PICOSECOND TIME-RESOLVED FLUORESCENCE OF RIBONUCLEASE T1

A pH and Substrate Analogue Binding Study

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ABSTRACT The tryptophyl fluorescence of ribonuclease T1 decays monoexponentially at pH 5.5, τ = 4.04 ns but on increasing pH, a second short-lived component of 1.5 ns appears with a midpoint between pH 6.5 and 7.0. Both components have the same fluorescence spectrum. Acrylamide quenches both fluorescence components, and the short-lived component is quenched fivefold faster than the predominant long component. Binding of the substrate analogue 2'-guanylic acid at pH 5.5 quenches the fluorescence by 20% and introduces a second decay component, τ = 1.16 ns. Acrylamide quenches both tryptophyl decay components, with similar quenching rates. The fluorescence anisotropy decay of ribonuclease T1 was consistent with a molecule the size of ribonuclease T1 surrounded by a single layer of water at pH 7.4, even though the anisotropy decay at pH 5.5 deviated from Stokes–Einstein behavior. The fluorescence data were interpreted with a model where the tryptophyl residue exists in two conformations, remaining in a hydrophobic pocket. The acrylamide quenching is interpreted with electron transfer theory and suggests that one conformer has the nearest atom ~3 Å from the protein surface, and the other, ~2 Å.

INTRODUCTION

The much improved precision and resolution of fluorescence techniques in the time and frequency domain (1, 2) has encouraged new interest in the use of tryptophan as a probe of protein structure and dynamics. The photophysics of tryptophan is complex (3–6), and although progress has been made in resolving individual contributions in two tryptophan-containing proteins (7, 8), it seems appropriate to concentrate initial efforts on single tryptophan proteins. In this paper we report studies on ribonuclease T1 (RNase T1), a single tryptophan-containing protein from *Aspergillus oryzae,* which has been widely studied.

RNase T1, discovered by Sato and Egami (9), is a single-chain protein with 104 amino acids. At neutral pH, it performs a specific two-stage endonucleolytic cleavage of single-stranded RNA to 3'-phosphomono and oligonucleotides ending in Gp, with 2',3'-cyclic phosphate intermediates (10). The primary structure of the enzyme was first determined in 1965 (11) and was refined recently (12). The enzyme contains a single tryptophan, nine tyrosines, and four phenylalanines (12). A crystal structure for the 2'-guanylic acid (2'-GMP) inhibitor complex of RNase T1 is known at 2.5-Å resolution (13). Among many important residues in the molecule, glutamate 58, histidine 92 and 40, and arginine 77 are directly involved in the substrate binding site (10, 13). Tryptophan 59 in RNase T1 is next to Glu 58 in the primary sequence, but the indole ring of tryptophan is on the side of the molecule opposite from the binding site. Although there is no evidence to show that tryptophan 59 is directly involved in the substrate binding, its integrity is essential for folding the peptide chain into the active position (10). The x-ray structure of the RNase T1–2'-GMP complex (12) indicates that the tryptophan is "buried" in the core region of the molecule but the side chain is near the periphery and surrounded by hydrophobic residues, including proline, tyrosine, and phenylalanine. Because of the critical role of RNase T1 in nucleotide sequencing, this enzyme has been studied extensively (10, 14–16). The fluorescence properties of RNase T1 have been studied by steady-state measurements (17, 18) as well as time-resolved measurements (19–21). Grinvald and Steinberg (19) obtained fluorescence decay times of 3.3 and 1.5 ns, respectively. Eftink and Gihron (22–24) studied fluorescence quenching of RNase T1 by acrylamide and measured the fluorescence decay time for the single tryptophan in the protein and fitted the decay with a single exponential with a lifetime of 3.5 ns. Gratton et al. (20) recently measured the fluoresence decay by frequency modulation; the results showed that the fluorescence decay at 4.9°C might be more complex than a triple exponential decay. While our own studies were in progress, James et al. (21) reported their results on fluorescence decay and anisotropy for RNase T1 at the single pH of 5.5 At this pH, a single exponential decay was observed with a lifetime

of 3.87 ns. The anisotropy measurements by Lakowicz et al. (25) and Eftink (26) gave a rotational correlation time of 5–6 ns at 25°C, whereas James et al. (21) obtained a value of ~7 ns. In this paper, we describe the pH dependence of the fluorescence decay, anisotropy of tryptophan in RNase T1, and the effect of substrate analogue binding on the fluorescence properties of the enzyme. Based on our experimental results, we propose a two-state model for tryptophan in RNase T1 at neutral pH, which is supported by the acrylamide quenching experiments. Finally, we present a schematic diagram of tryptophan residue dynamics in RNase T1.

MATERIALS AND METHODS

The RNase T1 was a gift of Professor F. G. Walz of Kent State University, prepared by the method of Hooverman (27). Gel electrophoresis exhibited a single band, and the UV absorption spectrum showed that there was no observable contamination. Acrylamide was obtained from Sigma Chemical Co. (St. Louis, MO) and was recrystallized from ethylacetate. tRNA, 2'-GMP, and Trisma salt were purchases from Sigma Chemical Co. and were used without further purification.

