Studies on the Nature of Guanine Nucleotide Binding with Ribonuclease T_1†

Frederick G. Walz, Jr.

ABSTRACT: The binding of ribonuclease T_1 with a series of guanine nucleotides and some of their methyl esters was studied using ultraviolet difference spectroscopy from pH 3.0 to pH 8.5. Similar experiments using γ-CM-Glu-58-ribonuclease T_1 were conducted under the same conditions at pH 5.0. At most pH values the characteristic difference spectrum and the association constant were determined for each ligand. At pH 5.0 the order of affinities for enzyme binding was: Guo-2′-P ≫ Guo-3′-P ≫ P-5′-dGuo ≥ dGuo-3′-P > P-5′-Guo ≫ (Me)-P-5′-Guo = dGuo-3′-P-(Me) > (Me)-P-5′-dGuo. At the extreme pH values tested, the affinities for all ligands decreased to approximately equal values. The order of affinities for γ-CM-Glu-58-ribonuclease T_1 binding at pH 5.0 was: P-5′-Guo ≥ P-5′-dGuo = Guo-2′-P ≥ Guo-3′-P > dGuo-3′-P ≥ (Me)-P-5′-Guo. The binding free energy change for all nucleotides can be completely accounted for in terms of enzyme binding loci for guanine, phosphate, and 2′-hydroxyl groups of the ligand. The interactions of the enzyme with the phosphomonoester groups of Guo-3′-P, dGuo-3′-P, and P-5′-dGuo were apparently similar and distinct from comparable interactions for P-5′-Guo and Guo-2′-P. The results suggest the existence of a phosphomonoester group binding locus at the active site which normally prefers binding with a guanosine 3′-phosphate group by virtue of an adjacent enzyme interaction with the ligand 2′-hydroxyl group (Walz, F. G. (1976), *Biochemistry 15,* 4446). On the other hand, the enzyme preferentially interacts with ligand 3′-phosphodiester groups regardless of its interaction with the guanosine 2′-hydroxyl group. In general, it appears that phosphate group binding requires ionization of a carboxyl and an imidazole group at the active site and probably involves hydrogen bonding and/or steric factors to a greater extent than simple Coulombic interactions. Comparisons of the binding data for native ribonuclease T_1 and γ-CM-Glu-58-ribonuclease T_1 are used to discuss guanine nucleotide binding in terms of specific models of the active site.

An initial approach toward understanding the detailed nature of RNase[1] T_1-RNA interactions requires a mapping of specific substrate binding loci and their enzyme complements that are involved in complex formations. A number of studies have indicated the existence of a primary recognition site for RNase T_1 where binding of an RNA guanine group determines the scissile bond for catalytic transesterification to a guanosine 2′:3′-phosphate product (Takahashi et al., 1970; Epinatjeff and Pongs, 1972; Oshima and Imahori, 1972; Walz and Hooverman, 1973). In addition, secondary recognition of other RNA base groups at specific subsites on RNase T_1 has recently been reported (Walz and Terenna, 1976; Zabinski and Walz, 1976) and was shown to play a significant role in enzyme catalysis as indicated from kinetic studies using oligomeric RNA substrates (Zabinski and Walz, 1976; Osterman and Walz, 1977). Nevertheless, the most pertinent interactions of the enzyme regarding substrate recognition and catalysis will most likely occur at or adjacent to the active site.

The nature of the binding locus at the active site for interacting with the 2′-hydroxyl group of a complexed guanosine moiety (Walz and Hooverman, 1973) was elucidated in recent pH dependent binding studies of RNase T_1 with guanosine and deoxyguanosine which led to a specific proposal that Glu-58 and an active site His residue were involved in ribose recogition (Walz, 1976). In regard to the interaction of phosphate groups at the active site, a number of enzyme-guanine nucleotide binding studies have been reported (Walz and Hooverman, 1973, and references therein); however, in most cases the determination of enzyme-ligand binding constants was at a limited number of pH values and ligands studied contained only phosphomonoester groups. More recently, the active site binding of phosphodiester groups (i.e., which represent phosphate groups on RNA substrates) was obliquely studied in an investigation of RNase T_1 base group specific subsites which used dinucleoside monophosphates and dinucleotides as ligand probes (Walz and Terenna, 1976). It was tentatively concluded from this work that phosphodiester group binding occurred when this group was on the 3′ position of a complexed guanine nucleoside moiety and that essentially no binding of isomeric 5′-phosphodiester groups was evident. However, these conclusions demanded further demonstration using simpler nucleotide diesters.

The present investigation was undertaken to systematically investigate phosphomonoester and phosphodiester group binding at the active site of RNase T_1 as a function of pH. It was hoped that these results would elucidate the nature of phosphate group binding with the enzyme as well as corroborate some aspects of secondary base group recognition (Walz and Terenna, 1976). In this study ultraviolet difference spectroscopy was used to determine the association constant and characteristic difference spectra for RNase T_1 binding with Guo-2′-P, Guo-3′-P, P-5′-Guo, (Me)-P-5′-Guo, dGuo-3′-P, dGuo-3′-P-(Me), P-5′-dGuo, and (Me)-P-5′-dGuo in the pH range 3.0–8.5. Similar experiments using CM-RNase T_1

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[1] Abbreviations used are: TLC, thin-layer chromatogrophy; NMR, nuclear magnetic resonance; RNase, ribonuclease; γ-CM-Glu-58-RNase T_1, RNase T_1 having a γ-carboxymethyl group on glutamate-58; (Me)-P-5′-Guo, (Me)-P-5′-dGuo, dGuo-3′-P-(Me), methyl esters of guanosine 5′-phosphate, deoxyguanosine 5′-phosphate, and deoxyguanosine 3′-phosphate, respectively; other nucleotide designations follow the recommendations of the IUPAC-IUB commission as reported ((1970), *Biochemistry 9,* 4025); Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; UV, ultraviolet.

