structure when considered phenomenologically. For example, the most dramatic qualitative deviations from more "typical" difference spectra were found for the cyclic deoxyribose dinucleotides which exhibited significantly decreased minima when compared with their open chain homologues (see Figures 1c and 1d). Furthermore, in the series ApG, CpG, and UpG, the shapes of the difference spectra for UpG and CpG were essentially identical; whereas, that for ApG differed significantly from these in the region 240 to 255 nm (see Figure 1a). Regarding the difference spectra quantitatively, two major correlations were noted: (1) the magnitudes of the difference spectra for a pair of homologous dinucleotides and dinucleoside phosphates were similar and differed from other pairs; e.g., for $\Delta\epsilon_{290}$ from Table I, pdGpdT $\geq$ dGpdT $\gg$ pdGpdA $\geq$ dGpdA; and (2) for the three pairs of sequence isomers of dinucleotides and dinucleoside phosphates, the values of $\Delta\epsilon_{290}$ from Table I showed (p)dXpdG $\gg$ (p)-dGpdX (X = A or T). It was also noted that the value of $\Delta\epsilon$ centered around 265 nm decreased in the order dGpdX > pdGpdX > pdGpdX (X = A, G, or T; see Figures 1b–d and values of $\Delta\epsilon_{290}$ from Table I). Some of these phenomena will be discussed below in the context of subsite interactions evidenced by ligand affinities.

It was hoped that initial evidence for the existence of RNase T_1 subsites could be deduced from relative values of $\Delta G°$ for enzyme binding of a given series of ligands under a defined set of experimental conditions. For a variety of reasons, such evidence for an enzyme subsite will necessarily be circumstantial and the dependence of $\Delta G°$ on temperature, pH, etc., as well as corroborative evidence using different physical techniques, would be required to conclusively certify whether a given subsite exists. In any event, compelling circumstantial evidence from the present work regarding the existence or nonexistence of particular RNase T_1 subsites warrants the following discussion.

Interactions at the p1 and 1p Positions. Previous binding studies of RNase T_1 with guanine nucleosides and nucleotides indicated at least one phosphate binding locus at the active site of the enzyme (Walz and Hooverman, 1973, and references therein). Nevertheless, these results could not distinguish specific binding sites for phosphate groups at the 3′ and 5′ positions of a bound guanosine residue (i.e., corresponding to the p1 and 1p groups, respectively: see Scheme I). A specific RNase T_1 subsite for the 1p group has recently been proposed by Sawada et al. (1973). However, this suggestion was based on estimated binding constants from experiments conducted at a single ligand concentration using 6-thioguanosine diphosphates whose 2′:3′-(cyclic)-5′-diphosphate derivative was not a hydrolytic substrate of RNase T_1. In view of these shortcomings, it was determined that additional experiments would be required to elucidate the existence of this proposed 1p subsite. The presence of a 1p subsite on pancreatic RNase A (i.e., for a phosphate group at the 5′ position of a bound pyrimidine nucleoside residue) was recently evidenced by the fact that $\Delta G°$ values characterizing the binding of the hydrolytic substrate, P-5′-Urd-2′:3′-P (Li and Walz, 1974), and inhibitor, P-5′-Urd-2′(3′)-P (Sawada and Irie, 1969), were both ~1.6 kcal/mol more *negative* than those for their corresponding mononucleotides, Urd-2′:3′-P and Urd-2′(3′)-P, respectively. A similar methodology for probing a possible 1p subsite on RNase T_1 was employed in the present study in