The concentration of the protein was determined from UV absorption at 278 nm with a Lambda-5 spectrophotometer (Perkin-Elmer Corp., Norwalk, CT; ϵ_{278} = 21,200 M^{-1} cm^{-1}, Takahashi [28]). Experiments were carried out at an RNase T1 concentration of 2×10^{-5} M.

The enzymatic activity was assayed by monitoring the absorption changes at 260 nm during the 20-min incubation of the same enzyme solution with tRNA at pH 7.4, 37°C, before and after 10 h of irradiation. No obvious difference between the change in A260 resulting from the production of soluble nucleotides was found for the two assays. In addition, over a long period of exposure to laser excitation at 295 nm, the lifetime measurement results for RNase T1 in solution remain unchanged. These results eliminate the role of any significant photodegradation of the protein during the course of our measurements.

The acrylamide fluorescence quenching experiments were performed by adding a small volume of 7 M acrylamide solution to the known volume protein solution. The protein was dissolved in 0.01 M Tris-HCl buffer, and the pH of the solution was adjusted by adding HCl.

The amount of inhibitor 2'-GMP required to bind 99% of enzyme was calculated using a dissociation constant, K = 6.5 μM (29). A threefold excess of that amount of 2'-GMP was added in the enzyme–inhibitor complex measurements.

The fluorescence lifetime and anisotropy measurements were performed with a time-correlated single photon counting technique as described in detail elsewhere (1). The excitation was set at 295 nm for all the measurements to avoid excitation of the tyrosine in the protein. A repetition rate of 75.7 KHz was used. The laser was frequency doubled with a $LiIO_3$ crystal, and residual visible light was filtered by a UG-11 filter (Schott America Glass & Scientific Products, Inc., Yonkers, NY). The fluorescence was collected through a sheet polarizer with the appropriate orientation for parallel, perpendicular, or magic angle of 54.7° from the vertical excitation polarization. The fluorescence signals were detected with a microchannel plate (model F4129; ITT Electro Optical Products Div., Fort Wayne, IN). To prevent contamination of the fluorescence signal with scattered light, J-Y H10 (Instrument SA Inc., Metochen, NJ) ion-etched holographic grating monochromater with 8-nm pass band centered at 340 nm and Schott W335 cutoff filters were placed in front of the microchannel plate. A control experiment using only buffer was run for the same amount of time required to collect the sample signal. The maximum signal from scattering was <20 counts. Even the non-dairy creamer solution, which is the scattering light source for the instrument response function, has only about a couple hertz average count rate at 340 nm under our experimental conditions via an average count rate of 1.5 KHz for the protein sample. Thus scattering can be neglected in our experimental results. The instrument function in the experiment was ~90 ps full width half maximum, and a time scale of 23.5 ps/channel was used on the multichannel analyzer. The anisotropy measurements were performed by collecting the fluorescence with parallel and perpendicular analyzer orientation separately. Scaling was carried out with an intensity integrator that monitored a portion of the excitation beam separating from excitation radiation beam splitter (30). Details of the data analysis for the fluorescence decay and anisotropy are given by Cross and Fleming (30). A nonlinear least-square fitting method was used. The quality of the fit was judged by reduced χ^2 and by the runs criterion.

RESULTS

pH Dependence of the Fluorescence Lifetimes of RNase T1

The fluorescence lifetimes of RNase T1 in 0.01 M Tris-HCl buffer solution are dependent on the pH of the solution. pH changes alter both lifetimes and the amplitude of the components (see Table I). Between pH 5.5 to 8.5 the fluorescence decay undergoes a transition from single to double exponential decay. The short-lifetime component increases slightly, while the long lifetime decreases over this pH interval. The midpoint for the transition is at pH 6.5–7.0, where the amplitude of the second component changes most drastically. No data for double exponential fits at pH 6.0 and 5.5 are shown in the table. When the shorter lifetime component has a weight of <5%, our method of data analysis does not give reliable values.

Several previous measurements of the RNase T1 fluorescence decay have been made and are summarized in Table II. Our results at pH 7.4 are similar to those of Grinvald and Steinberg (19). Our data at pH 5.5 are very similar to those of James et al. (21). It is possible that the short-lifetime component observed at pH 6.5 is due to an impurity or the product of photodegradation. However, when we used the same sample to titrate from pH 8.5 to 5.5, the transition from double to single exponential decay was clearly evident. Thus the short-lifetime component appears to be an intrinsic property of the protein rather than an impurity or a photodegradation product. Also no

TABLE I
pH DEPENDENCE OF THE FLUORESCENCE DECAY RESULTS OF RNase T1

	Single exponential		Double exponential			
pH	τ	χ^2	A_1	τ_1	τ_2	χ^2
	ns		%	*ns*	*ns*	
5.5	4.04 ± 0.01	1.12	—	—	—	—
6.0	4.02 ± 0.01	1.21	—	—	—	—
6.5	4.00 ± 0.01	1.32	95.5	4.06 ± 0.01	1.0 ± 0.1	1.01
7.0	3.85 ± 0.01	1.36	90.0	3.88 ± 0.01	1.4 ± 0.1	1.11
7.4	3.81 ± 0.01	1.41	90.8	3.95 ± 0.01	1.6 ± 0.1	1.05
8.0	3.62 ± 0.01	1.60	89.1	3.78 ± 0.03	1.5 ± 0.1	1.04
8.5	3.59 ± 0.01	1.52	91.3	3.71 ± 0.01	1.2 ± 0.1	1.03

All measurements were at 20°C in 0.01 M Tris buffer.

