Fluorescence Lifetime Quenching and Anisotropy Studies of Ribonuclease T1†

Douglas R. James,‡ David R. Demmer,§ Ronald P. Steer, and Ronald E. Verrall*
Department of Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada
Received February 19, 1985

ABSTRACT: The time-resolved fluorescence of the lone tryptophanyl residue of ribonuclease T1 was investigated by using a mode-locked, frequency-doubled picosecond dye laser. The fluorescence decay could be characterized by a single exponential function with a lifetime of 3.9 ns. The fluorescence was readily quenched by uncharged solutes but was unaffected by iodide ion. These observations are interpreted in terms of the electrostatic properties of the amino acid residues at the active site of the protein, which would appear to restrict the access of solute species to the tryptophanyl residue. The temperature dependence of the fluorescence lifetime and anisotropy decay time could be rationalized in terms of a model which postulates a significant ordering of the solvent layer immediately surrounding the surface of the protein.

Ribonuclease T1 (RNase T1)[1] from *Aspergillus oryzae* is a monomeric enzyme composed of 104 amino acid residues with a molecular weight of 11 100 (Longworth, 1971). In nature, it digests RNA by cleaving the polynucleotide chain at the 3′-phosphodiester bridge of a guanosine base (Uchida & Egami, 1971). A very high degree of specificity for the binding of guanosine nucleotide derivatives has been observed and is thought to be due to the presence of His_{40}, His_{92}, Glu_{58}, and Arg_{77} in an appropriate geometrical arrangement near the active site (Takahashi, 1973). 3′-Guanosine monophosphate binds very strongly to the active site and acts as a competitive inhibitor to the digestion of RNA (Arata et al., 1979). There is no evidence for allosteric regulation of this enzyme.

Of the 104 amino acid residues which make up the polypeptide chain, 11 are tyrosinyl (Tyr) residues (Takahashi, 1971). However, there is some disagreement as to the exact number of Tyr residues, and several authors have stated that only nine are present (Longworth, 1971). In addition, there are four phenylalanyl (Phe) residues and one tryptophanyl (Trp) residue (Longworth, 1971). The tryptophanyl residue is at position 59 and is therefore very near to the active-site Glu_{58} (Takahashi, 1971). Although both Phe and Tyr absorb at excitation wavelengths less than ca. 290 nm, very little fluorescence is expected to arise from these residues because of efficient long-range energy transfer to tryptophan and to their intrinsically low fluorescence quantum yields (Longworth, 1968; Yamamoto & Tanaka, 1970). At excitation wavelengths greater than 295 nm, only Trp_{59} is expected to contribute to the fluorescence which is observed. RNase T1 is thus an ideal candidate for examining in detail the fluorescence properties of tryptophanyl residues.

The photophysical properties of Trp_{59} in RNase T1 have not been extensively investigated, but some data are available. Longworth (1971) has reported a value for the fluorescence quantum yield of 0.44. However, aqueous tryptophan was used as a standard and assumed to have a quantum yield of 0.20. More recent estimates of the fluorescence yield of tryptophan solutions are 0.14, and when this is taken into account, the value of the fluorescence quantum yield of Trp_{59} becomes 0.31. Various steady-state fluorescence quenching studies (O'Grady, 1984; Eftink & Ghiron, 1976, 1977) have indicated that Trp_{59} is partially buried in the protein but is accessible to acrylamide. The activation energy for acrylamide quenching has been reported to be 36 kJ/mol (Eftink & Ghiron, 1977). Eftink (1983) reported a single fluorescence decay lifetime of 3.6 ns determined by phase fluorometry, similar to the 3.7-ns lifetime reported by Lakowicz et al. (1983). However, these results are in disagreement with those of Grinvald & Steinberg (1976), who measured a double-exponential decay with lifetimes of 3.3 and 1.5 ns. Rotational correlation times derived from the decay of the fluorescence anisotropy have also been measured at 25 °C by Eftink (1983) and Lakowicz et al. (1983), with values of 5.2 and 5.9 ns, respectively, being reported.

In this paper, we report the results of extensive studies of solute and thermal quenching of RNase T1 and the temperature dependence of the fluorescence anisotropy decay. These results can be interpreted in terms of the nature of the residues which lie close to the tryptophanyl residue, due to the protein's tertiary structure, and they provide insight to the nature of the protein–solvent interface.

Materials and Methods

K_2HPO_4, KI, KCl, CsCl, and Na_2SO_3 were used as received. Acrylamide was recrystallized 3 times from ethyl acetate, dried, and stored under vacuum over P_2O_5. CsCl solutions exhibited weak emission due to an impurity, and experiments where it was used should be interpreted cautiously. 2,2,2-Trichloroethanol (TCE) was freshly vacuum distilled prior to use. Water was purified by a Millipore Super-Q system and yielded less than 25 counts s^{-1} of fluorescence at 380 nm when illuminated at 300 nm under the same conditions that yielded well over 20 000 counts s^{-1} in the protein experiments. Any fluorescence at levels greater than this was taken as an indication of contaminated water. When this occurred, the water purification system was disinfected with commercial bleach and flushed with clean water, and the cartridges were replaced.

† Supported by the Natural Sciences and Engineering Research Council of Canada.
‡ Present address: Department of Chemistry, The University of Western Ontario, London, Ontario N6A 5B7, Canada.
§ Present address: Department of Chemistry, Lash Miller Chemical Laboratories, University of Toronto, Toronto, Ontario M5S 1A1, Canada.

[1] Abbreviations: RNase T1, ribonuclease T1; Tyr, tyrosine; His, histidine; Glu, glutamic acid; Arg, arginine; Phe, phenylalanine; Trp, tryptophan; RNA, ribonucleic acid; Br-TMIT, 4-bromo-2,2,3,3-tetramethylindanthione; TCE, 2,2,2-trichloroethanol; TCSPC, time-correlated single photon counting; IEF, isoelectric focusing.

0006-2960/85/0424-5517$01.50/0

Solutions of salts and quenchers, except CsCl, displayed 50 counts s^{-1} or less of background fluorescence.

RNase T1 was supplied as a lyophilized material by Dr. F. Walz of Kent State University. It had been extensively purified, and analysis by isoelectric focusing (IEF) techniques and electrophoresis showed no evidence of contamination by other polypeptides. A few milligrams of protein was dissolved and dialyzed at <10 °C in one or two changes of 0.005 M acetate buffer of pH 5.5 and 0.3 M ionic strength, maintained with KCl. The pH was chosen on the basis of results for the enzyme activity (Epinatjeff & Pongs, 1971), which display a plateau between pH 4.5 and 7.5 (Zabinski & Walz, 1976; Osterman & Walz, 1978, 1979). Quenching experiments were conducted similarly to those reported previously (Demmer et al., 1985; James et al., 1985), with constant pH, temperature, and ionic strength being maintained. Protein concentrations were typically 6×10^{-7} M as determined from the absorption at 278 nm, using a molar extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} at this wavelength (Walz & Hooverman, 1973). Absorption measurements were carried out on a Cary 118 spectrophotometer.

Fluorescence lifetime measurements were performed in the same manner as described previously (Demmer et al., 1985; James et al., 1985). The excitation source was a synchronously pumped, cavity-dumped, frequency-doubled dye laser. Fluorescence curves were collected by the time-correlated single photon counting technique (TCSPC) and analyzed by using the mimic technique (James et al., 1983). *trans*-Stilbene and 4-bromo-2,2,3,3-tetramethylindanthione (Br-TMIT) were used as reference compounds. The protein sample was flowed through a fluorescence cell so as to eliminate photochemical degradation (Demmer et al., 1985). The temperature was regulated by a specially constructed cell holder and a Haake F3/K circulating water bath and was maintained to within ±0.1 °C.

Magic angle polarization conditions were used in all experiments to eliminate artifacts due to the slow rotation of the protein molecules, unless specifically stated otherwise.