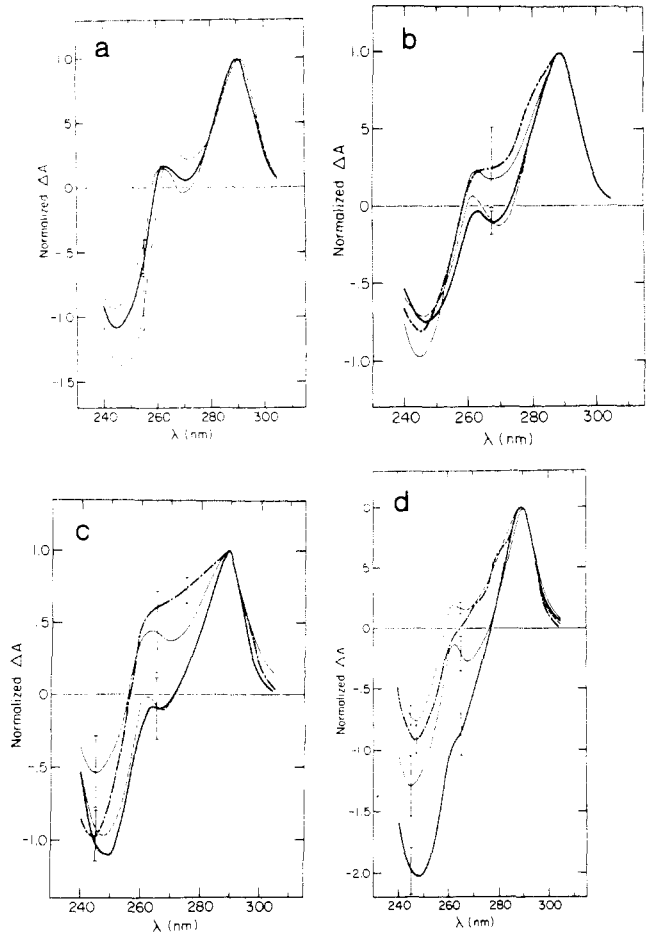

FIGURE 1: Normalized difference spectra for RNase T_1 binding with dinucleotides and dinucleoside phosphates. Experiments were conducted at pH 5.0, 0.2 M ionic strength, and 25 °C; other conditions were as described under Materials and Methods. Difference spectra were normalized to λ_{max} for each ligand; error bars represent standard deviations: see text for further details. (a) (Heavy solid line) UpG; (light solid line) CpG; (dashed line) ApG. (b) (Heavy solid line) pdGpdT; (light solid line) dGpdG; (dashed line) dGpdC; (dot-dashed line) dGpdT. (c) (Heavy solid line) pdApdG; (light solid line) dTpdG; (dashed line) pdTpdG; (dot-dashed line) dGpdA. (d) (Heavy solid line) c(pdGpdA); (light solid line) c(pdGpdG); (dashed line) pdGpdG; (dot-dashed line) pdGpdA.

binding experiments of the enzyme with P-5′-Guo-3′-P. The value of $\Delta G°$ for RNase T_1 binding of this ligand (see Table I) was 0.56 kcal/mol more *positive* than that of Guo-3′-P (Walz and Hooverman, 1973). In contrast to the findings with RNase A, this result strongly suggests that a specific 1p subsite does not exist on RNase T_1. The fact that the affinity of P-5′-Guo-3′-P is lower than that for Guo-3′-P could reflect an increased electrostatic repulsion in the complex due to the additional phosphate group at the 5′ position. Additional evidence against an RNase T_1 1p subsite is shown in comparisons 1, 2, and 3 listed in Table II. These differences in $\Delta G°$ indicate that the enzyme affinity for dTpdG, CpG, and UpG (which have phosphodiester groups in the 1p position) is significantly *less* than that for the parent guanine nucleosides. In view of this evidence against a 1p subsite, the previous observation that $\Delta G°$ for binding Guo-3′-P was ~1.1 kcal/mol more negative than that of P-5′-Guo (Walz and Hooverman, 1973) is consistent with the proposal of a single phosphate binding locus at the active site that *prefers* binding a group at the p1 position. The observation that the $\Delta G°$ values in Table I for the series dGpdX (X = A, C, and T) were more negative than that of dGuo ($\Delta G°$ = −3.71 kcal/mol; Walz and Hooverman, 1973) by 0.6 to 0.8 kcal/mol could be primarily due to a favorable interaction of the phosphodiester group at the proposed p1 subsite.