TABLE II
FLUORESCENCE DECAY RESULTS OF RNase T1

τ_1	A_1	τ_2	A_2	τ_3	A_3	pH	T	Reference
ns	%	*ns*	%	*ns*	%		°C	
3.3	*	1.5	*			—	r. t.	19
3.5	100					7.0	20–25	22
3.96	92.4	0.49	7.6			—	4.9	20
4.24	82.5	1.76	14.0	0.064	3.6	—	4.9	20
3.87	100					5.5	25	21
4.04	100					5.5	20	This paper
3.95	90.8	1.6	9.2			7.4	20	This paper

*Indicates amplitude was not specified.
—, Indicates pH was not specified.

difference in enzymatic activity was found after completion of a fluorescence decay measurement.

Tryptophanyl Fluorescence Quenching with Acrylamide

In the presence of an external fluorescence quencher, the fluorescence decay of a chromophore in the protein can be expressed in terms of the Stern–Volmer equation (21):

$$\tau_0/\tau = 1 + K_{sv}[Q] = 1 + k_q\tau_0[Q], \qquad (1)$$

where K_{sv} is the Stern–Volmer constant in M^{-1}, k_q is the quenching rate constant in $M^{-1}s^{-1}$, and τ_0 and τ are the fluorescence lifetimes without and with external quencher.

Fluorescence quenching experiments with acrylamide were performed at pH 5.5 and 7.4. At pH 5.5, the fluorescence decay of tryptophan residue is monoexponential. The acrylamide quenching at 20°C gives $K_{sv} = 0.675$ M^{-1} and $k_q = 1.69 \times 10^8$ $M^{-1}s^{-1}$. This value of k_q is much smaller than that of free indole derivatives, which is $\sim 7 \times 10^9$ $M^{-1}s^{-1}$ (23) and implies that the tryptophan is buried in the protein. At pH 7.4, the fluorescence decay for the tryptophan residue in the protein is double exponential. As shown in the Stern–Volmer plot (Fig. 1), the acrylamide quenching rates for the two components are very different. For the longer lifetime component, K_{sv1} is 0.592 M^{-1} and $k_{q1} = 1.55 \times 10^8$ $M^{-1}s^{-1}$. For the shorter lifetime component, $K_{sv2} = 1.455$ M^{-1}, and $k_{q2} = 8.89 \times 10^8$ $M^{-1}s^{-1}$. Acrylamide quenches the short-lifetime component about six times faster than it does the long life-time component. It is also interesting to notice that the preexponential factors of the two components are approximately constant over the range of quencher concentrations used in the experiment. Since neither impurity nor photodegradation causes the appearance of the second lifetime component, the results from the quenching experiment at pH 7.4 produce further confirmation of the existence of an intrinsic short-lifetime component. An impurity should react with the quencher in a different way from RNase T1, and a photodegradation product will increase during the course of the experimental process; in either case, the ratio of the weights for the two components will change, which was not observed.