The fluorescence lifetime(s), τ_i, and preexponentials, a_i, were determined by iterative reconvolution from the mimic and sample fluorescence decays utilizing sums of exponentials as trial decay functions:

$$I(t) = \sum_i a_i e^{-t/\tau_i} \quad (1)$$

Fluorescence anisotropies were determined directly from the data by

$$R(t) = (I_V - GI_H)/(I_V + 2GI_H) \quad (2)$$

where I_V is the emission intensity monitored through a polarizer oriented parallel to the excitation polarization, I_H is the emission intensity monitored through a perpendicular polarizer, and G is a factor used to correct for the polarization sensitivity of the detection system. Fluorescence decay curves collected for anisotropy decay measurements contained at least 40 000 counts in the peak channel for the least sensitive polarization. The G factor was determined from the relative intensities observed from 1,3,5-triphenylbenzene in acetonitrile. Complete experimental details of the instrumental arrangement have been published elsewhere (James et al., 1985). The anisotropy decay function was determined by fitting a sum of exponential decays to $R(t)$. This procedure precluded the observation of any very short-lived anisotropy components because of convolution artifacts, but the longer lived protein rotational correlation time was unperturbed. It was found that only a single-exponential term was needed to fit $R(t)$:

$$R(t) = r_0 \exp(-t/\phi) \quad (3)$$

where r_0 is the fluorescence anisotropy of the initially prepared excited state and ϕ is the rotational correlation time for overall tumbling of the protein.

Rotational correlation times were used to extract the apparent molecular volume by means of the Stokes–Einstein–Debye equation (Einstein, 1906):

$$V = kT\phi/\eta \quad (4)$$

where V is the volume of the particle, T is the absolute temperature, k is the Boltzmann constant, and η is the bulk solution viscosity.

Steady-state fluorescence measurements were performed by using a SPEX Fluorolog 222 spectrophotofluorometer. This instrument has a 450-W Xe arc source which produces radiation of useful intensity in the spectral region above 230 nm. Wavelength selection is achieved with double-grating excitation and emission monochromators, each with a reciprocal linear dispersion of 2 nm/mm. Emission is detected by an RCA C31034A photomultiplier tube operated in the single photon counting (SPC) mode. All aspects of the spectrometer operation were controlled by a microcomputer-based data acquisition system, a SPEX Datamate. This allowed digital collection and processing of the spectra, including corrections for the instrument sensitivity, background subtraction, and lamp intensity fluctuations. Consequently, the emission spectra reported in this work are fully corrected for these effects.

Samples were contained in a two-compartment unit which allowed spectra to be recorded in double-beam mode. Right-angle emission was collected simultaneously from both the sample and blank solutions to enable the Datamate to perform on-line background corrections. A higher level of Rayleigh scattering was obtained from the protein samples than from the blank, due to the larger size of the protein molecules. Consequently, the final corrected spectra contained a small contribution from scattered exciting light when the emission monochromator was scanned across the excitation wavelength. As long as the excitation and emission bandpasses were relatively narrow, this could be manually removed by inspection without introducing significant error.

Fluorescence quantum yields of dilute solutions were measured by the method of Parker & Rees (1960) as modified by Demas & Crosbie (1971), using the integrated intensities of corrected emission spectra. This method relies on the availability of a suitable reference compound with a known fluorescence quantum yield. Many workers have measured the fluorescence yields of proteins relative to that of an aqueous solution of tryptophan since the emission spectra are very similar. This allows uncorrected emission spectra to be used without introducing significant error. However, the value of the fluorescence quantum yield of tryptophan has been the subject of some disagreement, and it was deemed more suitable to use a solution of quinine sulfate in 0.1 N H_2SO_4 ($\phi_f = 0.52$) as a standard (Meech & Phillips, 1983). Though the fluorescence spectrum of quinine sulfate lies considerably to the red of tryptophanyl emission, the microcomputer-based data acquisition system of the SPEX Fluorolog allowed the necessary spectral corrections to be performed with good speed and precision.

The temperature dependence of the fluorescence of RNase T1 was investigated by using a special cell obtained from Hellma Inc. (162F). This cell is constructed so that a rapid flow of temperature-controlled water circulates through channels fashioned in the back of the cell. The temperature was regulated by a Haake F3/K circulating water bath and

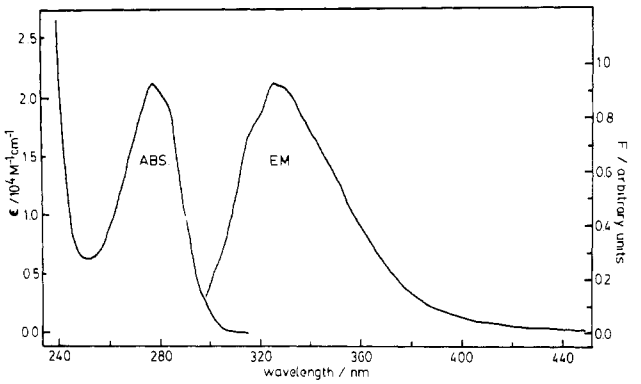

FIGURE 1: Absorption (ABS) and corrected emission (EM) spectra of RNase T1 in 0.05 M acetate buffer, pH 5.5, ionic strength 0.3 M, and 25 °C. Fluorescence was excited at 295 nm, and the excitation and emission band-passes were 2 nm.

monitored by using a calibrated thermistor placed directly in the solution through a hole in the cell stopper. The cell was placed in a temperature-regulated cell holder supplied by SPEX. Experiments were performed by setting the water bath to a temperature near 0 °C and allowing the system to equilibrate. The nominal temperature setting was changed to 60 °C, and at the same time, data acquisition was begun. The resulting temperature gradient was roughly +0.8 °C/min. Emission was monitored at the wavelength of maximum emission and recorded in the Datamate as a function of time. The resistance of the thermistor was monitored continuously and simultaneously by a digital voltmeter equipped with an analogue output which was fed into a millivolt chart recorder. After equilibration at the maximum temperature (near 50 °C), the process was repeated while the temperature fell to 0 °C. Emission spectra were recorded before and after the experiment to ensure that any changes in the protein were reversible. The excitation wavelength was 295 nm with a band-pass of 1 nm, and emission was monitored at 327 nm with an 8-nm band-pass.

RESULTS

The corrected steady-state fluorescence spectrum of RNase T1 was determined and is presented in Figure 1.

The maximum of the fluorescence emission lies at 327 nm, while indole compounds in water generally display emission maxima around 350 nm (Longworth, 1971). The spectrum shows evidence of vibrational structure on both sides of the maximum which is absent for indole derivatives in water.

The fluorescence quantum yield obtained upon excitation of the tryptophanyl residue in RNase T1 was 0.28 ± 0.05 at 298 K. This agrees well with a value of 0.31 (Longworth, 1971) and is in keeping with the general observation that indole derivatives display high quantum yields in nonaqueous solvents.

The fluorescence decay of RNase T1 was measured at several excitation and emission wavelengths. In all cases, it was found to be very well described by a single-exponential decay function at excitation wavelengths of 295 nm or greater. The data were analyzed from the base line preceding the rise of fluorescence intensity to the base line following the decay and were closely examined to determine whether a second exponential component could be extracted from the decay curves. Every attempt led either to a second component with a vanishingly small preexponential or to two components with identical lifetimes. These results are in disagreement with those of Grinvald & Steinberg (1976), who found evidence for a minor second component in the fluorescence decay. The protein used in this work had been extensively purified and

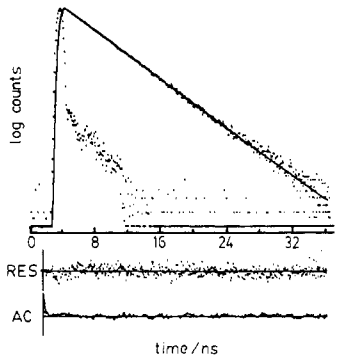

FIGURE 2: Fluorescence decay at RNase T1 analyzed by using a single-exponential decay function. The points represent experimental data and the solid curves the best-fit line. RES and AC are the residual and autocorrelation functions for the fit, as defined in James et al. (1983). Excitation was at 295 nm, and emission was monitored at 350 nm. The sample was prepared in 0.05 M acetate buffer, pH 5.5, ionic strength 0.5 M. The decay time was 3.87 ns for this fit, and the χ_ν^2 value was 0.917.