TABLE I: Binding Parameters for Various Ligands with RNase T_1 and CM-RNase T_1 at pH 5.0, 0.2 M Ionic Strength, and 25 °C.

Enzyme	Ligand	$\Delta\epsilon_{290nm}$ $\times 10^{-3}$ (M^{-1} cm^{-1})	λ_{max} (nm)	$K \times 10^{-3}$ (M^{-1})	$-\Delta G°$ (kcal/mol)
RNase T_1	Guo[a]	3.40	290.5 ± 0.3	3.42	4.82
	dGuo[a]	3.90	289.0 ± 0.4	0.55	3.74
	Guo-2′-P	4.00	290.1 ± 0.2	209	7.25
	Guo-3′-P	4.05	290.5 ± 0.3	52.0	6.43
	dGuo-3′-P	4.55	289.9 ± 0.2	12.0	5.56
	dGuo-3′-P-(Me)	3.73	289.5 ± 0.4	2.09	4.53
	P-5′-Guo	3.70	289.5 ± 0.2	8.66	5.34
	(Me)-P-5′-Guo	3.15	289.5 ± 0.5	2.02	4.51
	P-5′-dGuo	3.83	289.4 ± 0.2	15.7	5.72
	(Me)-P-5′-dGuo	2.95	288.7 ± 0.5	1.15	4.17
CM-RNase T_1	Guo[a]	3.30	289.8 ± 0.3	6.90	5.23
	dGuo[a]	3.10	289.7 ± 0.2	6.51	5.20
	Guo-2′-P	2.75	288.8 ± 0.3	20.0	5.86
	Guo-3′-P	2.85	290.0 ± 0.2	16.0	5.73
	dGuo-3′-P	3.00	289.4 ± 0.2	7.74	5.30
	P-5′-Guo	2.90	289.2 ± 0.2	26.7	6.03
	(Me)-P-5′-Guo	3.13	289.5 ± 0.3	6.03	5.15
	P-5′-dGuo	3.00	289.0 ± 0.2	22.0	5.92
	(Me)-P-5′-dGuo	3.10	289.4 ± 0.3	6.14	5.16

[a] Walz (1976).

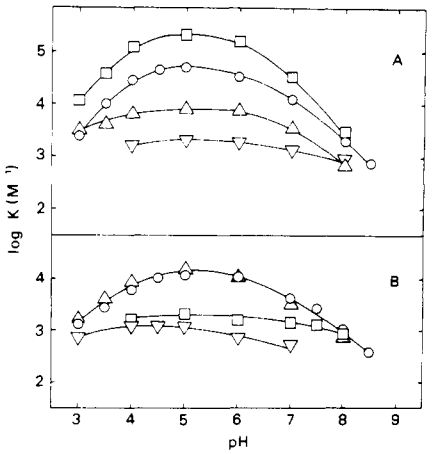

FIGURE 1: Plot of the association constant for nucleotide binding with RNase T_1 vs. pH. (A) Ribose containing nucleotides: (□) Guo-2′-P, (○) Guo-3′-P, (△) P-5′-Guo, (▽) (Me)-P-5′-Guo. (B) Deoxyribose containing nucleotides: (○) dGuo-3′-P; (△) P-5′-dGuo; (□) dGuo-3′-P-(Me); (▽) (Me)-P-5′-dGuo. All experiments were conducted at 0.2 M ionic strength at 25 °C. See details under Materials and Methods.

(Takahashi et al., 1967) were performed with these ligands at pH 5.0. The results are treated in terms of free energy contributions for enzyme binding of different ligand groups and are discussed in reference to previous models of the active site.

Materials and Methods

Ribonuclease T_1 was prepared as described previously (Walz and Hooverman, 1973). γ-CM-Glu-58-RNase T_1 was prepared according to the method of Takahashi et al. (1967) and had the same residual activity as reported before (~0.03%; Walz, 1976). The concentrations of RNase T_1 and γ-CM-Glu-58-RNase T_1 were both determined using 2.1×10^4 M^{-1} cm^{-1} as a molar extinction coefficient at 278 nm. All nucleotides were obtained from P-L Biochemicals, Inc. The nucleotide methyl esters, dGuo-3′-P-(Me), (Me)-P-5′-Guo, and (Me)-P-5′-dGuo were prepared and purified according to the method of Holy (1969). These compounds were shown to be homogeneous when chromatographed on cellulose TLC plates (Eastman) in two 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)). In each case a single spot was observed for the methyl ester that migrated with a different R_f value than the parent nucleotide. The concentrations of all nucleotides was calculated using 13 700 M^{-1} cm^{-1} as a molar extinction coefficient at pH 7.0 and 252 nm.

The ultraviolet difference spectra for RNase T_1–nucleotide binding were obtained in the same manner as previously reported (Walz and Terenna, 1976). 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 the desired pH with acetic acid. At pH 4.0 and lower pH values, the same buffer was used with sodium lactate–lactic acid replacing sodium acetate–acetic acid. The concentrations of RNase T_1 and γ-CM-Glu-58-RNase T_1 were ~4.5×10^{-5} M and the concentrations of nucleotides were varied in the range ~4×10^{-5} to ~2×10^{-4} M. The pH dependence of the molar extinction coefficients of RNase T_1, Guo-3′-P, and P-5′-Guo were determined at 290 nm using the same buffers as described above for the difference spectra.