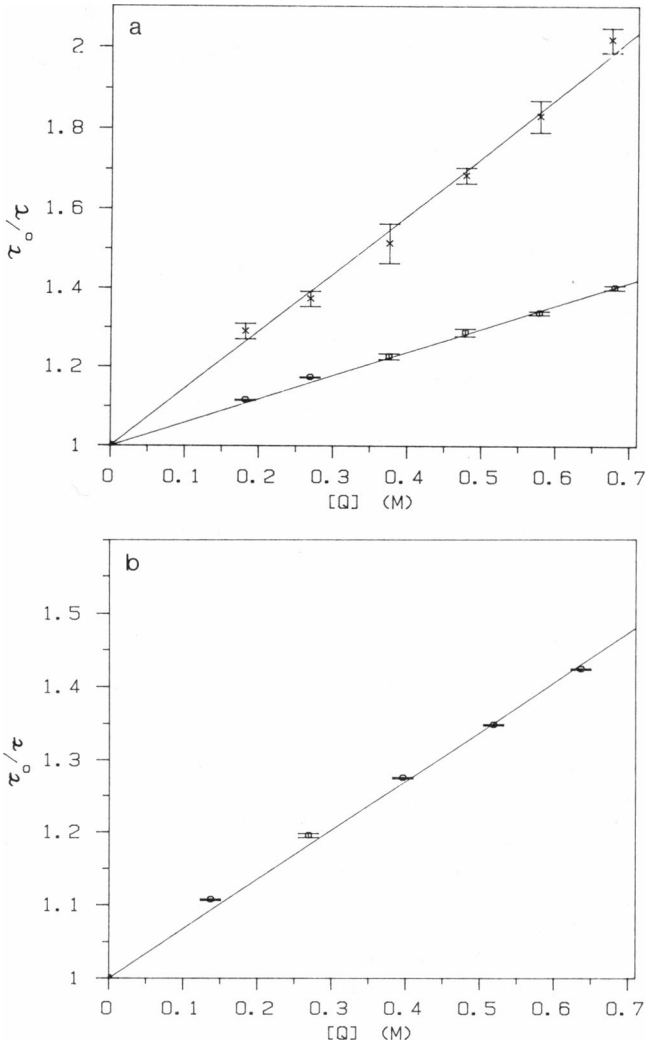

FIGURE 1 (*a*) Stern–Volmer plot of RNase T1 quenched by acrylamide, 20°C, pH 7.4, 0.01 M Tris-HCl buffer. ×, the short-lifetime component; O, the long-lifetime component. (*b*) pH 5.5.

The acrylamide quenching rate k_q at pH 5.5 is ~9% larger than that of the long-lifetime component at pH 7.4. Since the differences are significantly larger than the experimental error, the above results indicate that the tryptophan local environment in RNase T1 at pH 5.5 is similar to that of the long-lifetime component at pH 7.4 but is more exposed to the quencher. This similarity was also consistent with the fact that little alteration of steady-state fluorescence and UV absorption spectrum was observed between the two pH values.

Apoazurin from *Alcaligenes denitrificans* is a protein that contains two tryptophans, with one largely exposed and the other completely buried. The fluorescence decay measurements of Petrich et al. (8) showed that the ratio of the fluorescence decay components corresponding to the

two tryptophans changed drastically as the fluorescence was collected at different wavelengths. Therefore, if the two components in RNase T1 have different exposures to water, the relative weight of the short-lifetime component should increase at longer emission wavelength. To test this assumption, we conducted the measurements of fluorescence decay at different wavelengths from 320 to 380 nm. Unconstrained fits result in the weight of the short-lifetime component increasing from ~10 to 16% over the wavelength range 320–380 nm, but at the same time the lifetime of this component increases from 1.2 to 1.9 ns. In interpreting these small changes, the possibility of compensation between the lifetime and the weight of the component must be considered (31). If the lifetimes of the two components were fixed and the weights of the two components were allowed to change, the change in the relative weight of the two components at different emission wavelengths were within the experimental error. Therefore, there is no evidence for a wavelength dependency in the relative weight of the two components.

Effect of Substrate Analogue Binding to RNase T1

The enzyme–substrate complex for RNase T1 is not stable enough at room temperature to permit fluorescence decay studies. Stable inhibitor complexes between RNase T1 and 2′- or 3′-guanosine monophosphate were found by Sato and Egami (32). The binding of these inhibitors to RNase T1 was confirmed through UV absorption difference spectra and gel-filtration studies. The RNase T1–2′-GMP complex was chosen in this study for the following reasons: (*a*) the x-ray coordinates are available for the complex; (*b*) kinetic experiments show that the substrate analogue binds at the same site as the substrate. Therefore, the properties of the substrate analogue–enzyme complex are expected to be similar to those of the substrate–enzyme complex; and (*c*) 2′-GMP has the highest affinity for the enzyme, therefore only a small amount of 2′-GMP is required to ensure complete binding. Larger amounts of 2′-GMP will absorb significant amounts of light at the excitation wavelength. A pH of 5.5 was used, because at pH 7.5, the optimum pH for RNA digestion, the affinity of 2′-GMP to RNase T1 is much lower.

The fluorescence quantum yield of the complex is ~80% of that for pure enzyme (18). The fluorescence decay of the complex is a triple exponential instead of a single exponential in the pure enzyme. There was a substantial amount of very short-lifetime component (τ = 20–30 ps). This short component was not due to scattered light, as it was absent in a blank buffer comparison experiment. However, the 2′-GMP in the same buffer, without enzyme, gives an apparent fluorescence lifetime of ~30 ps. Fluorescence from 2′-GMP is therefore probably the predominant source of the very short-lifetime component. The remaining fluorescence decay of the complex can be expressed as a double exponential with $\tau_1 = 3.37 \pm 0.02$ ns, $\tau_2 = 1.16 \pm 0.1$ ns and weights of ~90 and 10%, respectively.

