Spectrophotometric Titration of a Single Carboxyl Group at the Active Site of Ribonuclease T_1†

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ABSTRACT: Low-pH-induced difference spectra for ribonuclease T_1, which were determined using a reference solution at pH 6, consisted of a shorter wavelength component from 270 to 285 nm that reflected an ionization having a pK_a of 3.54 and a longer wavelength component above 285 nm that reflected an ionization having a pK_a of 4.29. The temperature dependence of the pK_a value for data at 300 nm is consistent with its representing the dissociation of a carboxyl group. In addition, the pK_a determined at this wavelength significantly decreased at lower ionic strength. Similar experiments which were conducted using catalytically inactive γ-carboxymethyl-Glu-58-ribonuclease T_1 gave difference spectra having only the shorter wavelength component and were characterized by a single pK_a of 3.53. It is concluded that the longer wavelength component of the difference spectra is due to the ionization of Glu-58. The pK_a determined for this residue in the present study agrees with one found previously from kinetic studies which supports a role for Glu-58 in catalysis. Furthermore, the results suggest a model for the interaction of Glu-58 with histidine and tryptophan residues at the active site.

An outstanding feature of ribonuclease[1] T_1 is its large percentage of aromatic (13.5%) and carboxyl (10.6%) side chains (Uchida and Egami, 1971). The significance of Glu-58 in catalysis was first suggested by its unique reactivity with iodoacetate which specifically yields catalytically inactive CM-RNase T_1 (Takahashi et al., 1967). Primarily on the basis of these results, this residue has been proposed to participate in its ionized form as a general base species in a mechanism for RNase T_1 catalyzed transesterification (Takahashi, 1970). In support of this view, steady-state kinetic studies of RNase T_1 with GpA, GpG, GpC, and GpU as substrates suggested the catalytic involvement of an unprotonated group on the free enzyme having an apparent pK_a of 4.15 ± 0.3 (Zabinski and Walz, 1976; Osterman and Walz, in preparation). Furthermore, the specific role for the γ-carboxylate of Glu-58 in deprotonating a substrate guanosine 2′-hydroxyl group was supported in recent studies which indicated its participation as part of a binding locus for the 2′-hydroxyl group of guanosine (Walz, 1976). Considering this evidence in favor of a critical role for Glu-58 in catalysis and substrate recognition, it became of interest to study the ionization process of its γ-carboxyl group. In this regard, it is known that Glu-58 is flanked by Trp-59 and Tyr-57 in the primary structure of the enzyme (Takahashi, 1965), and it has been reported that the fluorescence of Trp and Tyr residues is perturbed in the pH range 2 to 5 (Pongs, 1970). These observations led us to consider the effect of pH on the ultraviolet absorption properties of the enzyme.

Materials and Methods

The preparation and concentration assay of RNase T_1 and CM-RNase T_1 were as previously described (Walz, 1976). It was critical in the present study that traces of enzyme-bound guanine nucleotides be removed; for this purpose, the enzyme preparations were exhaustively dialyzed in a Bio-Rad hollow-fiber dialyzer against ~300 volumes of distilled water. The ratios of absorbances of the maximum (278 nm) to the minimum (251.5 nm) were 3.58 and 3.34 for RNase T_1 and CM-RNase T_1, respectively (the ratio for RNase T_1, which was not corrected for scattered radiation, is in agreement with the value of 3.68 reported previously (Uchida and Egami, 1970)).

In order to provide reproducible pH-induced difference spectra, the following procedure was employed: slotted, split compartment cells (Pyrocell, Inc.) having a total path length of 0.88 cm were used; the reference cell contained 1 mL of an enzyme solution buffered at pH 6.0 in one compartment and 1 mL of of the pH 6.0 buffer in the other compartment, whereas the sample cell contained 1 mL of the pH 6.0 enzyme solution in one compartment and 1 mL of a more acidic buffer solution in the other compartment. The baseline for the difference spectrum was determined by scanning from 325 to 270 nm using a Cary 118C spectrophotometer at a constant slit width of 0.2 nm. The components in both cells were then mixed by inversion and the difference spectrum was recorded. The pH of the solutions in the sample and blank cells was then determined using a Radiometer PHM-26 pH meter (at the same temperature the difference spectra were determined). The standard buffers used consisted of 0.1 M KCl, 0.05 M Tris, 0.05 M sodium acetate adjusted to the desired pH with acetic acid. In experiments where the final pH after mixing was less than pH 4.3, the same buffer with sodium lactate–lactic acid substituted for sodium acetate–acetic acid was employed. Solutions for studying the effect of ionic strength on the difference spectra were all 0.005 M in potassium acetate and potassium lactate adjusted to the desired pH with lactic acid and contained varying concentrations of KCl to give different ionic strengths. Except where it is noted, the difference spectra were determined at 25 ± 0.03 °C. Temperature was monitored in the cell with a thermistor probe using a YSI Model 26 Tele-Thermometer.