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

Results and Treatment of the Data. The difference spectra for RNase T_1–nucleotide binding were determined using a constant concentration of protein (RNase T_1 or γ-CM-Glu-58-RNase T_1) and at least ten different concentrations of each ligand at a given pH value. The analysis of the difference spectra obtained in terms of the difference molar extinction coefficient at 290 nm, $\Delta\epsilon$, the wavelength for maximal difference absorbance, λ_{max}, and the association constant characterizing 1:1 protein–ligand binding, K, was the same as previously reported (Walz, 1976). Values for these parameters and the standard free energy change for binding, $\Delta G°$ (i.e., $\Delta G° = -RT \ln K$), for RNase T_1 and γ-CM-Glu-58-RNase T_1 nucleotide binding at pH 5.0 are presented in Table I. Values of K for RNase T_1 binding at other pH values are plotted in Figure 1.

For all of the ligands used in the present study the observed difference spectra for RNase T_1 binding from pH 3.0 to pH 8.5 virtually had the same shapes as those previously reported

TABLE II: Apparent Maximal Free Energy Changes for Binding Specific Loci of Guanine Nucleotides with RNase T_1.

Nucleotide moiety	Comparisons	Interpretation[a]	Designation	Value[b] (kcal/mol)
Phosphomonoester	Guo-3′-P − Guo dGuo-3′-P − dGuo P-5′-dGuo − dGuo	Intrinsic	ΔG_{int}^{P}	−1.67 ± 0.12
2′-Hydroxyl	Guo − dGuo	Intrinsic	$\Delta G_{int}^{2'OH}$	−1.01 ± 0.09
5′-Phosphomonoester of P-5′-Guo	P-5′-Guo − Guo	Apparent	ΔG_{app}^{P}	−0.60 ± 0.12
2′-Hydroxyl of P-5′-Guo	P-5′-Guo − P-5′-dGuo	Apparent	$\Delta G_{app}^{2'OH}$	+0.25 ± 0.12
5′-Phosphodiester	(Me)-P-5′-dGuo − dGuo	Intrinsic	$\Delta G_{int}^{5'MP}$	−0.46 ± 0.02
5′-Phosphodiester of (Me)-P-5′-Guo	(Me)-P-5′-Guo − Guo	Apparent	$\Delta G_{app}^{5'MP}$	+0.37 ± 0.09
3′-Phosphodiester	dGuo-3′-P-(Me) − dGuo	Intrinsic	$\Delta G_{int}^{3'MP}$	−0.71 ± 0.08

[a] See text for details. [b] Represents the means and standard deviations for maximal values of $\delta\Delta G°$ taken from the data in Figure 3 generally between pH 4 and pH 6.

around pH 5 (Oshima and Imahori, 1972: Epinateff and Pongs, 1972; Walz and Hooverman, 1973). Nevertheless, the magnitudes of these difference spectra in terms of $\Delta\epsilon$ at 290 nm showed a considerable variation with pH in some cases. This pH dependence was analyzed using:

$$\Delta\epsilon = \epsilon_{EL} - \epsilon_E - \epsilon_L \quad (1)$$

where ϵ_{EL}, ϵ_E, and ϵ_L are the molar extinction coefficients at 290 nm for the enzyme–ligand complex, the free enzyme and the free ligand, respectively. The values of ϵ_E at 290 nm showed insignificant changes from pH 3.0 to pH 8.5 (Walz, 1977), whereas, those for ϵ_L showed anticipated changes in this pH range. Values of ϵ_L at 290 nm for Guo-3′-P, dGuo-3′-P, and P-5′-Guo were essentially the same in the pH range 3 to 9 and are plotted as a representative curve in Figure 2. Since the major UV absorption change in the complex results from perturbations of the guanine chromophore (Sawada et al., 1973), values of the molar extinction coefficient of the complexed ligand, ϵ_L', were calculated from

$$\epsilon_L' = (\epsilon_{EL} - \epsilon_E) = \Delta\epsilon + \epsilon_L \quad (2)$$

Plots of ϵ_L' are shown as a function of pH for each ligand in Figure 2.

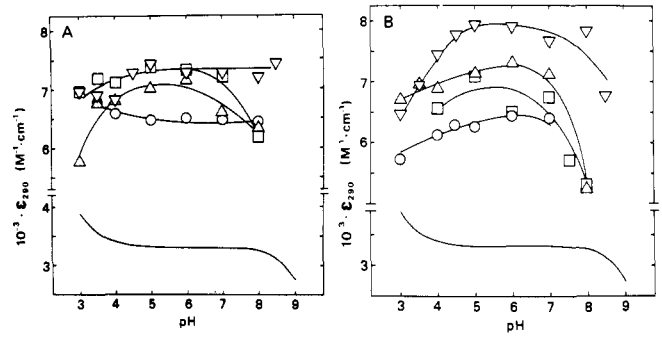

FIGURE 2: Plot of the molar extinction coefficients at 290 nm for free and RNase T_1 bound nucleotides vs. pH. (A) Ribose containing nucleotides (molar extinction coefficients for enzyme bound ligands, ϵ_L'): (□) Guo-2′-P; (▽) Guo-3′-P; (△) P-5′-Guo; (○) (Me)-P-5′-Guo; lower curve is a composite for the molar extinction coefficients of free Guo-3′-P and P-5′-Guo, ϵ_L. (B) Deoxyribose containing nucleotides (molar extinction coefficients for enzyme bound ligands, ϵ_L'): (▽) dGuo-3′-P; (△) P-5′-Guo; (□) dGuo-3′-P-(Me); (○) (Me)-P-5′-dGuo. Lower curve is for free dGuo-3′-P, ϵ_L. All measurements were at 0.2 M ionic strength and 25 °C at 290 nm. See text for details.