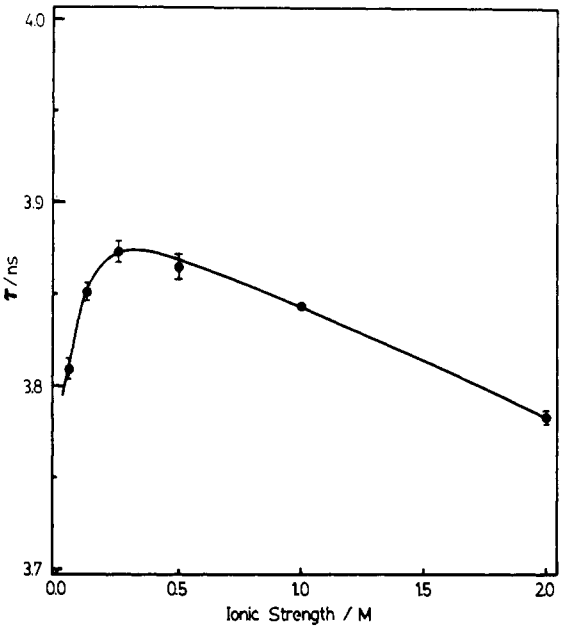

FIGURE 3: Fluorescence lifetime of RNase T1 as a function of ionic strength in 0.025 M acetate buffer, pH 5.5, at 25 °C. The ionic strength was adjusted by the addition of KCl.

showed no evidence of contamination by other polypeptides. All preparations were extensively dialyzed before use, so it is also certain that no appreciable contamination by smaller aromatic molecules was present. In contrast, the protein employed by Grinvald and Steinberg was used as received from a commercial supplier (Worthington Biochemical Corp.) and could very well have contained fluorescent impurities. Consequently, it is highly likely that their results are in error. A representative fluorescence decay curve is shown in Figure 2, together with the residual and autocorrelation functions obtained for a single-exponential fit to the data. The quality of the fit is self-evident. From these experiments, the fluorescence lifetime of RNase T1 was found to be 3.88 ± 0.01 ns at 0.3 M ionic strength and 3.87 ± 0.01 ns at 0.5 M ionic strength. The reproducibility of these values was found to be excellent, and all fluorescence lifetimes reported in this work for RNase T1 are of similar precision. These values are in agreement with those reported by previous authors (Lakowicz et al., 1983; Eftink, 1983).

Both the fluorescence quantum yield and the lifetime of RNase T1 were found to be relatively insensitive to the com-

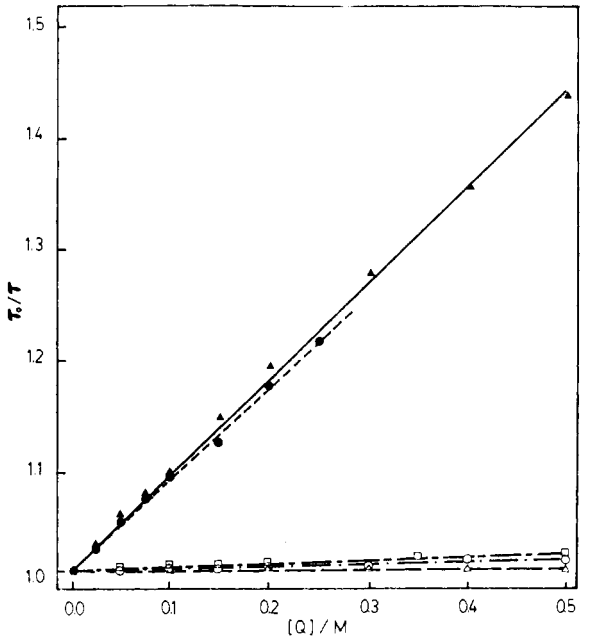

FIGURE 4: Stern–Volmer plots for the quenching of RNase T1 fluorescence by a variety of species: (○) CsCl; (□) KI; (△) acetate; (●) TCE; (▲) acrylamide. Note the very low quenching efficiency of the ionic species.

Table I: Summary of the Bimolecular Quenching Rate Constants Obtained for Quenching of RNase T1 Fluorescence[a]

quencher	k_q ($\times 10^7$ M^{-1} s^{-1})	pH
CsCl	0.54 ± 0.08	5.5
KI	0.76 ± 0.08	5.5
KI	2.9 ± 0.3	3.0
acetate	0.19 ± 0.06	5.5
TCE	20.9 ± 0.01	5.5
acrylamide	21.8 ± 0.01	5.5

[a] In all cases, the temperature was 25 °C, and the pH was 5.5 in 0.05 M acetate buffer. Results for ionic quenchers are for 0.5 M total ionic strength, 0.3 M for acrylamide and TCE.

position and ionic strength of the buffer in which the samples were prepared. Slight variations were observed, however, as illustrated for the lifetime in Figure 3. This dependence parallels that observed in the relative quantum yield (O'Grady, 1984).

The fluorescence properties of RNase T1 were also found to be relatively insensitive to the nature of the ionic solute which was present. This was not unexpected for KCl since this is not an efficient fluorescence quencher (Altekar, 1977a,b), but it was interesting to note that even normally efficient quenchers such as KI had virtually no effect on the measured lifetime as long as care was taken to maintain constant ionic strength. This was true even at temperatures as high as 50 °C. Thus, though KI usually quenches indole fluorescence at diffusion-controlled rates, its bimolecular quenching rate constant with RNase T1 must be less than 10^7 M^{-1} s^{-1}, the lower limit measurable by our techniques when the fluorescence lifetime lies in the nanosecond range. Similar results were obtained for two other ionic species, CsCl and acetate ion. Stern–Volmer plots for these experiments are presented in Figure 4, and the bimolecular quenching rate constants obtained from weighted least-squares analyses of the data in Figure 4 are summarized in Table I.

When uncharged quenchers were employed, the situation changed dramatically, as is readily apparent from Figure 4 and Table I. Thus, both acrylamide and TCE were found to quench the tryptophanyl fluorescence with reasonable efficiency. Both rate constants are roughly an order of magnitude less than those expected for diffusion-controlled bimolecular quenching of protein fluorescence, which are generally of the order of 10^9 M^{-1} s^{-1} (Lakowicz, 1983).

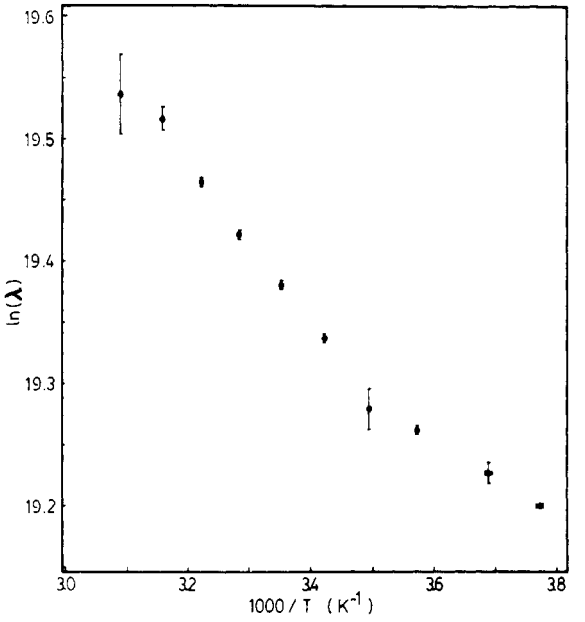

FIGURE 5: Fluorescence decay rate ($\lambda = 1/\tau$) of RNase T1 plotted against temperature. Note that the choice of scale exaggerates the temperature dependence of the decay rate.