Temperature-jump experiments used an apparatus that was described previously (Walz, 1971).

Results

Some examples of the pH-induced difference spectra for RNase T_1 and CM-RNase T_1 are presented in Figure 1. The

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[1] Abbreviations used are: RNase, ribonuclease; CM-RNase T_1, γ-carboxymethyl-Glu-58-ribonuclease T_1; oligonucleotide designations follow the IUPAC–IUB recommendation as reported in *Biochemistry 9*, 4025 (1970); Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; UV, ultraviolet; NMR, nuclear magnetic resonance.

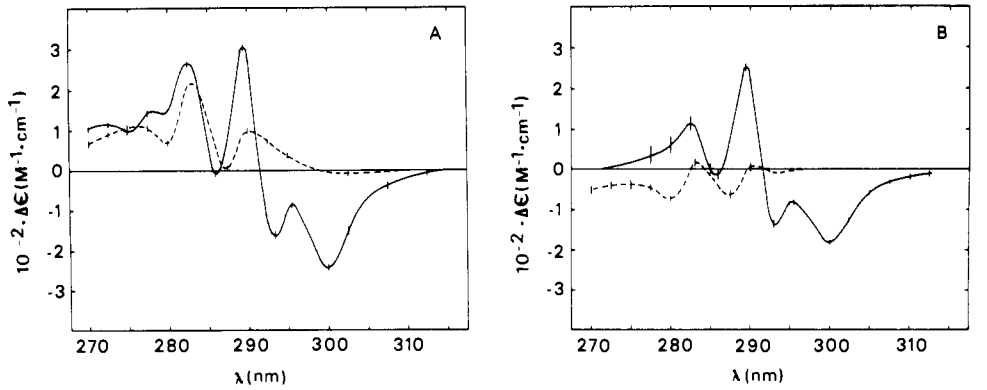

FIGURE 1: The pH-induced difference spectra of RNase T_1 and CM-RNase T_1. RNase T_1 (—) at a concentration of 5.58×10^{-5} M; CM-RNase T_1 (- - -) at a concentration of 4.08×10^{-5} M. (A) Final pH of the sample was 2.84; (B) final pH of the sample was 3.82; reference pH was 6.0. Error bars represent duplicate spectra. Experiments were conducted at 25 °C using the standard buffer solution. Other conditions are described under Methods.

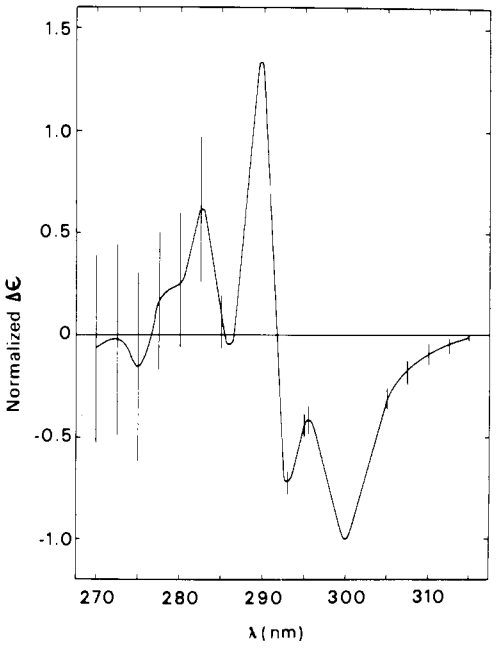

FIGURE 2: The normalized pH-induced difference spectrum for RNase T_1. Sixteen pH-induced difference spectra using a reference pH of 6.0 and samples from pH 2.8 to 5.4 and covering a range of RNase T_1 concentration from 1.99×10^{-5} to 9.97×10^{-5} M were normalized to 300.2 nm. Other conditions were the same as those in Figure 1. The error bars represent standard deviations.