Tryptophyl fluorescence quenching by acrylamide on the RNase T1–2′-GMP complex showed that the fraction of the two longer components at different quencher concentrations varies by ~2%. Since the presence of 2′-GMP fluorescence creates difficulties in the accurate determination of the smaller weighted 1-ns component, the data for this component should be treated with caution. The quenching rates for the 3.3- and 1-ns components are $K_{sv1} = 0.544\ M^{-1}$, $k_{q1} = 1.61 \times 10^8\ M^{-1}s^{-1}$, and $K_{sv2} = 0.226\ M^{-1}$, $k_{q2} = 1.95 \times 10^8\ M^{-1}s^{-1}$, respectively. The average quenching rate is $K_{sv} = 0.553\ M^{-1}$ and $k_q = 1.75 \times 10^8\ M^{-1}s^{-1}$. The Stern–Volmer plot for RNase T1–2′-GMP is shown in Fig. 2.

The binding of 2′-GMP to RNase T1 at pH 5.5 causes three principal changes: (*a*) reduction of the fluorescence lifetime of Trp in the protein from 4 ns to an average lifetime of 3 ns; (*b*) generation of nonexponentiality in the fluorescence decay of Trp similar to that observed at pH 7; and (*c*) a slight (~4%) increase in the average value of k_q. The reduction of the fluorescence lifetime of the long component is very likely due to quenching by bound 2′-GMP through electron or energy transfer. An increase in the concentration of 2′-GMP above the value required for saturation of binding does not change the fluorescence lifetime of tryptophan in RNase T1–2′-GMP. Thus, the excess free 2′-GMP merely screens absorption of tryptophan at 295 nm and binding is necessary for 2′-GMP to influence the tryptophan fluorescence decay.

Fluorescence Anisotropy of RNase T1

The fluorescence anisotropy of a single fluorophore-containing protein can be expressed in the following equation (33):

$$r(t) = r_1(0) \exp(-t/\tau_1) + r_2(0) \exp(-t/\tau_2). \quad (2)$$

The flexibility of the fluorophore can be described by the order parameter, S^2, which is defined as (30–34),

$$S^2 = \frac{r(t)}{r(0)} e^{t/\tau_r} = \frac{r(0^+)}{r_{eff}(0)}. \quad (3)$$

τ_r and $r(0^+)$ are the parameters describing the long-time behavior; $r_{eff}(0)$ is the effective initial anisotropy measured in the experiment (33). If the motion of the dipole moment of the fluorophore can be described as a vector moving in a cone with a semiangle θ_0, the following relation holds (34),

$$S = \tfrac{1}{2} \cos\theta_0 (1 + \cos\theta_0). \quad (4)$$

The fluorescence anisotropies of RNase T1 were measured at both pH 7.4 and 5.5, respectively. Up to 28,000 counts in the peak channel were accumulated in some of the measurements. All anisotropy results gave a satisfac-

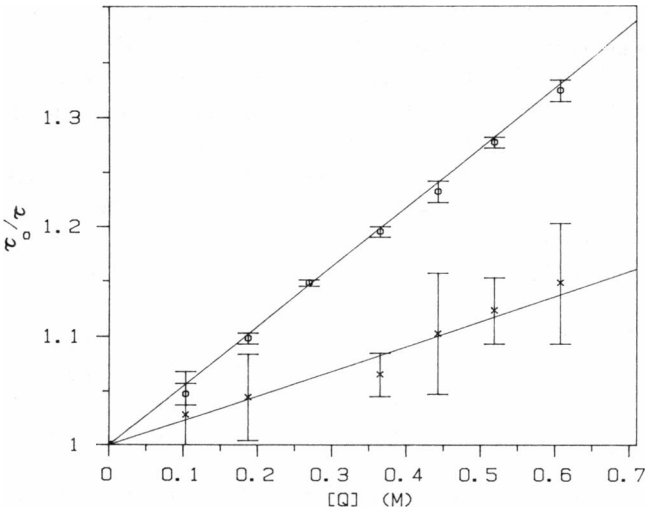

FIGURE 2 Stern–Volmer plot of RNase T1–2′-GMP quenched by acrylamide. 20°C, pH 5.5, 0.01 Tris-HCl.

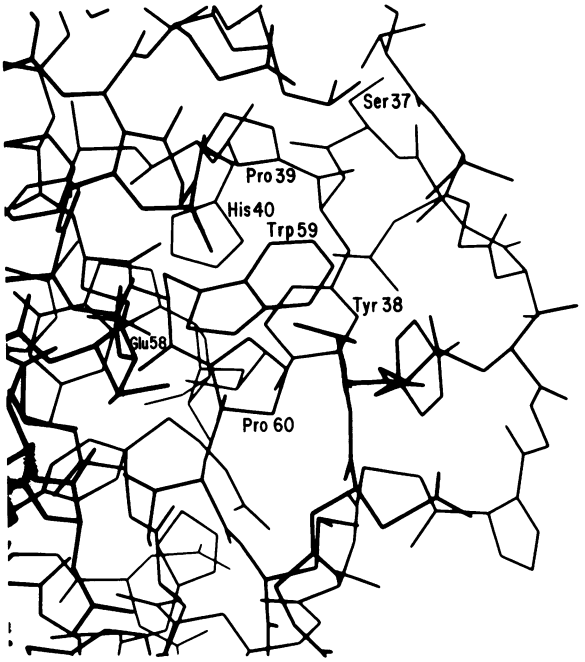

FIGURE 3 The x-ray structure of the environment around Trp 59 in RNase T1–2′-GMP (from the coordinates of reference 13).

tory fit to a single exponential decay. Attempts to reach higher counts in the peak channel produced distortions of the decay data, resulting from long-term instabilities in the laser and detection electronics.