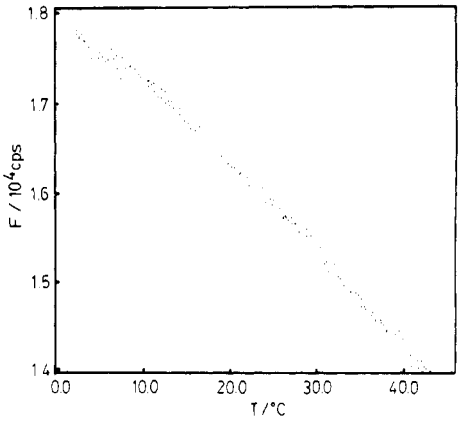

FIGURE 6: Fluorescence intensity of RNase T1 plotted as a function of temperature. Excitation was at 295 nm to avoid interference from tyrosinyl residues, and emission was monitored at 327 nm.

The fluorescence lifetime of RNase T1 was found to be only slightly sensitive to the temperature of the surrounding medium over the range from 0 to 60 °C. The results are shown in Figure 5 as a plot of the decay rate, $\lambda = 1/\tau$, and λ changes by only 20% between 0 and 40 °C. By comparison, the fluorescence quantum yield of tryptophan in aqueous solution decreases by a factor of 5 over the same temperature range (Kirby & Steiner, 1970). Indeed, aqueous solutions of indoles usually display a very marked temperature dependence due to the photoionization decay pathway, which is assisted by solvent and has an activation energy of roughly 30 kJ/mol (see below).

This presents difficulties in analysis, however, since the bulk of the decay takes place by temperature-independent pathways. It is instructive to determine whether the decrease in fluorescence lifetime is paralleled by a decrease in quantum yield, as it should if the decrease is caused by a thermally dependent nonradiative decay pathway. Accordingly, the steady-state fluorescence intensity was monitored as a function

of temperature over the range from roughly 2 to 40 °C, and the results are plotted in Figure 6.

It is apparent from a comparison of this figure with Figure 5 that the fluorescence intensity, which is proportional to the quantum yield, is also directly proportional to the decay rate. Fluorescence spectra of the protein recorded before and after the experiment at 25 °C were identical, demonstrating that whatever changes occurred to the protein were completely reversible.

The decay of S_1 (lowest single state) for indole compounds in nonpolar solvents is primarily by fluorescence and ISC (intersystem crossing) to T_1 (lowest triplet state). Over the modest temperature range used here, both k_R (radiative rate constant) and k_{ISC} should be constant, since the proportion of highly vibrationally excited molecules in S_1 should remain negligible. These processes account for the bulk of the decay of indole in nonpolar solvents so that the overall temperature dependence is not very pronounced. Consequently, the activation energy of the thermally activated process is difficult to extract directly in this case.

Analysis of the data presented in Figure 6 was performed according to the following scheme. The steady-state fluorescence intensity at a given wavelength and at any temperature, $F(T)$, is proportional to the fluorescence quantum yield, ϕ_f:

$$F(T) = a\phi_f(T) \tag{5}$$

Using the standard definition that $\phi_f(T) = k_R/(k_R + k_{NR})$, and assuming k_{NR} to be the sum of two terms, only one of which, $k'(T)$, depends on temperature while the other, k^0_{NR}, is temperature independent, one may write

$$\phi_f(T) = \frac{k_R}{k_R + k^0_{NR} + k'(T)} = \frac{k_R}{k^0 + k'(T)} \tag{6}$$

where both temperature-independent terms in the denominator have been collected into k^0. Substituting eq 5 into eq 6 and rearranging, one obtains the result

$$F^{-1}(T) = \frac{k^0}{ak_R} + \frac{k'(T)}{ak_R} \tag{7}$$

The first term in eq 7 can often be neglected, in which case it is readily shown that a plot of ln F^{-1} vs. T^{-1} should be linear with a slope of $-E_a/R$, if $k'(T)$ is of the Arrhenius form. However, this treatment is inadequate for indole derivatives where k_R and k^0_{NR} are of comparable magnitudes. In this case, the temperature dependence can be extracted by taking the derivative of F^{-1} with respect to T, in which case the first term drops out quite neatly. Alternatively, the derivative with respect to T^{-1} can be used, which leads to a particularly convenient result. Differentiating eq 7 in this way, one obtains

$$\frac{dF^{-1}(T)}{dT^{-1}} = 0 + \frac{1}{ak_R}\left(\frac{dk'(T)}{dT^{-1}}\right) \tag{8}$$

Substituting the Arrhenius form for k' into eq 8 and carrying out the indicated operations yield

$$\frac{dF^{-1}}{dT^{-1}} = \frac{-AE_a}{aRk_R}e^{-E_a/RT} \tag{9}$$

Taking the logarithm of both sides of eq 9 linearizes the problem. Both sides of eq 9 will be less than zero since F decreases with increasing T, so that eq 9 is first multiplied by −1. The final expression is

$$\ln\left(\frac{-dF^{-1}}{dT^{-1}}\right) = \ln\left(\frac{AE_a}{aRk_r}\right) - \frac{E_a}{RT} \tag{10}$$

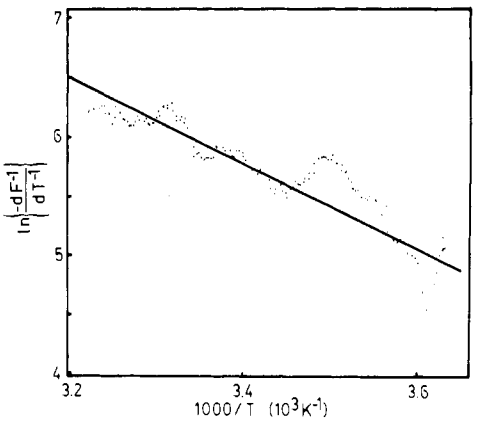

FIGURE 7: Plot of the left-hand side of eq 10 against inverse temperature. The slope of this plot yields an activation energy of 30 kJ/mol.

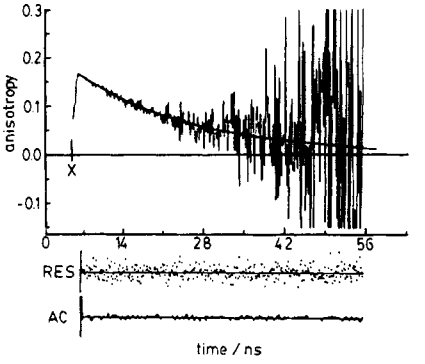

FIGURE 8: Time-resolved fluorescence anisotropy decay of RNase T1 in buffered aqueous solution at −1.5 °C, pH 5.5. RES and AC are as defined in James et al. (1983).

a result first obtained by Longworth (1971). Thus, a plot of the left-hand side of eq 10 vs. T^{-1} should be linear, with a slope of $-E_a/R$.

The data presented in Figure 6 can be treated in this way if the value of the derivative at each temperature, T_i^{-1}, is taken to be the slope of a linear fit for 20 data points distributed equally on both sides of the ith datum in a plot of F_i^{-1} vs. T_i^{-1}. The results of this analysis are shown in Figure 7. Note that there is a systematic ripple in the plot, but the overall trend is not in doubt, and the activation energy of 30 kJ/mol obtained from the slope has a statistical error of only ±2 kJ/mol. However, systematic errors may be important, and so a safer error limit is estimated to be ±5 kJ/mol.

At temperatures between −2 and 45 °C in buffered aqueous solution, the fluorescence anisotropy decay of RNase T1 can be adequately described by a single-exponential decay function. However, only quite strong departures from this behavior would be resolvable (Lakowicz, 1983). The results of a representative experiment are shown in Figure 8.

Note the very high degree of scatter in the experimental fluorescence anisotropy decay curve at long times, a consequence of the very low intensity of fluorescence in this region. Nevertheless, the residual and autocorrelation plots are excellent and show that the data are well described by a single rotational correlation time, ϕ, of 20.9 ± 1.0 ns at this temperature. The value of r_0 is 0.18 ± 0.007. This is relatively high and indicates that Trp is in a relatively rigid environment.