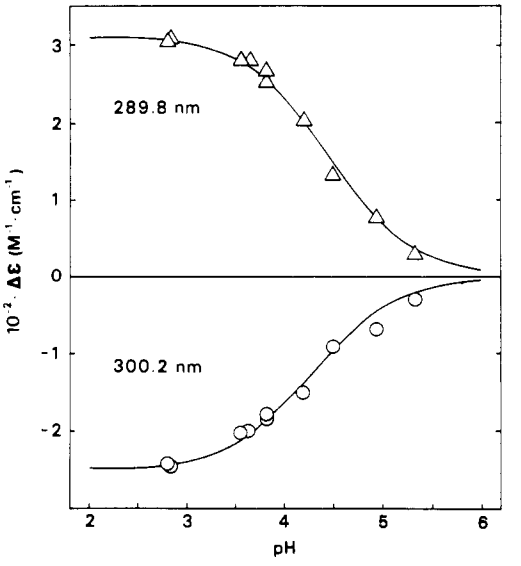

FIGURE 3: Plot of the difference extinction coefficient at two wavelengths for the pH-induced difference spectra of RNase T_1 as a function of pH. RNase T_1 concentration was 5.08×10^{-5} M. Reference pH was 6.0. Other conditions were the same as those in Figure 1. Theoretical curves were calculated as described under Results.

difference spectrum for RNase T_1 above 285 nm changed slightly from pH 2.84 to 3.82, whereas below this wavelength a significant change occurred over this pH interval. These observations suggested two independent components of the difference spectrum that reflected different ionization processes. The characteristic difference spectrum for RNase T_1 in Figure 2 was determined by normalizing difference absorbance values to those at 300.2 nm for 16 experiments covering a pH range from 2.8 to 5.4 and an enzyme concentration range from 1.99×10^{-5} to 9.97×10^{-5} M. The mean crossover wavelength was 291.6 ± 0.3 nm; a similar precision was found for the maximum at 289.8 ± 0.3 nm and minimum at 300.2 ± 0.2 nm. The increased standard deviations for the normalized difference extinction coefficients below 285 nm result from a different pH dependence of the different spectra in this wavelength range (see below). As shown in Figure 1, the entire shape of the difference spectra with respect to the baseline for CM-RNase T_1 varied with pH and, therefore, a meaningful normalized difference spectrum could not be obtained in this manner. This lack of a normalized "shape" for the difference spectra of CM-RNase T_1 could be due to the presence of trace amounts of bound nucleotide, as indicated by control pH-induced difference spectra determined in the presence of added nucleotide (guanosine 3'-phosphate at one-fifth the concentration of the enzyme); however, the maxima and minima in the CM-RNase T_1 difference spectra are not due to the presence of contaminant nucleotide.

For RNase T_1, values of the difference molar extinction coefficient, $\Delta\epsilon$, varied in a systematic manner with pH as shown in Figure 3 for data above 285 nm and in Figure 4A for data below 285 nm. These plots are consistent with the titration of a single dissociating group according to:

$$K_a = a_{H^+}(\Delta\epsilon_0 - \Delta\epsilon)/\Delta\epsilon \quad (1)$$

where K_a is the acid dissociation constant, a_{H^+} is the hydrogen ion activity measured with a glass electrode, and $\Delta\epsilon_0$ is the maximal value of $\Delta\epsilon$ at low pH. Best fit values of K_a and $\Delta\epsilon_0$ were determined for the data by finding the value of $\Delta\epsilon_0$ that minimized the coefficient of variation for K_a (Walz and Hooverman, 1973). The values of $\Delta\epsilon_0$ and K_a were found to