To evaluate the detectability of a second component in the fluorescence anisotropy of RNase T1, we carried out some simulations. The simulations showed that for an anisotropy decay that contains 80% of a 5-ns component and 20% of a 100-ps component, the lowest peak count required to have χ^2 as large as 1.4 is ~50,000 counts.

Inspection of the x-ray structure of RNase T1–2′-GMP showed the region around the tryptophan is very crowded. Using an Evans and Sutherland graphics system and the x-ray coordinates of RNase T1–2′-GMP (See Fig. 3), we found that the tryptophan side chain could only move ~10° or less in either its χ_1 or χ_2 angle without colliding with other groups. If this residue is assumed to move in a cone with semiangle 10°, S^2 will be 0.96, which is undetectable with our current precision. Our failure to detect internal motion of the tryptophan is not unreasonable since large amplitude motion of the tryptophan must involve not only a change of the local environment, but also larger scale motion. This may require a partial refolding and the time scale may be beyond the time window set for fluorescence anisotropy measurement by the fluorescence decay time. Our results are in agreement with previous studies that all report a single exponential anisotropy decay (21, 25, 26).

The shape of the RNase T1 molecule is a distorted tetrahedron, with all three dimensions close to 31 Å. Therefore, the molecule can be considered as an approximately spherical molecule with a volume of ~1.6×10^4 Å^3. The fluorescence anisotropy results for RNase T1 at pH 5.5 and 7.4 are shown in Table III. Based on the Stokes–Einstein hydrodynamics law, $\tau_r = V_h\eta/k_BT$, where τ_r is the rotational correlation time, V_h, the hydrated volume, η, the viscosity of the solution, k_B, the Boltzmann constant, and T, the temperature in Kelvin. The last column of Table III gives the rotational correlation time for an unhydrated sphere with a volume of 1.6×10^4 Å^3. The experimental values of τ_r are always longer than these calculated values. A plot of τ_r vs. η/T is shown in Fig. 4. At pH 7.4, the experimental results fall on a straight line corresponding to a hydrated volume of 2.1×10^4 Å^3 or a hydrated radius of 17 Å, 1.5 Å larger than the estimated radius without hydration. In contrast, the results of τ_r at pH 5.5 do not fall on a straight line. The τ_r values at 5° and 20°C deviate from the values at pH 7.4 by a considerable amount. At pH 7.4, RNase T1 follows the Stokes–Einstein law well, with no detectable change in hydrated volume over a rather wide temperature range (5°–46°C). The hydrated volume is

TABLE III
FLUORESCENCE ANISOTROPY RESULTS OF RNase T1

pH	T	η/T	r_0	τ_r (Measured)	τ_r (Calculated)
	°C	10^{-3} kgm^{-1}s^{-1}K^{-1}		ns	ns
7.4	5.0	5.462	0.21 ± 0.01	8.1 ± 0.6	6.2
	20.0	3.430	0.21 ± 0.01	5.3 ± 0.4	3.9
	35.0	2.346	0.21 ± 0.01	3.3 ± 0.1	2.7
	44.5	1.905	0.18 ± 0.01	2.8 ± 0.2	2.2
5.5	5.0	5.462	0.26 ± 0.01	11.6 ± 0.2	6.2
	20.0	3.430	0.24 ± 0.01	6.0 ± 0.4	3.9
	33.0	2.458	0.21 ± 0.01	3.5 ± 0.3	2.8
	46.0	1.844	0.20 ± 0.04	2.7 ± 0.1	2.1

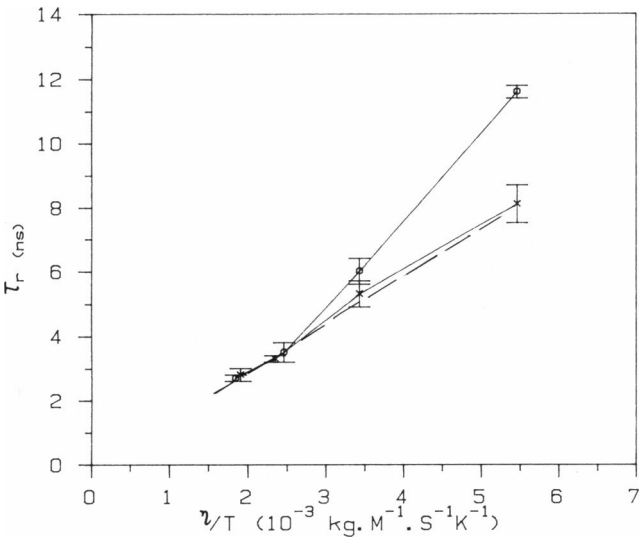

FIGURE 4 Rotational correlation time τ_r of RNase T1 vs. η/T. ×, pH 7.4; O, pH 5.5.