Discussion

In this model study of RNase T_1–substrate binding, it was of interest to elucidate the nature of enzyme interactions with ligand phosphomonoester and phosphodiester groups, as well as the relationship of these interactions with those for the ribose 2′-hydroxyl and guanine base groups. A valid approach for assessing the binding contributions of different nucleotide moieties is to determine the difference in $\Delta G°$ values for binding of appropriate ligand pairs (Jencks, 1975). It is understood that the values of $\delta\Delta G°$ obtained from such comparisons may not only reflect the intrinsic binding free energy change for specific ligand sites but could also include other coupled free energy and entropy terms (Jencks, 1975). The following analysis will assume that the gross conformational features of the various ligands investigated are not significantly different. Therefore, with these provisions in mind, the discussion that follows should be considered only as an initial attempt toward understanding the complicated binding topography at the active site of RNase T_1.

Binding of Phosphomonoester Groups. The differences in $\Delta G°$ values for RNase T_1 binding of nucleotide phosphomonoesters and their related nucleosides are plotted as a function of pH in Figure 3A. These plots immediately suggest that the phosphate groups of Guo-3′-P, dGuo-3′-P, and P-5′-dGuo interact with the enzyme in a similar manner. The average of the maximal values of $-\delta\Delta G°$ in Figure 3A for these three ligands (i.e., values between pH 4 and pH 6) will be considered to represent the intrinsic free energy change for enzyme binding of a guanine nucleotide phosphomonester group (ΔG_{int}^{P}) and is listed in Table II. In order to rationalize the different results for phosphate group binding of RNase T_1 with P-5′-Guo and Guo-2′-P the existence of other interaction loci at the active site is considered which include: (1) the primary recognition site for binding a guanine residue (gua-site) (Oshima and Imahori, 1972; Epinatjeff and Pongs, 1972; Walz and Hooverman, 1973); and (2) the ribose recognition site for binding the 2′-hydroxyl of a guanosine moiety (2′-OH-site) (Walz and Hooverman, 1973; Walz, 1976). The apparent maximal, intrinsic free energy change for binding at the 2′-OH-site ($\Delta G_{int}^{2'OH}$) is ~1.01 kcal/mol (Walz, 1976; see below) and that for binding at the gua-site can be estimated as at least ca. −4 kcal/mol which is $\Delta G°$ for RNase T_1 binding of 9-methylguanine (Oshima and Imahori, 1972; Walz, unpublished). (It is interesting to note that the affinity of 9-methylguanine with RNase T_1 is somewhat greater than that for deoxyguanosine (i.e., $\Delta G°$ for binding deoxyguanosine is −3.7 kcal/mol; see Table I). Therefore, it appears that guanine nucleoside sugar binding with the enzyme only occurs with the 2′-hydroxyl group.)

The observation that phosphate group binding for P-5′-Guo is different when compared with that of Guo-3′-P suggests the involvement of the 2′-hydroxyl group in this discrimination since the phosphate groups of dGuo-3′-P and P-5′-dGuo ap-

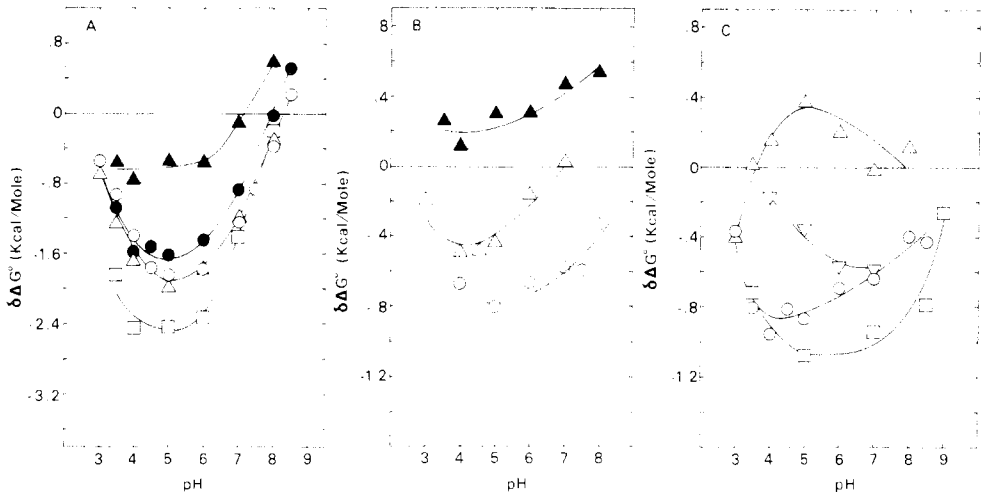

FIGURE 3: Plot of the difference in $\Delta G°$ for RNase T_1 binding of ligand phosphate and 2′-hydroxyl groups vs. pH. (A) Phosphomonoester group binding: (▲) P-5′-Guo minus Guo; (●) Guo-3′-P minus Guo; (○) dGuo-3′-P minus dGuo; (△) P-5′-dGuo minus dGuo; (□) Guo-2′-P minus Guo. (B) Methyl phosphodiester group binding: (▲) (Me)-P-5′-Guo minus Guo; (△) (Me)-P-5′-dGuo minus dGuo; (○) dGuo-3′-P-(Me) minus dGuo. (C) 2-Hydroxyl group binding: (△) P-5′-Guo minus P-5′-dGuo; (▽) (Me)-P-5′-Guo minus (Me)-P-5′-dGuo; (○) Guo-3′-P minus dGuo-3′-P; (□) Guo minus dGuo. The $\Delta G°$ values for Guo and dGuo were calculated from previous data (Walz, 1976). All experiments were conducted at 0.2 M ionic strength at 25 °C.

parently bind equivalently (see Figure 3A). A likely explanation for this effect is that the binding of the ribose nucleotide at the gua-site and 2′-OH site restricts the interaction of the ligand 3′ or 5′ phosphomonoester group such that the binding of Guo-3′-P has complementary enzyme interaction with the phosphate and 2′-hydroxyl groups, whereas these interactions "compete" and are mutually exclusive for binding P-5′-Guo. The notion of competing, mutually exclusive interactions for enzyme binding of the phosphate and 2′-hydroxyl groups of P-5′-Guo predicts that the apparent contribution to $\Delta G°$ for binding of the phosphate group, $\Delta G_{app}{}^{5'P}$, can be approximately expressed as

$$\Delta G_{app}{}^{5'P} = \Delta G_{int}{}^{P} - \Delta G_{int}{}^{2'OH} \quad (3)$$

Substitution for $\Delta G_{int}{}^{P}$ and $\Delta G_{int}{}^{2'OH}$ in eq 2 using the values in Table II yields a calculated value of -0.66 ± 0.26 kcal/mol for $\Delta G_{app}{}^{5'P}$ which agrees very well with the observed value listed in Table II which was determined from the data in Figure 3A.