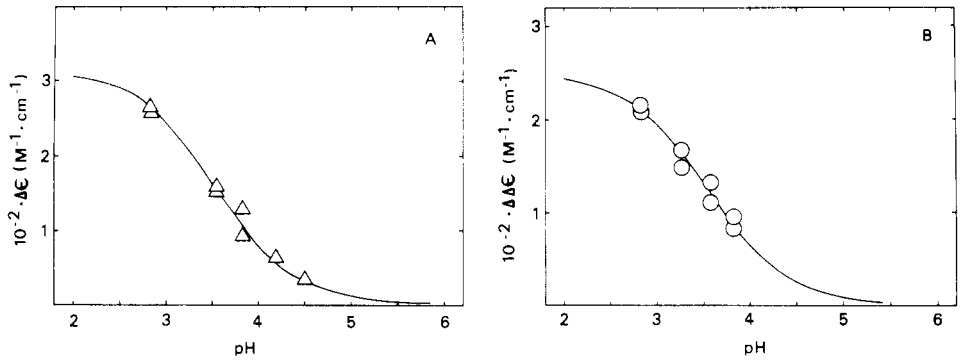

FIGURE 4: pH-induced difference spectral titration plots for RNase T_1 and CM-RNase T_1. (A) Plot of the difference extinction coefficient at 282.8 nm for RNase T_1 as a function of pH using pH 6.0 as a reference; (B) plot of the difference in the difference extinction coefficients at 283 and 287 nm for the CM-RNase T_1 difference spectra using a reference pH of 6.0. Other conditions were the same as those in Figure 1. Theoretical curves were calculated as described under Results.

be 314 M^{-1} cm^{-1} and 3.56×10^{-5} M ($pK_a = 4.45$) at 289.8 nm and -252 M^{-1} cm^{-1} and 5.18×10^{-5} M ($pK_a = 4.29$) at 300.2 nm, respectively. At 282.8 nm the value of $\Delta\epsilon_0$ was found to be 316 M^{-1} cm^{-1} and K_a was 2.91×10^{-4} M ($pK_a = 3.54 \pm 0.1$). The theoretical curves in Figure 3 were constructed using these values of K_a and the appropriate values of $\Delta\epsilon_0$ with eq 1. The theoretical curve in Figure 4A for the data at 282.8 nm was calculated in the same manner.

The analysis of the pH dependence of the difference spectra for CM-RNase T_1 was not straightforward (e.g., some values of $\Delta\epsilon$ at a given wavelength were either positive or negative, depending on pH). Nevertheless, it was noticed that the difference in $\Delta\epsilon$ between the maximum and minimum (i.e., at 283 and 287 nm, respectively; see Figure 1), defined as $\Delta\Delta\epsilon$, changed in a systematic way with pH as shown in Figure 4B. This data was analyzed in the same manner as $\Delta\epsilon$ for RNase T_1 and gave a value of 255 M^{-1} cm^{-1} for $\Delta\Delta\epsilon_0$ (i.e., the maximum value of $\Delta\Delta\epsilon$ at low pH) and 2.98×10^{-4} M for K_a ($pK_a = 3.53 \pm 0.2$). An empirical justification of this analysis used the data at comparable maxima and minima wavelengths for RNase T_1: i.e., $\Delta\Delta\epsilon$ was calculated as the difference in $\Delta\epsilon$ between 282.8 and 286.3 nm; in this case, the best-fit values for $\Delta\Delta\epsilon_0$ and K_a were 324 M^{-1} cm^{-1} and 2.51×10^{-4} M ($pK_a = 3.60 \pm 0.2$). It should be noted that these values are similar to those for CM-RNase T_1 and agree very well with those determined in the conventional manner at 282.8 nm for RNase T_1. The theoretical curve in Figure 4B was constructed using values for $\Delta\Delta\epsilon_0$ and K_a appropriately substituted in eq 1.

The effect of RNase T_1 concentration on the pH-induced difference spectra in the standard buffer was determined at pH 2.84 in six experiments over the concentration range 1.99×10^{-5} to 9.97×10^{-5} M. The shapes of the difference spectra regarding the wavelengths for the maxima, minima, and crossover were constant; however, the value of $\Delta\epsilon$ decreased slightly (~13% at 300.2 nm) from the highest to the lowest concentrations tested.

The effect of temperature on the pH dependence of the difference absorbance at 300 nm for RNase T_1 was tested at 10, 25, and 40 °C and yielded pK_a values which are plotted in Figure 5. The values of $\Delta H°$ and $\Delta S°$ at 25 °C were calculated from this Van't Hoff plot (i.e., using $pK_a = (\Delta H°/2.30RT) + C$ and $\Delta S° = (\Delta H° - \Delta G°)/T$) and were found to be 2.4 ± 0.8 kcal/mol and -11 ± 4 cal/mol·deg, respectively.