approximately that of the protein molecule with a single layer of water molecules on the surface. However, at pH 5.5 the protein deviates from the Stokes–Einstein law with its apparent hydrated volume changing over the temperature interval in the experiments. The longer τ_r at pH 5.5 at the lower temperatures might arise because of the binding of extra water molecules or from partial aggregation of RNase T1. At 5°C and pH 5.5, τ_r corresponds to a hydrated volume that is twice that of the unhydrated volume for a single RNase T1 molecule. Typically, protein aggregation occurs at a concentration as low as 10^{-4} M, but the experiments were conducted at 2×10^{-5} M. Therefore, only a partial aggregation effect is likely. Since RNase T1 bears more charges at pH 5.5 than at pH 7.4, increased binding of water at pH 7.4 and at 5°C probably also contributes to the great volume of the protein.

DISCUSSION

The fluorescence data described above have two principle points of interest for discussion: (*a*) the nature of the pH-induced transition in RNase T1 and its connection (if any) with substrate binding and enzymatic activity, and (*b*) the connection between the time-resolved anisotropy studies and the acrylamide quenching experiments, and their implications for protein mobility studies.

RNase T1 has optimum activity at neutral pH, but has higher affinity for inhibitors such as 2′-GMP at pH 5. The change in tryptophyl fluorescence observed in binding 2′-GMP at pH 5.5 is very similar to that observed on increasing the pH to 7.5 in the free enzyme. Although highly speculative, it is possible that an equilibrium between two configurations (see below) similar to those existing at pH 7.4 might be necessary for enzymatic activity.

The fluorescence quantum yield and spectrum have been investigated as a function of pH near neutral pH and show little dependence on pH (18). A small but distinct inflection in fluorescence intensity occurs at pH 7.5 (35), which was associated with a salt bridge involving Glu 58 and Arg 77. The aromatic amino acid ultraviolet absorption (36) and circular dichroic spectra (37) show little change near neutral pH values. These studies suggest only subtle changes in structure occur between pH 5 and 8 in RNase T1.

In intepreting the fluorescence decay data we consider three hypotheses. (*a*) At higher neutral pH the protein exists in two distinguishable conformations, whereas at pH 5.5 there is a single conformation (the possibility of a broad distribution of conformations is discussed below). (*b*) There are two conformations at all pH values, but they only become distinguishable when the effectiveness of an internal quencher is enhanced after deprotonation of specific residues. (*c*) A single, distinct conformation exists at both pH values but they differ in solvent accessibility.

To discuss these points we turn to our previous study of buried and exposed tryptophyl residues in a series of homologous azurins (8). The buried tryptophan (W48) in apoazurin from *Pseudomonas aereginosa* exhibits single exponential decay, whereas the exposed tryptophan (W118) in apoazurin from *Alcaligenes faecalis* has a fluorescence decay that fits well to a sum of two exponentials. The W48 fluorescence is considerably blue shifted from that of W118. The blue fluorescence from W59 in RNase T1 and the lack of any pH-dependent spectral shifts argue against the third proposal. The acrylamide quenching experiments argue for the first proposal over the second proposal, since if the two configurations have substantially different quenching rates at pH 7.4 it seems likely that they will behave accordingly at pH 5.5. We thus conclude that the most likely explanation for our data is that W59 in RNase T1 can occupy two different environments at pH 7.5. The distinction between the two environments is most likely related to the separation of W59 from an internal quencher such as a peptide bond or other effective electron acceptors (4, 8). Naturally, both W59 and the internal quencher could change position in the two environments.

Sugio et al. (39) report a crystal structure for RNase T1–2′-GMP at pH 4.0, which shows little difference in folding pattern and side-chain conformation from the structure at pH 5.3. Yet there are subtle changes in side-chain conformation from the pH 5.3 structure, which accompany a significant difference in inhibitor conformation and the nature of the H-bonds it makes with the protein side-chains. Subsequently, Sugio et al. (39) solved the crystal structure for a bound substrate, 3′-GMP, at pH 4.0 where the enzyme is inactive. Again the overall pattern of the backbone folding remains strikingly similar to the inhibitor complexes. Now there is a shift in the position of a surface loop, adjacent to the active site region. Sugio et al. (39) suggest that the disorder they observed in the ribose-

3-phosphate moeity of the substrate arises from binding of different substrate conformers and is associated with subtle differences in protein side-chain conformation. Clearly, changes in the ionization state of side-chains causes small, but distinct changes in the structure of RNase T1.