The values of ϕ obtained at each of several temperatures are given in Table II, along with the limiting anisotropies. The theoretical relationship for the rotational correlation time of a rigid sphere in a fluid continuum was given by eq 4 and

Table II: Rotational Correlation Times, ϕ, and Limiting Anisotropies, r_0, Obtained for RNase T1 at a Number of Temperatures[a]

T (°C)	η/T ($\times 10^{-3}$ kg m^{-1} s^{-1} K^{-1})	r_0	ϕ (ns)
−1.5	6.63	0.183 ± 0.007	20.9 ± 1.1
1.5	6.18	0.176 ± 0.003	15.6 ± 0.8
15.4	3.92	0.184 ± 0.004	9.6 ± 0.6
29.9	2.63	0.187 ± 0.004	6.0 ± 0.4
44.4	1.89	0.197 ± 0.003	3.7 ± 0.2

[a] Also listed are the calculated values of η/T used to plot Figure 9: η is the solution viscosity, taken to be that of pure water.

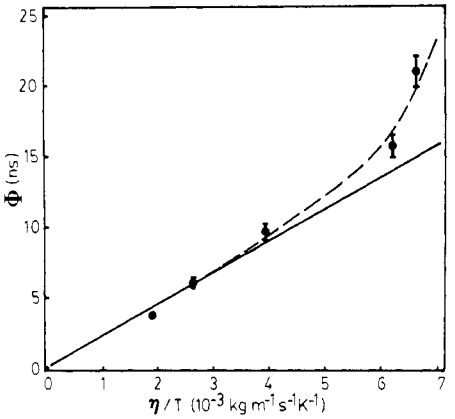

FIGURE 9: Rotational correlation time of RNase T1 plotted against η/T. See text and Table II for details.

shows in principle that the effective volume of a protein molecule can be calculated from the slope of a plot of ϕ vs. η/T. Such a plot is illustrated in Figure 9. There is significant upward curvature in the plot, but by use of the roughly linear portion of the curve at high temperatures, a value of $V = 19$ L/mol is obtained. Note that this treatment is quite approximate, and the data will be considered more fully below.

Discussion

Fluorescence Lifetime Decay. The relatively structured fluorescence spectrum of RNase T1 is significantly blue shifted relative to indoles in water. These features suggest that the tryptophanyl residue of RNase T1 is shielded from the aqueous solvent by the folding of the polypeptide chain. The emission maxima of indole compounds in solvents such as cyclohexane are generally found at wavelengths shorter than 320 nm, however, so that it would be inappropriate to suggest that it lies in a completely nonpolar environment; in fact, the observed maximum is at a wavelength close to that observed in alcohol solutions (Lumry & Hershberger, 1978). Nevertheless, on the basis of the fluorescence Stokes shift and the structured emission spectrum, it can be asserted that the lone Trp residue lies within the protein matrix where it has little direct contact with the aqueous solvent.

It is important to note that the fluorescence decay of RNase T1 is clearly single exponential, since this represents one of the few known cases where the fluorescence decay of a single tryptophanyl protein can be completely described in such a simple way (Grinvald & Steinberg, 1976; Lakowicz, 1983). This implies that no complications are present due to dual fluorescence from the ^1L_a and ^1L_b states of indole. This may appear surprising in view of the fact that dual fluorescence has been observed for many indole derivatives in nonpolar solvents (Lakowicz, 1983). However, it would appear that the tryptophanyl residue is only partially shielded from interactions with polar groups, and under these circumstances, dual emission is generally not observed.

Given this situation, it is clear that RNase T1 comprises an ideal system for investigating the dynamic properties of protein structure, since complications due to dual emission are absent. Accordingly, it is appropriate to examine rather more closely the results of the fluorescence quenching experiments reported above.

Recall that both acrylamide and TCE were found to quench the fluorescence of Trp_{59} with reasonably high efficiency. This was attributed to dynamic quenching of the partly buried residue by diffusion into the protein matrix, but it should be noted that static quenching cannot be resolved by measurements of the time-resolved fluorescence decay when there is only a single emitting species (Demmer, 1984; James et al., 1985). Thus, steady-state fluorescence experiments are necessary to determine the presence of static quenching for the case of RNase T1. Such measurements have been performed with acrylamide as a quencher (O'Grady, 1984; Eftink & Ghiron, 1976, 1977), but no evidence for static quenching was observed. Normally, static quenching is expected to accompany dynamic quenching since it can be regarded as a consequence of the time dependence of the bimolecular quenching rate constant (Eftink & Ghiron, 1981). The absence of static quenching therefore implies that the steady-state local concentration of acrylamide near the tryptophanyl residue is abnormally low (Longworth, 1968). Thus, it would appear that an acrylamide molecule is normally excluded from the vicinity of the tryptophanyl residue by the folding of the polypeptide chain and must diffuse into proximity during the residue's excited-state lifetime.

This hypothesis is further supported by the observation that the bimolecular quenching rate constants for acrylamide and TCE are substantially less than those expected on the basis of diffusion through the solvent. Since both of these quenchers are thought to quench indole fluorescence on every encounter, the relatively low quenching rate constants imply that their approach to the tryptophanyl residue is partially hindered. Consequently, the rate-limiting step would appear to be diffusion into the protein matrix. Indeed, the extremely low rate of quenching by ionic species would indicate that the polypeptide chain substantially limits their diffusion, so much so that no quenching is observed.

It is also of interest to note that acrylamide is a relatively polar molecule while TCE is essentially hydrophobic; yet both appear to penetrate the matrix with similar facility. The activation energy for the bimolecular quenching rate constant of RNase T1 by acrylamide has been found to be roughly 36 kJ/mol (Eftink & Ghiron, 1977). This value is roughly 20 kJ/mol greater than expected for free diffusion of molecules of this size through water, lending additional support to the assertion that there is restricted access for diffusion of the quencher into proximity with Trp_{59}.

When KI is introduced to a solution of RNase T1, no appreciable flurescence quenching is observed, even at temperatures as high as 50 °C. Thus, it was not possible to extract an activation energy for this system. This implies that the activation energy is extremely high, and it is likely that an ionic species such as I^- cannot penetrate the protein matrix in the absence of very large-scale unfolding of the polypeptide chain. Thus, it appears that the folding of the polypeptide chain very effectively shields the tryptophanyl residue from any direct interaction with the solvent. Note that this is true even for temperatures very close to the denaturation temperature. As expected, when RNase T1 is denatured, its tryptophanyl residue is found to undergo diffusion-controlled fluorescence

quenching by a number of solutes (Grinvald & Steinberg, 1976).

It has been found that Trp_{59} undergoes slight absorption spectral changes in the presence of uncharged solutes analogous to, but less pronounced than, those produced by the same solutes with *N*-acetyl-L-tryptophan ethyl ester (Campbell et al., 1976). The solutes used provided a range of effective molecular diameters: for example, D_2O (2.0 Å), methanol (2.8 Å), glycerol (5.2 Å), and sucrose (9.4 Å). The results for each of these solutes were very similar. If the solutes had to penetrate the matrix to any great extent in order to affect the tryptophanyl residue, one would expect a very strong dependence on the molecular dimensions of the perturbant.

Thus, it appears that the tryptophanyl residue is partially exposed to the bulk solvent. However, charged solutes appear to be rigorously excluded from any contact with Trp_{59}, and its emission spectrum shows that the indole ring is in a predominantly nonpolar environment. These results appear at first sight to be mutually exclusive, but they can be accounted for by a hypothesis which is based on structural features peculiar to the RNase T1 molecule.