The data in Figure 3A also suggest that the phosphate group of Guo-2′-P interacts in a unique manner with the enzyme. This observation is in agreement with previous studies where qualitative differences were observed for RNase T_1 binding of Guo-2′-P when compared with other nucleotides (Rüterjans et al., 1969; Epinatjeff and Pongs, 1972). The present view of the active site predicts that the phosphate group of Guo-2′-P would abut with the 2′-OH site which was recently proposed to consist, in part, of an ordered carboxylate–imidazolium pair with a carboxylate oxygen serving as a hydrogen bond acceptor for the 2′-hydroxyl group of a bound ligand (Walz, 1976). Considering this hypothesis, it may be speculated that the juxtaposition of an adjacent 2′-phosphate group would "disorder" enzyme groups at the 2′-OH site and/or facilitate phosphate group ionization which could explain the observation that the binding of RNase T_1 with Guo-2′-P is accompanied by a positive entropy change, whereas, binding of Guo, Guo-3′-P, and P-5′-Guo exhibits negative entropy changes (Epinatjeff and Pongs, 1972). The decrease in binding of phosphomonoester groups with RNase T_1 from pH 6 to pH 8 (Figure 3A) is consistent with the proposal that His-40 directly interacts with the phosphate group of Guo-3′-P (Arata et al., 1976). The observation that phosphomonoester group binding is not significant or is actually destabilizing at pH values near 8 could result, in part, from secondary ionization of the complexed phosphate group: this view is supported by the observation that the decrease in phosphodiester group binding (Figure 3B) is less precipitous in the alkaline pH range. All three His residues of RNase T_1 are associated with carboxyl groups as indicated by proton NMR studies (Rüterjans and Pongs, 1971; Markley, J. L., Walz, F. G., and Westler, F. M., unpublished experiments); therefore, the decreased binding of phosphate groups at low pH may be due to protonation of a carboxyl group associated with His-40.

Binding of Phosphodiester Groups. Plots of the differences in $\Delta G°$ for RNase T_1 binding of nucleotide phosphodiesters and their corresponding nucleosides are presented in Figure 3B. Comparing these results with those in Figure 3A indicates some remarkable differences for RNase T_1 binding of corresponding monoester and diester phosphate groups. In the first place, the maximal values of $-\delta\Delta G°$ were much smaller in all cases for the phosphodiester ligands. Furthermore, there is a significant preferential binding of dGuo-3′-P-(Me) over (Me)-P-5′-dGuo which was not the case for the parent nucleotide monoesters. On the other hand, the finding that $\delta\Delta G°$ values in Figure 3B were positive for (Me)-P-5′-Guo and negative for (Me)-P-5′-dGuo is consistent with the proposal of mutually exclusive active site interactions with ligand 5′-phosphodiester and 2′-hydroxyl groups as was discussed above for P-5′-Guo binding. For example, in this case

$$\Delta G_{app}{}^{5'MP} = \Delta G_{int}{}^{5'MP} - \Delta G_{int}{}^{2'OH} \quad (4)$$

where $\Delta G_{app}{}^{5'MP}$ is the apparent free energy contribution for binding the methyl phosphodiester group of (Me)-P-5′-Guo and $\Delta G_{int}{}^{5'MP}$ represents the intrinsic value for binding the 5′-methyl phosphodiester group which is assumed to equal the maximal value of $-\delta\Delta G°$ for (Me)-P-5′-dGuo in Figure 3B. The calculated value for $\Delta G_{app}{}^{5'MP}$, using eq 4 and the values of $\Delta G_{int}{}^{5'MP}$ and $\Delta G_{int}{}^{2'OH}$ from Table II, was 0.55 ± 0.12 kcal/mol which agrees with the observed value (i.e., 0.37 ± 0.09 kcal/mol; see Table II).

The results for the binding of guanine nucleotide methyl phosphodiesters in the present study are in agreement with those from a previous report (Walz and Terenna, 1976) that used dinucleoside monophosphate diesters as ligand probes for

RNase T_1-subsite interactions. For example, the values of $-\Delta G°$ for RNase T_1 binding in Table I and those determined previously under the same conditions (Walz and Terenna, 1976) show: dGuo-3′-P-(Me) = dGpdX (X = A, C, T) ≫ (Me)-p-5′-dGuo > dXpG (X = A, T) = dGuo. Similar relations were also true for ribose containing ligands which show the following order for $-\Delta G°$: GpX (X = A, C, G, U) ≫ Guo > UpG = CpG = (Me)-P-5′-Guo. The apparent $-\Delta G°$ values for binding GpC and GpU (Zabinski and Walz, 1976) and GpA and GpG (Osterman and Walz, in preparation) were determined from Michaelis' constants (i.e., $\Delta G° = RT \ln K_m$) and those for UpG and CpG were determined from equilibrium binding constants (Walz and Terenna, 1976). Therefore, an apparent rule for the ligands tested so far is that, in the absence of specific nucleoside subsite interactions (e.g., for A with ApG; Walz and Terenna, 1976), guanine nucleotide 3′-phosphodiesters preferentially bind with RNase T_1 when compared with their 5′ isomers.