A preliminary test on the ionic-strength dependence of the pK_as observed at 282.8 and 300 nm for RNase T_1 was accomplished in experiments employing ten times diluted standard buffers and the pK_a values found were 3.48 ± 0.1 and 3.60 ± 0.1, respectively. The results of a more systematic study of

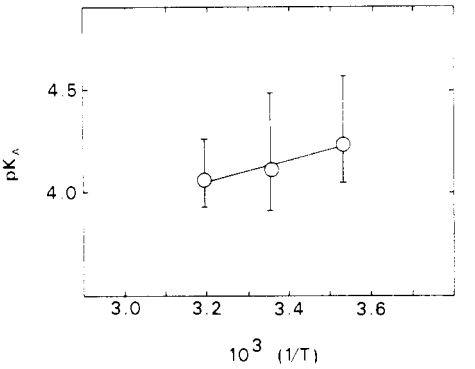

FIGURE 5: Plot of the pK_a value determined at 300 nm for RNase T_1 vs. reciprocal absolute temperature. Experiments were conducted in standard buffer. The difference spectrum for a given pH was determined at 10, 25, and 40 °C. The pHs of the solutions were measured at the same temperatures. The concentration of RNase T_1 was 5.51×10^{-5} M. Other conditions were as described under Methods. The line is a linear least-squares best fit to the data. Error bars represent standard deviations.

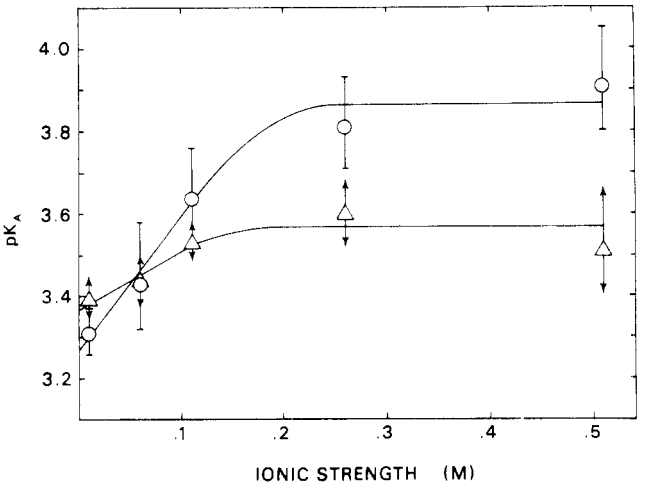

FIGURE 6: Plot of pK_a determined at 300 and 282.8 nm for RNase T_1 vs. ionic strength. (○) pK_a values from data at 300 nm; (△) pK_a values from data at 282.8 nm. Error bars represent standard deviations. The lines have no theoretical meaning. RNase T_1 concentration was 6.3×10^{-5} M. Temperature was 25 °C. Other conditions are described under Methods.

ionic-strength effects on pK_a values are shown in Figure 6. At low ionic strengths (i.e., at 0.01, 0.06, and 0.11 M) pK_a values showed a linear relationship with ionic strength. The slope in this range for data at 300 nm was ca. two times greater than that for data at 282.8 nm, whereas the extrapolated pK_a values,

which possibly represent pK_a at zero ionic strength, were 3.26 and 3.37 for data at 300 and 282.8 nm, respectively. In experiments where either the ionic strength or the temperature was varied the values of $\Delta\epsilon$ did not systematically vary and deviated less than 8% from those determined at 25 °C in the standard buffers. In addition, the qualitative nature of the difference spectra did not significantly change under these conditions.

Temperature-jump relaxation kinetic studies conducted with RNase T_1 at pH 4.5 in the standard buffer, at either 10 or 25 °C, indicated that absorption changes associated with enzyme ionization (i.e., decreased absorption at 286 nm and increased absorption at 300 nm) are very fast and occur during the 5.5 °C heating pulse (i.e., $\tau < 8$ μs).