The position adjacent to Trp_{59} in the polypeptide chain is occupied by Glu_{58}, which is known to be at the active site and thought to be important in substrate recognition. The γ-carboxyl group of Glu has a pK_a of 4.25 (Mahler & Cordes, 1971), and at pH 5.5, it would be expected to bear a negative charge. Two other charged residues are thought to be at the active site: His_{40} and His_{92}. The pK_a of the imidazole system is 6.0 (Mahler & Cordes, 1971), and so these residues would be expected to bear positive charges. Thus, it is reasonable to suppose that the observations presented above can be explained by the presence of nearby charged groups which shield the approach of ions from the bulk solution into proximity with Trp_{59}.

This conjecture is consistent with the high-resolution optically detected magnetic resonance (ODMR) experiments of Hershberger et al. (1980). At temperatures near 5 K, the ODMR signal due to the lowest triplet state of Trp_{59} was found to be susceptible to hole burning, indicating that the ODMR absorption line was inhomogeneously broadened. This implies the existence of multiple conformations of the indole ring system within the protein matrix which are relatively rigid, at least on a microsecond time scale. The heterogeneity in the transition frequencies of each conformer was attributed to the presence of an anisotropic electrostatic field near the indole ring. This is consistent with the spectral properties of Trp_{59} which were discussed earlier, in particular the assertion that the largely nonpolar environment of Trp_{59} was perturbed by the presence of a polar group, thereby shifting the emission maximum to 327 nm from the roughly 320 nm expected for indole in a purely nonpolar medium.

It is of interest to note that a maximum in the fluorescence lifetime occurs at an ionic strength of roughly 0.25 M. The tertiary structure of a protein is expected to be highly dependent on the solvent shell which is bound to the protein surface. In turn, this depends on the state of the polar and charged groups which are found on the surface, and whether ionic species are present in solution to interact with them. Thus, it is not unexpected to find the lifetime to be slightly dependent on ionic strength, since this reflects the minor conformational variations resulting from the interactions between the surface residues and the surrounding aqueous solvent.

As a result, a likely explanation for the inability of I^- to quench the fluorescence is to imagine that Trp_{59} is selectively "protected" from interactions with charged solutes by an electrostatic barrier composed of some combination of these amino acid side chains. Though the histidinyl residues are known to be important in conferring substrate specificity, they may not be sufficiently close to Trp_{59} to account for the observed behavior, and it is possible that Glu_{58} plays the only important role. Neutral species will not interact as strongly with the charged side chains and may diffuse past them due to fluctuations in their orientations. Negative charged ionic species such as I^- will be repelled from the vicinity of the tryptophanyl residue, and the degree of quenching will be correspondingly reduced (Lakowicz, 1983; Lakowicz & Weber, 1973). This hypothesis is consistent with the observation that at a pH of 3.0, where the carboxyl group of Glu_{58} is expected to be uncharged, the bimolecular quenching rate constant increases by a factor of 3. This increase is estimated to be outside the limits of error, but it is very small, and both measurements are very close to the lower limit that can be measured. If only the γ-carboxyl group of Glu_{58} were responsible for the low quenching efficiency of I^-, it is likely that the increase would have been much more pronounced.

Furthermore, if this model is correct, it would be expected that quenching by Cs^+ should be enhanced. While the present data show it to be very low, it should be noted that CsCl is a very inefficient quencher. In the case of LADH, the bimolecular quenching rate constant for CsCl is approximately an order of magnitude slower than that for acrylamide, in the absence of any apparent steric or electrostatic restrictions (James et al., 1985). If the same ratio of quenching efficiencies were observed for the case of RNase T1, the bimolecular quenching rate constant for CsCl would be expected to be 2×10^6 M^{-1} s^{-1}, whereas it has been measured to be greater than 5×10^6 M^{-1} s^{-1}. Though these values lie at the lower limit of resolution, they are not inconsistent with this hypothesis.

With regard to uncharged quenchers, it would appear that on a time-averaged basis the charged groups deny them access to the volume immediately surrounding the tryptophanyl residue, thereby lowering the local steady-state concentration of quenching species and prohibiting static quenching.

In this context, it is of interest to note the results of Bushueva et al. (1980), who found that the fluorescence of the lone tryptophanyl residue of ribonuclease C2 was effectively quenched by KI at a pH of 6.0. RNase C2 is genetically related to RNase T1 (Bushueva et al., 1980) and is expected to have a very similar amino acid sequence and overall conformation. Therefore, it is likely that the marked difference in the quenching behavior of these two proteins is due to some relatively minor difference in the amino acid sequence, such as the presence of the glutamic acid residue in RNase T1. However, the amino acid sequence of RNase C2 is not available, and until such work has been done, an unambiguous interpretation of these results cannot be made. In this regard, it is also obvious that X-ray crystallographic data for RNase T1 are urgently needed.

Nevertheless, the picture presented here would also be consistent with the relatively high activation energy (36 kJ/mol) measured by Eftink & Ghiron (1977) for acrylamide quenching. The diffusion of acrylamide to the tryptophanyl residue would involve the disruption of the electrostricted solvation shell surrounding the charged residues to at least some degree, a process which would be expected to have an activation energy rather larger than that for diffusion through bulk water (Bushueva et al., 1978, 1980). An alternative explanation might be that the observed activation energy may be due to the requirement for local motion of the polypeptide

folding in order to open, transiently, an access channel.

Note that a similar activation energy was obtained in this work for the thermally induced fluorescence quenching of the tryptophanyl fluorescence of RNase T1, 30 ± 5 kJ/mol. The corresponding activation energy for tryptophan in aqueous solution has been measured by several workers, and values in the range from 28 to 35 kJ/mol have been reported (Galley & Edelman, 1962; Weinryb & Steiner, 1970; Barenboim et al., 1969; Stryer, 1966; Steiner & Kirby, 1970; Lehrer, 1970). The temperature dependence of the fluorescence quantum yield of tryptophan has been attributed solely to solvent-assisted photoionization, and, indeed, the yield of solvated electrons for aqueous tryptophan solutions has been found to be very sensitive to temperature (Bent & Hayon, 1975). Thus, the thermally dependent process in RNase T1 appears to have an activation energy similar to that of photoionization, and it is tempting to assign this as the process in question.

It is also true that the activation energy measured in this work for the thermal quenching of RNase T1 fluorescence is similar to that observed for the bimolecular quenching of the tryptophanyl fluorescence by acrylamide (see above). This was interpreted as the activation energy for the diffusion of acrylamide past the charged groups which lie between Trp_{59} and the bulk solvent or the opening of a transient access channel. Thus, it is possible that the value measured here for the thermal quenching also reflects in part the energetic requirements for diffusion of water molecules into proximity with the tryptophanyl residue, where they could solvate the electron subsequently ejected from the excited indole moiety. However, the latter mechanism would require the presence of several water molecules at the time the electron was ejected, so that the electron could be stabilized by the formation of a solvent cage. In this case, the measured activation energy would represent an average of those for the motion of the polypeptide chain and for solvation of the electron.

However, it has been shown that ϕ_{e^-} decreases by a factor of 2 (from 0.08 to 0.04) when tryptophan is incorporated into oligopeptides (Bent & Hayon, 1975) and that the fluorescence quantum yield of indole derivatives in nonpolar solvents is virtually independent of temperature. This is thought to be due to the inability of these solvents to stabilize the electron which would otherwise be ejected. Both of these observations can be used to account for the relative insensitivity of the fluorescence of RNase T1 to temperature if it is assumed that Trp_{59} is not directly accessible to the bulk solvent. Consequently, to attribute the temperature-dependent process in RNase T1 to photoionization would be to ignore the evidence presented earlier, which suggested that it was shielded from the aqueous medium. It would also ignore the fact that the cluster of water molecules around the ejected photoelectron must occupy a significant volume within the protein, and it is very unlikely that this volume would be available due to the close packing of the amino acid residues.

The second possibility, and one that appears to be more likely, is to regard the measured activation energy as a reflection of the general mobility of the surface of the protein. This mobility is likely to be influenced by secondary bonding interactions between portions of the polypeptide chain, but also between the residues at the surface of the protein and the solvation layer which surrounds them. In this view, the quenching process would be due to "intramolecular collisions" between the indole ring system and, for example, the γ-carboxyl group of Glu_{58}.