Assuming the additivity of free energy contributions for substrate binding (Jencks, 1975) and accepting the maximal value of $-\delta\Delta G°$ for dGuo-3′-P-(Me) in Figure 3B as characterizing the intrinsic free energy contribution for binding a guanine nucleotide 3′-phosphodiester group, we can calculate the expected value of $\Delta G°$ for binding a dinucleoside monophosphate substrate (GpX) as the sum of $\Delta G°$ for binding guanosine (−4.81 kcal/mol) and a 3′-phosphodiester group (i.e., −0.71 kcal/mol; for $\Delta G_{int}^{3'MP}$ in Table II) which is −5.52 kcal/mol. This value is similar to the apparent value for binding GpA (−5.84 kcal/mol) and GpC (−5.25 kcal/mol) but significantly less negative than that for GpU (−6.23 kcal/mol) and GpG (−6.25 kcal/mol). Therefore, it is possible that these differences for GpU and GpG represent the binding of the uridine and guanosine moieties with a specific enzyme substite (Zabinski and Walz, 1976; Osterman and Walz, in preparation). In this regard, it is interesting to note that $\Delta G°$ values for binding the series dGpdX (X = A, C, G, T) showed only small differences (Walz and Terenna, 1976) which suggests that discriminating interactions of the enzyme with the X residue of GpX require an interaction with a guanosine 2′-hydroxyl group. However, this analysis depends on the validity of K_m values representing true dissociation constants. Studies on the binding of RNase T_1 with GpcX (X = C, U) phosphonate substrate analogues are planned which should resolve this issue.

The pH dependence of phosphodiester group binding is apparently bell shaped as was the case for corresponding monoester groups but is considerably less steep at extreme pH values. Since the phosphodiester group does not ionize from pH 6 to pH 8, the decline in binding over this pH range could reflect ionization of His-40 which was implicated in binding phosphomonoester groups (Arata et al., 1976).

Considering the ionized states of phosphate groups on the free nucleotides from pH 3 to pH 6, the affinity of RNase T_1 for the phosphomonoester monoanion ($ROPO_3H^-$) appears to be considerably greater than that for the corresponding phosphodiester monoanion ($ROPO_3CH_3^-$) and this difference approaches zero at high pH where the free monoester group exists on a dianion ($ROPO_3^{2-}$) (Figure 1). If a simple electrostatic interaction is the primary means for binding the nucleotide phosphate groups, it would be anticipated that the species $ROPO_3H^-$ would have an affinity that is similar to the $ROPO_3CH_3^-$ species. Therefore, it seems likely that hydrogen bonding and/or steric factors are important for RNase T_1 binding of phosphate groups at the active site.

The Binding of the 2′-Hydroxyl Group of Guanine Nucleotides. Considered from another perspective the results of the present study can be viewed in terms of the effect of binding nucleotide phosphate groups on the binding of the 2′-hydroxyl group. The difference in $\Delta G°$ values for binding comparable ribose and deoxyribose ligands is shown in Figure 3C. The $\delta\Delta G°$ values for Guo minus dGuo were calculated from a previous study (Walz, 1976) and are considered to represent the intrinsic binding of the 2′-hydroxyl group. The free energy contributions for RNase T_1 binding of nucleotide 2-hydroxyl groups are necessarily dependent on those already discussed for binding ligand phosphate groups. For example, it can be shown for the binding of P-5′-Guo that

$$\Delta G_{app}^{2'OH} = \Delta G_{int}^{2'OH} + \Delta G_{app}^{P} - \Delta G_{int}^{P} \qquad (5)$$

where $\Delta G_{app}^{2'OH}$ is the apparent value for binding the 2′-hydroxyl group of P-5′-Guo. Substituting the right side of eq 5 with the values in Table II yields a calculated value of 0.06 ± 0.38 kcal/mol for $\Delta G_{app}^{2'OH}$ which agrees, within experimental error, with the observed value of 0.25 ± 0.12 kcal/mol listed in Table II. The lower values of $\delta\Delta G°$ for the 5′-methyl phosphodiester comparisons in Figure 3C were also expected in accord with the model of mutually exclusive interactions for enzyme binding 2′-hydroxyl and 5′-phosphate groups of (Me)-P-5′-Guo as discussed above.

The binding of the 2′-hydroxyl group of Guo-3′-P is characterized by a maximal change in $\Delta G°$ of −0.88 ± 0.07 kcal/mol (i.e., between pH 4 and 5; see Figure 3C) which is the same, within experimental error, as $\Delta G_{int}^{2'OH}$ listed in Table II. This result was anticipated for complimentary binding of nucleotide 3′-phosphate and 2′-hydroxyl groups as discussed above. It is interesting that the apparent binding of the 2′-hydroxyl group of Guo-3′-P with the enzyme is similar to that of guanosine at pH 4–5 but shows a decline at higher pH values (see Figure 3C). This could result from proton dissociation of the complexed phosphate group in this pH range which electrostatically destabilizes the adjacent interaction of the enzyme with the ligand 2′-hydroxyl group. Such a rationale supports the view that negatively charged Glu-58 is directly involved in binding the 2′-hydroxyl group (Walz, 1976).