Discussion

The pH-induced difference spectra of RNase T_1 can be interpreted as resulting from changes in the ultraviolet adsorption properties of protein chromophores caused by the titration of two dissociable groups on the enzyme. The titration of one group, having an apparent pK_a of 3.54, causes a difference spectrum that predominates from 270 to 285 nm, and the other, having an apparent pK_a of 4.29, yields a difference spectrum that occurs from 285 to 310 nm. The component of the difference spectra characterized by a pK_a of 3.54 appears to be identical with that of the pH-induced difference spectra for CM-RNase T_1 for the following reasons: (1) Approximately the same maxima (at ~283 nm) and minima (at 286–287 nm) appear in both difference spectra, and (2) the values of pK_a and $\Delta\Delta\epsilon_0$ characterizing the RNase T_1 and CM-RNase T_1 difference spectra were almost identical for $\Delta\Delta\epsilon$ measured between comparable wavelengths. Since a significant portion of the RNase T_1 difference spectrum above 285 nm does not appear in that for CM-RNase T_1 (see Figure 1), it can be concluded that this component of the difference spectrum results almost exclusively from the titration of the γ-carboxyl group of Glu-58. Considering that virtually no difference absorbance was observed for CM-RNase T_1 at 300 nm, the data at this wavelength for RNase T_1 is deemed as exclusively representing the titration of Glu-58 which yielded an average pK_a value of 4.2 ± 0.2 at 25 °C using the standard buffers. Further evidence that the dissociation process monitored at 300 nm is indeed a carboxyl group was found in the values of $\Delta H°$ and $\Delta S°$ obtained from the data at 300 nm (see above) which agree with those previously reported for carboxyl ionizations (Cohn and Edsall, 1943).

The present results can be correlated with previous studies of RNase T_1 fluorescence as a function of pH where an increase in tryptophanyl fluorescence from pH 2 to 5 was characterized by an estimated pK_a of ~4.0 and an increase in tyrosyl fluorescence over this pH range was associated with an estimated pK_a of ~3.5 (Pongs, 1970). The observations that the apparent pK_a for titration of Glu-58 (i.e., 4.2) is similar to that estimated from the change in tryptophanyl fluorescence and that this dissociation causes absorption changes in the range 285 to 310 nm (Donovan et al., 1961; Ananthanarayanan and Bigelow, 1969) suggest that the pH-induced difference spectrum caused by γ-carboxyl ionization of Glu-58 results from a change in the absorption properties of the single Trp-59 residue. In addition, it was also suggested that the *increase* in tryptophanyl fluorescence caused by carboxyl ionization is due to the interaction of the carboxylate group (i.e., of Glu-58) with an imidazolium residue which was quenching tryptophanyl fluorescence (Pongs, 1970). A charge pair involving the γ-carboxylate of Glu-58 and an imidazolium group was previously evidenced from NMR studies (Ruterjans and Pongs, 1971) and was proposed to constitute part of an interaction site for the 2′-hydroxyl group of guanosine (Walz, 1976).

The dramatic effect of ionic strength on the pK_a value for Glu-58, particularly when compared with that for the other (putative) carboxyl group monitored at 282.8 nm (see Figure 5), is consistent with the view that this residue is adjacent to a cationic residue. The observation that the apparent pK_a values in the standard buffer (and ten times diluted standard buffer) were consistently greater when compared at similar ionic strengths with the simpler, dilute acetate–lactate buffer is currently not understood but could be related to the presence of Tris cation. In any event, these results are consistent with the proposal that an electrostatic interaction exists between Glu-58 and an active-site His residue. The formation of this putative charge pair and the resulting alteration of trytophanyl absorption properties probably do not entail large conformational changes, since the ionization-induced absorption changes are very rapid, as indicated by the temperature-jump kinetic experiments.

Steady-state kinetic studies of RNase T_1 using dinucleoside monophosphate substrates implicated a catalytic role for an ionized group having a pK_a of 4.15 ± 0.3 on the free enzyme (Zabinski and Walz, 1976; Osterman and Walz, in preparation); this value agrees very well with the pK_a value for ionization of Glu-58 which was determined under the same conditions in the present study. The results of kinetic experiments using GpU as a substrate for RNase T_1 (Osterman and Walz, in preparation) and binding studies of the enzyme with guanosine (Walz, unpublished experiments), which were conducted with ten times dilute standard buffer, indicated that the apparent pK_a for an enzyme group involved in these processes shifted from ~4 in the standard buffer to ~3.5 in the dilute buffer. In light of the present work, these findings further corroborate the role of Glu-58 in substrate binding (Walz, 1976) and catalysis (Takahashi, 1970; Zabinski and Walz, 1976). The discrete interaction of carboxyl, imidazole, and tryptophan groups at the active site of RNase T_1 will be the subject of future research.

Acknowledgment

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