Interaction at the 1N Position. Evidence for a purine and possibly an adenine-specific subsite on RNase T_1 at the 1N position can be deduced from comparisons 4, 5, and 6 in Table II. As indicated in comparisons 2 and 3, both UpG and CpG have lower affinities with the enzyme when compared with guanosine; on the other hand, the binding of ApG is characterized by a considerably more negative $\Delta G°$ than that of this nucleoside (comparison 4). This "quantitative" evidence for a specific 1N subsite is supported by the qualitative difference in the normalized difference spectrum of ApG *vis a vis* those of CpG and UpG (see Figure 1a). Further evidence for this subsite is presented in comparisons 5 and 6 in Table II which shows that a substitution of adenine in place of thymine at the X position of pdXpdG (probing a possible 1N subsite) results in a considerable increase in enzyme binding affinity; whereas a similar substitution in pdGpdX (probing a possible N1 subsite) results in a slight decrease in the binding affinity. The fact that a specific enzyme subsite for the 1N position was evidenced in comparisons of $\Delta G°$ values using either ribose dinucleoside phosphates or deoxyribose dinucleotides diminishes possible arguments that ligand conformation per se is the discriminating factor for these observed differences in affinity. Steady state kinetic studies are planned for RNase T_1 using ApGpC and ApGpU as substrates. The results of these studies in comparison with those already completed with GpC and GpU (Zabinski and Walz, submitted) should further clarify the role of the 1N subsite in terms of substrate binding and/or catalysis.

Interactions at the N1 Position. As shown in Table I only slight differences were found in $\Delta G°$ for the series pdGpdX (X = A, C, G, or T); this was also the case for the series pdGpdX (X = A, G, or T). These results for both series of deoxyribose dimeric ligands suggest that either the X nucleoside moiety does not interact with the enzyme or the enzyme is nondiscriminating at the putative N1 subsite. The fact that $\Delta G°$ values for a given dGpdX are more negative than that of dGuo could be mainly accounted for in terms of the interaction of the phosphodiester group of these ligands at the p1 subsite, as discussed above, rather than enzyme interactions with the X nucleoside residues. Therefore, there is no compelling evidence for an N1 subsite interaction with these *deoxyribose* substrate analogues. On the other hand, steady-state kinetic studies of the RNase T_1 catalyzed transesterification of GpC and GpU clearly suggested the existence of an N1 subsite because of the dramatic differences in the kinetic parameters for these *ribose* dinucleoside phosphates (Zabinski and Walz, submitted). For example, under experimental conditions identical with those used in the present study, it was found that the Michaelis constant (K_m) and the turnover number were 2.86×10^{-5} M and 33 s^{-1} for GpU and 1.75×10^{-4} M and 400 s^{-1} for GpC, respectively. Furthermore, if it is assumed that these K_m values represent dissociation constants, then $\Delta G°$ values for enzyme binding of these substrates (i.e., $\Delta G° = RT \ln K_m$) would be −6.17 kcal/mol for GpU and −5.12 kcal/mol for GpC. It is possible that the apparent order of RNase T_1 affinities with these deoxyribose and ribose dinucleoside phosphates (i.e., GpU ≫ GpC ≫ dGpdX (X = A, C, G, or T)) reflects the importance of a cooperative enzyme interaction with the Guo 2′-hydroxyl group (Walz and Hooverman, 1973) in facilitating a subsite interaction with the nucleoside residue at the N1 position; however, mainly because of the uncertainty in interpreting K_m values as affinity con-