In short, one cannot say with certainty which process is responsible for the thermally activated fluorescence quenching of RNase T1 using the data which are at hand, but it seems unlikely that photoionization plays a significant role in the deactivation of the excited Trp_{59} residue.

Fluorescence Anisotropy Decay. As indicated above, the value of r_0 was 0.18 ± 0.05, a relatively high value which indicates that Trp_{59} is in a farily rigid environment. This contention is in agreement with the work of Hershberger et al. (1980) since the anisotropy observed in the ODMR experiments would be "averaged out" if the indole ring were free to rotate during the roughly 10-μs lifetime of the triplet state. Thus, at least the same degree of rigidity will be present on the much shorter time scale involved in fluorescence anisotropy decay measurements, thereby accounting for the relatively high value of r_0 which was observed here. In addition, the immobility of Trp_{59} on a microsecond time scale implies that the measured values of ϕ are characteristic of the depolarizing rotations of the protein molecule as a whole, and not of any segmental mobility of the tryptophanyl residue within the protein.

However, the value of r_0 measured in this work is smaller than 0.3, the value measured for indoles in very viscous solutions for excitation into the ^1L_a band (Valeur & Weber, 1977). The maximum value of the limiting fluorescence anisotropy which has been observed for tryptophanyl residues in proteins is roughly 0.2 (Lakowicz, 1983). This fact is often attributed to rapid, small-scale librational motions of the indole ring system within a "pocket" formed by the protein matrix, but this has not been conclusively demonstrated, and so the origin of this lower value cannot be uniquely assigned at the present time.

Returning to the data for the dependence of the rotational correlation time on temperature, which was presented in Figure 9, a molecular volume of $V = 19$ L/mol was calculated from the slope of the plot by ignoring the curvature which was evident at low temperatures. This calculation does not take into account the shape of the molecule or the considerable degree of interaction between the surface of the protein and the surrounding solvent. Accordingly, the volume which is recovered represents an "effective" volume, which is expected to contain contributions from both the true molecular shape and specific protein–solvent interactions. However, RNase A is known to be relatively spherical (Dickerson & Geis, 1969), and RNase T1 can be reasonably expected to have the same shape. Thus, interpretation of ϕ in terms of an effective volume is less uncertain than for a protein with lower overall symmetry.

Making the reasonable approximation that, in the absence of denaturation, the molecular volume of the protein is only weakly dependent on temperature (and would decrease at lower temperature in any case), we can interpret the distinct curvature observed in Figure 9 as a change in the effective molar volume with temperature (Yguerabide, 1972).

For the case of RNase T1, the r_0 values were essentially independent of temperature, so it is plausible to assume that any changes in the packing density of the protein matrix were relatively minor. With this in mind, the apparent molecular volume, $V(T)$, can be written (Yguerabide, 1972)

$$V(T) = v + h(T) \tag{11}$$

where v is the specific volume, per mole, intrinsic to the folded polypeptide chain and h is the degree of hydration, expressed as the volume of water bound per mole of protein. Thus, $V(T)$ reflects both the displacement of the folded polypeptide chain and the volume of water which is hydrodynamically associated with the protein surface. The slope of the plot shown in Figure 9 increases with decreasing temperatures, so that the degree of hydration apparently increases at lower temperatures. The

effective molecular volume of RNase T1 was found to be 19 L/mol for temperatures higher than roughly 15 °C. If this volume were to be attributed solely to the polypeptide, and using a molecular weight of 11 100, the resulting density of the protein matrix would be approximately 0.6 kg/L. Protein densities (as determined from partial specific volumes) have been found to cluster in a narrow range about 1.33 kg/L (Bernhardt & Pauly, 1977). These results imply that a significant proportion of the effective volume of 19 L/mol is contributed by bound water at temperatures between 15 and 50 °C. This water appears to remain bound to the protein even at temperatures as high as 50 °C, since the graph in Figure 9 remains approximately linear over this range. Accordingly, it is reasonable to associate this with the primary solvation layer. A volume of 19 L/mol corresponds to an effective molecular radius of 20 Å. A protein of spherical symmetry with a molecular weight of 11 100 and a density of 1.3 kg/L would be expeected to have an intrinsic volume, v, of only 8.5 L/mol, corresponding to a radius of 15 Å. Thus, the depth of the bound water layer is approximately 5 Å. The O–O distance between two hydrogen-bonded water molecules can be estimated to be roughly 3 Å (Huheey, 1978), which implies that the primary solvation shell can be characterized as a layer, roughly one to two molecules thick. This result is in good agreement with earlier hydration studies of RNase [see Kuntz & Kauzmann (1974) and references cited therein].

Water bound to proteins is often described as being composed of two "shells" or "spheres". The inner or primary shell is thought to be very tightly held to the protein surface through direct interactions with the polar and charged amino acid side chains [see Kuntz & Kauzmann (1974) and references cited therein; Eagland, 1975; Berendsen, 1975]. This layer invariably accompanies the polypeptide chain when the protein is crystallized, and the relative immobility of the water molecules often results in their being visible in X-ray crystallographic studies. The secondary shell is only indirectly associated with the protein and is actually hydrogen bonded to the outside of the primary shell. The secondary shell effectively "links" the more highly structured primary shell with the bulk solution and is thus intermediate in its degree of structural order (Lumry, 1973).

It is of interest to consider the effective molar volume of RNase T1 in light of this model of protein hydration. The first observation is that since the primary hydration layer appears to remain firmly bound at high temperatures, it is not unreasonable to speculate that the similarity of the activation energy for acrylamide quenching to that for thermally induced quenching arises from the fact that both are intimately related to the activation energy for diffusion through the primary solvation layer. This would be possible if the orientational flexibility of the amino acid side chains which hinder the approach to Trp_{59} and possibility contribute to the thermally induced quenching were determined in large part by the bound solvation layer.

The second observation is that changes which occur as a function of temperature in the protein–solvent system that alter the secondary layer would be expected to appear as further changes in the effective molar volume. This is apparently the case at lower temperatures.

Below 15 °C, the plot begins to display significant curvature, which can be interpreted as a gradual increase in the molecular volume. It is possible to estimate the effective volume at a given temperature by calculating the slope of the of the tangent to the curve at that temperature. With only five experimental points, this procedure will not yield particularly accurate results but will allow some qualitative conclusions to be reached. Thus, at 0 °C one estimates that the molecular volume has increased roughly 4-fold to a value near 80 L/mol. Such a large increase could be due to a gradual decrease in the mobility of the secondary solvation layer, perhaps accompanied by an increase in its depth. If we attribute the volume increase to an increase in thickness of the primary layer, and again using an inter-oxygen distance of 3 Å for hydrogen-bonded water, it is possible to calculate that at 0 °C an additional layer of roughly three molecules is added to the primary layer which exists at higher temperatures. For this interpretation, the bulk solvent appears to be aggregating around the primary solvation layer. One alternative explanation is for a volume increase by protein aggregation on interaction. This seems unlikely although we cannot rigorously exclude this possibility from our data. Some evidence for this sort of behavior at high protein concentrations exists from ^{17}O NMR data (Halle et al., 1981), but our experiments were performed on solutions which were several orders of magnitude more dilute and are thus unlikely to show such long-range ordering effects. Another possibility is that we may be observing changes in the nature of the water structure itself as we near the freezing point. Thermodynamic evidence exists for hemoglobin exhibiting heat capacities, expansibilities, and compressibilities below 10 °C which might be attributable to the water (R. W. Lumry, personal communication). On balance, the behavior of ϕ below $\sim$10 °C appears to be due to the interactions of the solvent–protein system, resulting in an overall decrease in mobility, but an exact attribution of the observed behavior to a specific process is not possible with the data at hand.