Binding of the Guanine Group. The values of ϵ_L' plotted in Figure 2 should represent, for the most part, the absorption properties of the RNase T_1 complexed guanine residue which is presumably protonated at the N-7 position (Epinatjeff and Pongs, 1972; Oshima and Imahori, 1972; Walz and Hooverman, 1973). Even though the data for ϵ_L' at extreme pH values are less reliable, due to weak ligand binding, it is clear in some cases that ϵ_L' changes with pH; this is particularly noticeable for bound dGuo-3′-P from pH 3 to pH 5. On the other hand, the value of ϵ_L' for bound Guo-3′-P is virtually pH independent from pH 3 to pH 8.5. Considering the view that ϵ_L' reflects protonation of the bound guanine group, the pH dependence of ϵ_L' at 290 nm could indicate the degree of guanine protonation in the complex as well as direct or indirect interaction of the complexed guanine residue with enzyme acid–base groups; e.g., the increase in ϵ_L' from pH 3 to pH 5, in some cases, could reflect the ionization of an adjacent enzyme carboxyl group, whereas cases in which ϵ_L' decreases at high pH values could indicate N-7 deprotonation of the bound guanine group. The observation that the pH dependence of ϵ_L' depends on the nature of the bound nucleotide is not surprising since other quantitative (see Figure 2 and Walz and Hooverman, 1973) and qualitative (Walz and Terenna, 1976; Walz, 1976; and see λ_{max} values in Table I) aspects of the binding difference spectra are idiosyncratic regarding the nature of the bound ligand. In the absence of a theoretical basis for these differences, some phenomenological correlations can be made: e.g., as shown in Figure 2 the maximum values of ϵ_L' for 3′-nucleotides are

TABLE III: The Effect of γ-Glu-58 Carboxymethylation of RNase T_1 on the Difference in $\Delta G°$ for Binding of Various Ligand Groups

Comparison	$-\delta\Delta G°$ RNase T_1 (kcal/mol)	$-\delta\Delta G°$ CM-RNase T_1 (kcal/mol)
Ribose and deoxyribose		
Guo − dGuo	1.08	0.03
Guo-3′-P − dGuo-3′-P	0.87	0.43
P-5′-Guo − P-5′-dGuo	−0.38	0.11
(Me)-P-5′-Guo − (Me)-P-5′-dGuo	0.34	0.01
Nucleotide and nucleoside		
Guo-2′-P − Guo	2.43	0.63
Guo-3′-P − Guo	1.61	0.50
P-5′-Guo − Guo	0.52	0.80
(Me)-P-5′-Guo − Guo	−0.31	−0.08
dGuo-3′-P − dGuo	1.82	0.10
P-5′-dGuo − dGuo	1.98	0.72
(Me)-P-5′-dGuo − dGuo	0.44	−0.04

[a] Data were taken from Table I.

greater than those of 5′-nucleotides in all comparisons which could reflect the different binding interactions for 3′- and 5′-phosphate groups as discussed above. In addition, ϵ_L' is greater for nucleotide monoesters when compared with homologous diesters which again could reflect different binding of the respective phosphate groups.

Recent NMR studies of RNase T_1 (Arata et al., 1976) have led to the conclusion that the N-7 position of complexed Guo-3′-P is not protonated per se but instead is hydrogen bonded with a His-imidazolium group which was tentatively identified as His-92. Since this model suggests that the binding difference spectra for this ligand result from such an interaction, it is not compatible with the present results since ϵ_L' for Guo-3′-P is pH independent over a pH range in which the imidazolium residue, that is proposed to interact at the N-7 position of guanine group, is ~50% dissociated in the complex (Arata et al., 1976). Furthermore, Takahashi has concluded that His-92 is part of the "catalytic site" based on photo-oxidation (Takahashi, 1971) and other chemical modification studies (Takahashi, 1976) of RNase T_1 which were performed in the presence and absence of guanine ligands. Nevertheless, the observation that 7-methylguanosine and 7-deazainosine derivatives do not interact significantly with RNase T_1 (Takahashi et al., 1970) could reflect the requirement of hydrogen bonding at the N-7 position in the complex. However, at this point it does not seem likely that a His residue is involved in this interaction.

Nucleotide Binding with CM-RNase T_1. Previous binding studies of RNase T_1 and CM-RNase T_1 with guanosine and deoxyguanosine led to a hypothetical model for nucleoside binding at the active site (Walz, 1976). This proposal suggested that the γ-carboxyl group of Glu-58 interacts with one active site His residue (probably His-92 (Takahashi, 1976)) and serves as a binding locus for the guanosine 2′-hydroxyl group in the native enzyme, whereas the newly introduced carboxyl group of CM-RNase T_1 interacts with the other active site His residue (probably His-40 (Arata et al., 1976; Takahashi, 1976)) and serves as a binding locus for the 3′-hydroxyl group of either guanosine or deoxyguanosine. In the present study the effect of Glu-58 carboxymethylation on the binding of guanine nucleotide 2′-hydroxyl and phosphate groups was tested and the results are summarized in Table III. The observation that Glu-58 carboxymethylation significantly diminished the binding of the guanosine 2′-hydroxyl group, which is indicated in all "ribose and deoxyribose" comparisons, is consistent with the view that Glu-58 is involved in binding this group in nucleotides as well as nucleosides. Furthermore, with the exception of P-5′-Guo, phosphate group binding with CM-RNase T_1 was also decreased when compared with the native enzyme. This observation agrees with the proposal that the newly introduced carboxyl group of CM-RNase T_1 interacts with His-40 which is part of the phosphate group binding site (Arata et al., 1976). It is interesting to note that the order of affinities for guanosine nucleotides with CM-RNase T_1 is P-5′-Guo ≥ Guo-2′-P ≥ Guo-3′-P which compares with that of Guo-2′-P ≫ Guo-3′-P ≫ P-5′-Guo for the native enzyme (Table I); furthermore, the affinity of CM-RNase T_1 is significantly greater for P-5-dGuo compared with dGuo-3′-P which is not the case for the native enzyme. These results probably reflect the binding of the 3′-hydroxyl groups of P-5′-Guo and P-5′-dGuo with CM-RNase T_1 (Walz, 1976). As shown in Table I the values of $\Delta\epsilon$ at 290 nm for CM-RNase T_1-ligand binding are relatively constant and are similar in magnitude to that for the low pH induced difference spectra of the free nucleotides (Oshima and Imahori, 1972; Walz, F. G., unpublished experiments). This phenomenon could reflect less specific ligand binding of the carboxymethylated enzyme with sugar and phosphate groups which, in the native enzyme, result in more characteristic values of $\Delta\epsilon$ for a given ligand.