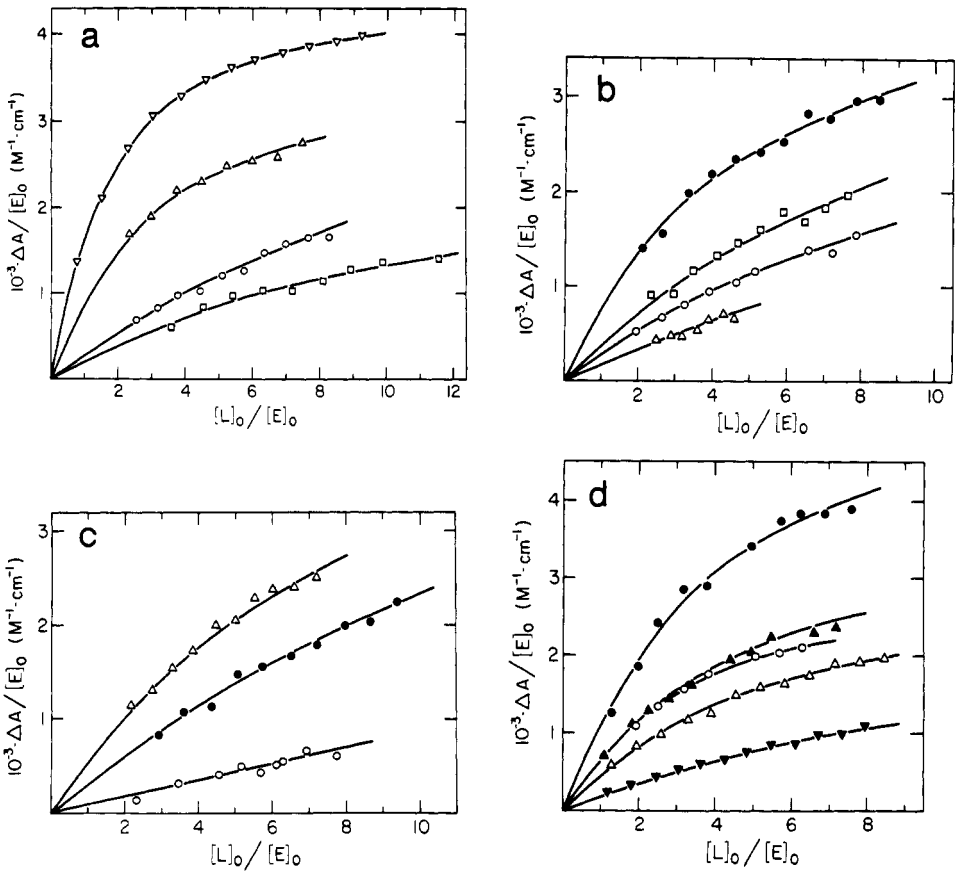

FIGURE 2: Plots of the difference absorbance at 290 nm divided by the total RNase T_1 concentration vs. the ratio of the total ligand and total enzyme concentrations. Experiments were conducted at pH 5.0, 0.2 M ionic strength, and 25 °C; other conditions were as described under Materials and Methods. Theoretical curves were calculated as described in the text. (a) (▽) p-5′-Guo-3′-p ($[E]_0 = 4.19 \times 10^{-5}$ M); (△) ApG ($[E]_0 = 4.27 \times 10^{-5}$ M); (○) CpG ($[E]_0 = 4.53 \times 10^{-5}$ M); (□) UpG ($[E]_0 = 4.2 \times 10^{-5}$ M). (b) (●) pdGpdT ($[E]_0 = 4.39 \times 10^{-5}$ M); (□) dGpdC ($[E]_0 = 4.12 \times 10^{-5}$ M); (○) dGpdT ($[E]_0 = 4.33 \times 10^{-5}$ M); (△) dGpdG ($[E]_0 = 4.15 \times 10^{-5}$ M). (c) (△) pdApdG ($[E]_0 = 4.47 \times 10^{-5}$ M); (●) dpTpdG ($[E]_0 = 4.39 \times 10^{-5}$ M); (○) dTpdG ($[E]_0 = 4.5 \times 10^{-5}$ M). (d) (●) pdGpdG ($[E]_0 = 4.15 \times 10^{-5}$ M); (▲) c(pdGpdA) ($[E]_0 = 4.18 \times 10^{-5}$ M); (○) c(pdGpdG) ($[E]_0 = 4.2 \times 10^{-5}$ M); (△) pdGpdA ($[E]_0 = 4.09 \times 10^{-5}$ M); (▼) dGpdA ($[E]_0 = 4.09 \times 10^{-5}$ M).

TABLE II: Difference in $\Delta G°$ Values for Ribonuclease T_1 Binding of Selected Ligands at pH 5.0, 25 °C, and 0.2 M Ionic Strength.

No.	Comparison[a]	$\Delta\Delta G°$ (kcal/mol)
1	dTpdG − dGuo	+0.27
2	CpG − Guo	+0.30
3	UpG − Guo	+0.57
4	ApG − Guo	−0.63
5	pdApdG − pdTpdG	−0.56
6	pdGpdA − pdGpdT	+0.11

[a] Values of $\Delta G°$ for dGuo and Guo were taken from Walz and Hooverman (1973); other values were taken from Table I.

stants, a definitive conclusion regarding the nature and specificity of the N1 subsite cannot be made at this time. Experiments are planned or in progress to further elucidate this subsite and include: (1) steady-state kinetic studies of RNase T_1 using GpA and GpG as substrates; (2) binding studies of the enzyme with GpcX (X = A, C, G, and U) which are dinucleoside phosphonate substrate analogues (Jones et al., 1970); and (3) binding studies of the series GpX (X = A, C, G, and U) with the catalytically inactive γ-CM-Glu-58 RNase T_1 which is still capable of binding guanosine-containing ligands (Takahashi, 1970).