Conclusions

It has been possible to characterize the environment of the lone tryptophanyl residue of RNase T1 in some detail. The vibrational structure and the relatively short wavelength of its emission maximum, coupled with its relatively high fluorescence quantum yield, provide firm evidence for its being in a predominantly nonpolar environment (Burstein et al., 1973). On this basis alone, it is appropriate to consider it to be buried in the hydrophobic interior of the protein, as has been assumed by some authors (Longworth, 1971; Pigauli & Gerard, 1984).

However, the observation that it is readily quenched by both acrylamide and TCE indicates that shielding from the solvent is far from complete. Indeed, the absorption spectral results of Campbell et al. (1976) show that Trp_{59} is readily accessible to a wide variety of uncharged molecules with little discrimination as to their molecular size and indicate that it is partly exposed to the solvent.

These difficulties can be explained by noting that the position of Trp_{59} in the polypeptide chain places it near the surface of the protein and adjacent to Glu_{58}. Thus, it may be shielded from direct interactions with the aqueous solvent or charged solutes by the charged side chain of the glutamic acid residue. The high r_0 value in combination with the ODMR results of Hershberger et al. (1980) indicates that it is held in place somewhat rigidly. Overall, it is appropriate to envision the tryptophanyl residue as residing in a hydrophobic pocket, but with part of the indole ring protruding into the active site and shielded from the solvent by the side chain of Glu_{58}.

Fluorescence anisotropy decay experiments have revealed the presence of water which remains relatively firmly bound to the protein at temperatures up to 50 °C. This can be identified with the primary solvation shell. Its immobility relative to that of bulk water has important implications for the fluorescence quenching of the tryptophanyl residue,

whether by solute species or by functional groups intrinsic to the protein. Thus, the activation energies associated with both of these processes contain significant contributions from the dynamic properties of this solvation shell and its influence on the structural flexibility of the surface regions of the protein (Bushueva et al., 1980).

ACKNOWLEDGMENTS

D.R.J. thanks the President's NSERC fund of the University of Saskatchewan for funding a postdoctoral fellowship. D.R.D. thanks NSERC for provision of a graduate fellowship.

Registry No. RNase T1, 9026-12-4; TCE, 115-20-8; Trp, 73-22-3; CsCl, 7647-17-8; KI, 7681-11-0; acetic acid, 64-19-7; acrylamide, 79-06-1.

REFERENCES

Altekar, W. (1977a) *Biopolymers 16*, 341.
Altekar, W. (1977b) *Biopolymers 16*, 369.
Arata, Y., Kimura, S., & Matsuo, H. (1979) *Biochemistry 18*, 18.
Barenboim, G. M., Domanskii, A. N., & Turoverov, K. K. (1969) *Luminescence of Biopolymers and Cells*, Plenum Press, New York.
Bent, D. V., & Hayon, E. (1975) *J. Am. Chem. Soc. 97*, 2612.
Berendsen, H. J. C. (1975) in *Water—A Comprehensive Treatise* (Franks, F., Ed.) Vol. 5, Plenum Press, New York.
Bernhardt, J., & Pauly, H. (1977) *J. Phys. Chem. 81*, 1290.
Burstein, E. A., Vedinkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263.
Bushueva, T. L., Busel, E. P., & Burstein, E. A. (1978) *Biochim. Biophys. Acta 534*, 141.
Bushueva, T. L., Busel, E. P., & Burstein, E. A. (1980) *Arch. Biochem. Biophys. 204*, 161.
Campbell, M. K., Shipp, S., & Jantzen, E. (1976) *Biochem. Biophys. Res. Commun. 72*, 1014.
Careri, G., Fasella, P., & Gratton, E. (1979) *Annu. Rev. Biophys. Bioeng. 8*, 69.
Demas, J. N., & Crosby, G. A. (1971) *J. Phys. Chem. 75*, 991.
Demmer, D. R., James, D. R., Steer, R. P., & Verrall, R. E. (1985) *Biochemistry* (submitted for publication).
Dickerson, R. E., & Geis, I. (1969) *The Structure and Action of Proteins*, Harper and Row, New York.
Eaglund, D. (1975) in *Water—A Comprehensive Treatise* (Franks, F., Ed.) Vol. 4, Plenum Press, New York.
Eftink, M. R. (1983) *Biophys. J. 43*, 323.
Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672.
Eftink, M. R., & Ghiron, C. A. (1977) *Biochemistry 16*, 5546.
Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199.
Einstein, A. (1906) *Ann. Phys. (Leipzig) 19*, 371.
Epinatjeff, C., & Pongs, O. (1971) *Eur. J. Biochem. 26*, 434.
Galley, J. A., & Edelman, G. M. (1962) *Biochim. Biophys. Acta 60*, 499.
Grinvald, A., & Steinberg, I. Z. (1976) *Biochim. Biophys. Acta 427*, 663.
Halle, B., Anderson, T., Forsén, S., & Lindman, B. (1981) *J. Am. Chem. Soc. 103*, 500.
Hershberger, M. V., Maki, A. H., & Galley, W. C. (1980) *Biochemistry 19*, 2204.
Huheey, J. E. (1978) *Inorganic Chemistry. Principles of Structure and Reactivity*, 2nd ed., Harper and Row, New York.
James, D. R., Demmer, D. R., Verrall, R. E., & Steer, R. P. (1983) *Rev. Sci. Instrum. 54*, 1121.
James, D. R., Demmer, D. R., Steer, R. P., & Verrall, R. E. (1985) *Biochemistry* (submitted for publication).
Kirby, E. P., & Steiner, R. F. (1970) *J. Phys. Chem. 74*, 4480.
Kuntz, I. D., Jr., & Kauzmann, W. (1974) *Adv. Protein Chem. 28*, 239.
Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
Lakowicz, J. R., & Weber, G. (1973) *Biochemistry 12*, 4161.
Lakowicz, J. R., Maliwal, B. P., Cherek, H., & Balter, A. (1983) *Biochemistry 22*, 1741.
Lehrer, S. S. (1970) *J. Am. Chem. Soc. 92*, 3459.
Longworth, J. W. (1968) *Photochem. Photobiol. 7*, 587.
Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) Plenum Press, New York.
Lumry, R. (1973) *J. Food Sci. 38*, 744.
Lumry, R., & Hershberger, M. V. (1978) *Photochem. Photobiol. 27*, 819.
Mahler, H. R., & Cordes, E. H. (1971) *Biological Chemistry*, 2nd ed., Harper and Row, New York.
Meech, S., & Phillips, D. J. (1983) *Photochemistry 23*, 193.
O'Grady, M. (1984) B.S. Honour's Thesis, University of Saskatchewan.
Osterman, H. L., & Walz, F. G., Jr. (1978) *Biochemistry 17*, 4124.
Osterman, H. L., & Walz, F. G., Jr. (1979) *Biochemistry 18*, 1984.
Parker, C. A., & Rees, W. T. (1960) *Analyst (London) 85*, 587.
Pigauli, C., & Gerard, D. (1984) *Photochem. Photobiol. 40*, 291.
Steiner, R. F., & Kirby, E. P. (1970) *J. Phys. Chem. 74*, 4480.
Stryer, L. (1966) *J. Am. Chem. Soc. 88*, 5708.
Takahashi, K. (1971) *J. Biochem. (Tokyo) 70*, 946.
Takahashi, K. (1973) *J. Biochem. (Tokyo) 74*, 1279.
Uchida, T., & Egami, F. (1971) *Enzymes, 2nd Ed. 4*, 205.
Valeur, B., & Weber, G. (1977) *Photochem. Photobiol. 25*, 441.
Walz, F. G., Jr., & Hooverman, L. L. (1973) *Biochemistry 12*, 4846.
Weinryb, I., & Steiner, R. F. (1970) *Biochemistry 9*, 135.
Yamamoto, Y., & Tanaka, J. (1970) *Biochim. Biophys. Acta 207*, 522.
Yguerabide, J. (1972) *Methods Enzymol. 26*, 498.
Zabinski, M., & Walz, F. G., Jr. (1976) *Arch. Biochem. Biophys. 175*, 558.