Hershberger et al. (40) investigated the triplet states of the tryptophyl residue of RNase T1 at pH 7.5 in a polyalcohol-water glass solution with optical detection of magnetic resonance at 1.1 K. These workers found evidence of small, but definite inhomogeneity in the spectral features of the triplet states. They ascribed this to microheterogeneity of the environment caused by small conformation changes at the tryptophyl residue. The phosphorescence emission spectra at 1.1 K have the most resolved spectral detail in this survey of several polypeptides and proteins (40). The electronic origin of phosphorescence shifted 0.4 nm on altering the excitation from 280 to 295 nm, which was outside the estimated instrument resolution of 0.1 nm. The magnetic resonance line-widths for the two zero-field microwave absorptions, though among the narrowest found in proteins (50 and 135 MHz), were still significantly broadened compared with indole in a Shipol'skii matrix of indan (10 MHz). At large microwave power, a hole could be "burnt" into the magnetic resonance line, with a width of 7 MHz. This observation provides conclusive evidence for an inhomogeneously broadened magnetic resonance line. Hershberger et al. (40) conclude that there is a small heterogeneity in the immediate microenvironment of the single tryptophyl residue of RNase T1.

Although the fluorescence decay at pH 7.5 fits well to a double exponential, it is possible that a wide range of conformations exist, and with them a wide range of tryptophyl lifetimes. A recent discussion of this point has been given by Gratton et al. (20). To test this point more carefully we have studied the simulations of fluorescence decay of distributions of lifetimes. We used a rectangular distribution of fluorescence decay rate with mean lifetime k_0 and full width at half maximum of Δk. The form of the fluorescence decay is (Cross, A. J., unpublished results):

$$I(t) = \frac{2}{\Delta kt} \exp(-k_0 t) \sinh\left(\frac{\Delta kt}{2}\right). \quad (5)$$

Curves calculated according to this expression fit very well to a double exponential decay ($\chi^2 = 1.1$) after convolution with an instrument function and having Gaussian noise added. In the quenching experiments we found that the weights of the two exponential components were unchanged with quencher concentration. This is consistent with the distribution model only if all members of the distribution are quenched in the same way. In this case the whole distribution is merely shifted an amount $k_q[Q]$ in the k-domain. Differential quenching rates will change the distribution width and alter the "mimic" double exponential weights as the quencher concentration increases. Since this latter case seems more likely, we again interpret our results as favoring a two-state model for W59 at pH 7.5.

The most likely candidates for initiating a structural change in the pH range 6.5–7.0 are histidine residues. However, two of the three histidines in RNase T1 have unusually high pK values (H40, $pK_a = 7.92$, H92, $pK_a = 7.80$) (35, 41). The x-ray crystal structures (13, 38, 39) place the active site glutamate E58 between histidine H40 and arginine R77. It appears that the group(s) titrating between 6.5 and 7.0 are associated with a disruption of the salt bridges involving the active site glutamate E58 (35, 41).

Acrylamide Quenching and Protein Mobility

Acrylamide quenching of tryptophan fluorescence is often used to assess protein mobility (22–24, 41). Calhoun et al. (42), using oxygen quenching of tryptophyl phosphorescence, criticized the use of acrylamide quenching to determine protein mobility, though subsequently Ghiron and Eftink (24) pointed out that the mechanism of phosphorescence quenching is significantly different from fluorescence quenching. The spectral overlap between protein fluorescence and acrylamide is small, and the acrylamide absorption is also small, hence the mechanism of acrylamide singlet quenching is more likely to be an electron transfer than an electronic energy transfer (43). The steady-state and wavelength-dependent time-resolved spectral data indicate little, if any, difference in two components observed at pH 7.5, such as would be caused by different interactions with water. This makes it difficult to accept a sixfold increase in the ability of acrylamide to penetrate the protein, or to postulate sixfold greater mobility in the conformation giving the shorter lifetime. The fluorescence anisotropy measurements give no evidence of substantial internal mobility on the fluorescence time scale, although it must be noted that a nonexponential anisotropy associated with the shorter lifetime component only would be difficult to detect. A more likely explanation for the different quenching efficiencies, we propose, is the distance of the indole ring from the surface of the protein (or the acrylamide binding site) in the two conformations. In the crystal structure of the RNase T1–2′-GMP complex (13) the tryptophan is in the antiperpendicular conformation, and the shortest distance to the surface (from C_{32}) is ~3 Å. If we assume that the quenching process is electron transfer with a distance dependence of the form $\exp(-2\alpha R)$ where $\alpha = 0.72$ Å^{-1} (8), then R would have to decrease from 3 to 1.8 Å for a sixfold increase in rate. These remarks are not inconsistent with the finding (43) that there is only a weak acrylamide quenching of W48 in apoazurin *Pseudomonas aeruginosa* where the tryptophan is at least 3 Å from the surface (45). The studies of Hershberger et al. (40) and Sugio et al. (38, 39) indicate that small but definite differences in side-chain conforma-

tion can exist in the structure of RNase T1, and that these can be altered by changing the pH.

On the basis of these remarks it seems that, at least for blue fluorescing ("dry") tryptophans where no mobility is detectable on the fluorescence time scale, the acrylamide quenching technique cannot be used to distinguish protein mobilities but simply senses the distance of closest approach to the tryptophan of the acrylamide.

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