Binding of (Open Chain) Deoxyribose Dinucleotides. As shown in Table I, the series of dinucleotides having the sequence pdGpdX (X = A, G, or T) exhibit $\Delta G°$ values for RNase T_1 binding that are from 1.5 to 1.3 kcal/mol more negative than those of their corresponding (homologous) dinucleoside phosphates of the series dGpdX. At first sight these differences in $\Delta G°$ suggest the existence of a 1p subsite which is contrary to the evidence documented above against such a specific subsite. An alternative explanation of these differences is that the pdG moiety of a given pdGpdX interacts with the enzyme in a manner similar to that of P-5′-dGuo. This hypothesis is consistent with the fact that P-5′-dGuo exhibits a similar affinity with the enzyme ($\Delta G° = -5.33$ kcal/mol; Walz and Hooverman, 1973) when compared with members of the series pdGpdX (see Table I). Additional support for this proposal is that the normalized difference spectrum for a given pdGpdX more closely resembles that of a typical guanine mononucleotide (such as P-5′-dGuo; Walz and Hooverman, 1973) than does the normalized difference spectrum of its homologous dinucleoside phosphate: this is particularly noticeable in the region around 265 nm (see Figures 1b–d). This rationale for explaining the relative affinities of RNase T_1 with pdGpdX and dGpdX homologues obviously cannot account for the fact that $\Delta G°$ for binding pdTpdG is ~0.7 kcal/mol more negative than that of dTpdG (see Table I). In this case, a subsite interaction at the 2p position would have to be considered a possibility at this time.

Binding of (Cyclic) Deoxyribose Dinucleotides. The values of $\Delta G°$ in Table I indicate that c(pdGpdG) and c(pdGpdA) bind with RNase T_1 having a similar (or slightly greater) af-

finity when compared with their open chain analogues, pdGpdG, pdGpdA, and pdApdG. This result was surprising considering the usual order of binding affinities originally observed by Sato and Egami (1965) which indicated that the affinity of RNase T_1 with a guanosine monoanionic phosphomonoester was much greater than that of a corresponding monoanionic phosphodiester. Data from the present report and previous studies (Walz and Hooverman, 1973), obtained under experimental conditions where phosphomonoester groups are predominately in their monoanionic form, have consistently corroborated this proposed relationship; i.e., the order of binding affinities and absolute differences in $\Delta G°$ were: dGuo-3′-P $\gg$ dGpdX (X = A, C, or T), $|\Delta\Delta G°|$ = 1.0–1.2 kcal/mol; P-5′-Guo $\gg$ XpG (X = U or C), $|\Delta\Delta G°|$ = 0.9–1.1 kcal/mol; P-5′-dGuo $\gg$ dTpdG, $|\Delta\Delta G°|$ = 1.9 kcal/mol. An apparent exception to this rule was in the case of ApG whose affinity with the enzyme was comparable to that of P-5′-Guo. However, this deviation can be accounted for by the specific 1N subsite interaction of the adenine moiety as discussed above. Therefore, considering this well-documented order of RNase T_1 binding affinities, the order, c(pdGpdX) $\geq$ pdGpdX, was not anticipated. The unexpectedly high enzyme affinities of the cyclic deoxyribose dinucleotides as well as their unique binding difference spectra (see Figure 1d) are currently not understood. However, it is likely that these atypical characteristics for RNase T_1-ligand binding reflect in some degree a binding preference for the relatively limited range of conformations available to these cyclic dinucleotides (Cantor et al., 1969). In any event, these results suggest the possible significance of ligand conformation on the RNase T_1-oligomer binding process.

The results so far are encouraging regarding the demonstration of nucleotide base group subsite interactions of RNase T_1. In this study, the effects of different base group substitutions were probed only at the 1N and N1 positions (see Scheme I) and there is reasonable evidence that enzyme subsite binding occurs at these locations. As indicated in specific cases above, the present results also provide a basis for designing future experiments that could further elucidate the nature of subsite binding at a given position in terms of its relations to other enzyme-ligand binding loci and catalysis. Future studies using trinucleotides and trinucleoside diphosphates will probe for more extensive subsite interactions at the 2N, 2p, p2, N2, and p3 positions. It is hoped that these studies will ultimately provide not only a greater insight into RNase T_1 action, but also some general principles regarding the detailed nature of the protein–nucleic acid recognition process.

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