Summary

Some tentative conclusions of this work can be summarized as follows: (1) the free energy change characterizing RNase T_1 binding with guanine nucleotides can be accounted for in terms of enzyme interactions with guanine, phosphate, and 2′-hydroxyl groups of the ligand which are typically characterized by $-\Delta G°$ contributions for binding of ~4, ~1.7, and ~1 kcal/mol, respectively; (2) the phosphate group binding site favors binding 3′-phosphomonoester groups vis-à-vis 5′-phosphomonoester groups by virtue of complementary interactions of the enzyme with the 2′-hydroxyl group, whereas preferential binding of the 3′-phosphodiester group when compared with the 5′-phosphodiester group is intrinsic to the phosphate binding site and independent of enzyme binding of the 2′-hydroxyl group; (3) maximal binding of ligand phosphate groups requires the ionization of His-40 and probably an associated carboxyl group; (4) hydrogen bonding and/or steric factors play a greater role than electrostatic attraction in binding phosphomonoester groups at the active site; (5) secondary ionization of the complexed phosphomonoester group destabilizes enzyme interactions with phosphate and 2′-hydroxyl groups; (6) the results are consistent with a model of the active site which proposes that Glu-58 and His-92 interact with the substrate 2′-hydroxyl group (Walz, 1976), whereas His-40 (Arata et al., 1976), and probably its associated carboxyl group, interact with substrate phosphate groups.

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Mechanism of Inactivation of γ-Cystathionase by β,β,β-Trifluoroalanine†

Richard B. Silverman‡ and Robert H. Abeles*

ABSTRACT: β,β,β-Trifluoroalanine irreversibly inactivates γ-cystathionase. The inactivator becomes covalently attached to the enzyme. Two moles of inactivator is incorporated per tetramer (Silverman, R. B., and Abeles, R. H. (1976), *Biochemistry 15,* 4718). The rate of inactivation, the rate of [^{14}C]trifluoroalanine incorporation into the protein, and the rate of release of F^- from trifluoroalanine are equal. F^-, 2.7 mol, is released per mol of inactivator which becomes covalently attached to the protein. In the course of the inactivation, the β carbon of trifluoroalanine is converted to an acyl group which is bonded to an amino group of the protein. (See Scheme I.) It is proposed that trifluoroalanine labels the functional group, probably a lysine ε-amino group, which protonates or deprotonates the α and β positions of the substrate in the normal catalytic process. Propargylglycine inactivates and covalently labels the native enzyme (Washtien, W., and Abeles, R. H. (1977), *Biochemistry 16* (in press)). Enzyme inactivated with trifluoroalanine does not react with propargylglycine. However, enzyme inactivated with 2 mol of propargylglycine per tetramer reacts with trifluoroalanine and 2 mol of trifluoroalanine become covalently bonded per mol of enzyme (tetramer). It is proposed that propargylglycine does not affect the functional group required for reaction with trifluoroalanine. Trifluoroalanine labels a group required for the activation of propargylglycine and thus prevents reaction of the enzyme with propargylglycine.

Recently, we reported our studies on the irreversible inactivation of several pyridoxal phosphate dependent enzymes, including γ-cystathionase, by polyhaloalanines (Silverman and Abeles, 1976). Some aspects of the mechanism of inactivation of γ-cystathionase were also described. When γ-cystathionase was inactivated with [1-^{14}C]trifluoroalanine and then subjected to gel filtration, ^{14}C was found with the protein fraction. Dialysis or gel filtration of the ^{14}C-labeled protein did not result in loss of ^{14}C. Two moles of inactivator was incorporated per mol of protein, which consists of 4 subunits and contains 4 mol of pyridoxal phosphate. Subsequent acid denaturation of this labeled protein released all of the radioactivity in the form of $^{14}CO_2$. Scheme I shows the mechanism which we proposed for this inactivation (Silverman and Abeles, 1976). In this paper, we report a detailed investigation of the mechanism of the inactivation of γ-cystathionase by trifluoroalanine.

Materials and Methods

Reagents. γ-Cystathionase was purified from rat liver by the procedure of Greenberg (1971). Enzyme activity is expressed in the same units as previously used (Greenberg, 1971), i.e., μmol of α-ketobutyrate produced in 60 min under standard assay conditions. Enzyme with specific activity of 350 showed a single band on acrylamide electrophoresis. β,β,β-Trifluoro-D,L-alanine and [1-^{14}C]trifluoro-D,L-alanine (specific activity 7.8×10^5 dpm/μmol) were synthesized by the procedure of Weygand et al. (1967). Potassium [^{14}C]cyanide was used to prepare the intermediate 2,2,2-trifluoro-1-[^{14}C]cyano-*N*-benzoylethylamine. Propargylglycine and [1-^{14}C]propargylglycine were generous gifts of Dr. W. Washtien. β-(*N*-Acetyl)aminoethyl thioacetate was kindly donated by Ms. S. Moore and glycine phenyl ester hydrobromide, by Mr. R. Suva. Pyridoxal 5′-phosphate (PLP)[1] and iodoacetic acid were purchased from Sigma Chemical Co. Urea (recrystallized from

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‡ Present address: Department of Chemistry, Northwestern University, Evanston, Illinois 60201.

[1] Abbreviations used: PLP, pyridoxal 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.