Journal of Fluorescence, Vol. 2, No. 3, 1992

Tryptophan Phosphorescence of Ribonuclease T_1 as a Probe of Protein Flexibility

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Received July 20, 1992; revised October 12, 1992; accepted October 15, 1992

The phosphorescence properties of Trp-59 of ribonuclease T_1 from *Aspergillus oryzae* were monitored as a function of temperature, pH, salt concentration, and complex formation with substrate analogues and, also, in the presence of glycerol as viscogenic cosolvent. The results establish a rough correlation between the internal flexibility of the macromolecule, as derived from the triplet lifetime, and its stability (ΔG or T_m) toward unfolding. Below 10°C or in 70% glycerol the triplet probe distinguishes at least two gross conformations for the protein, which are characterized by a large difference in phosphorescence lifetime. It is pointed out that such structural heterogeneity does not correspond with the heterogeneity inferred from fluorescence decays and acrylamide quenching rates. Further, implications of the phosphorescence data with regard to the interpretation of acrylamide quenching of fluorescence are discussed.

KEY WORDS: Tryptophan; phosphorescence; ribonuclease T_1; protein flexibility.

INTRODUCTION

Ribonuclease T_1 from *Aspergillus oryzae* (RNase T_1) is one of the best-characterized single tryptophan proteins. The crystal structure at high resolution is available for the free protein [1] and its complexes with inhibitors 2′-GMP and 3′-GMP [2–6] and NMR [7–8] and Raman [9] studies confirm a great similarity between the solution structure and X-ray coordinates. Because of its small size and the wealth of structural information available, RNase T_1 has been a model for numerous fluorescence studies, both experimental [10] and theoretical [18–20], most of them aimed at the difficult task of correlating the fluorescence properties of Trp residues in proteins to the molecular characteristics of their environment.

Trp-59 in RNase T_1 is characterized by a high fluorescence yield and a blue-shifted spectrum bearing some degree of vibrational fine structure [10], features which are consistent with its location in a buried nonpolar region of the macromolecule. Its fluorescence is quenched by acrylamide at a rate that is markedly reduced with respect to solvent exposed residues [12,13,17,21]. This property and a limiting fluorescence anisotropy nearly equivalent to that of indole derivatives in vitrified solutions [19,22,23] suggest that the chromophore is surrounded by rather compact, rigid segments of the polypeptide. At ambient temperature and pH 5.5 the fluorescence decay obeys an exponential law, but it becomes nonexponential at pH levels higher than 6.5 or temperatures above 30°C [12,15]. The biexponential decay at neutral pH and distinct bimolecular quenching rate constants for each lifetime component have been interpreted as evidence of conformational heterogeneity in the solution structure of this enzyme [12,15].

An analysis of multiple fluorescence lifetimes in terms of heterogeneity in the globular structure of the biopolymer may not always be straightforward. Trp itself and most of its analogues and short peptides invar-

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1053-0509/92/0900-0157$06.50/0

iably display nonexponential decays. It was also pointed out [15] that other origins of heterogeneity in the decay kinetics may be found in solvent relaxation and dynamic quenching reactions. Similarly, concerns have been expressed over the common interpretation of acrylamide quenching rate constants in terms of frequencies and amplitudes of fluctuations in the folded structure. Clearly, a quantitative appraisal of quenching rates in terms structural fluctuations of the macromolecule requires knowledge of both the quenching mechanism and the pathways of quencher migration. While it is generally assumed that quenching of buried Trp residues by acrylamide involves migration of the quencher through the protein matrix and collision with the indole ring, alternative models have been proposed that contemplate either efficient quenching at distances beyond van der Waals contact [24,25] or transient unfolding of the protein, exposing the indole ring to the quencher in the aqueous phase [25].

In recent years, the detection of Trp phosphorescence from proteins in fluid solution at room temperature has provided an alternative and, in some respects, complementary approach for studying protein dynamics and structure. In particular, the unique sensitivity of the triplet lifetime to the local viscosity [26] has proved to have interesting potentialities both for reporting on the dynamical features of the chromophore's environment and for resolving even subtle conformational differences that may be induced by binding of substrates and allosteric effectors or change in conditions of the medium [27–30].

It is the aim of the present investigation to obtain independent dynamic information on the environment of Trp-59 of RNase T_1 by means of its phosphorescence decay kinetics. We wish to inquire into the homogeneity of the solution structure and, more generally, what correlation exists between the flexibility of the native fold and its stability to unfolding. In addition, from a close comparison with acrylamide fluorescence quenching rates, we wish to test the validity of the proposed quenching models. In this paper we report the phosphorescence properties of Trp-59 as a function of temperature, pH, salt concentration, and binding of the inhibitors 2′-GMP and 3′-GMP and of the presence of glycerol as a viscogenic cosolvent. The results establish a good correlation between the internal flexibility and the stability of the macromolecule toward unfolding. At a low temperature/high solvent viscosity, the triplet probe distinguishes at least two conformations for the protein. These, however, do not correspond with the structural heterogeneity inferred from fluorescence studies.

MATERIALS AND METHODS

Lys-25-Ribonuclease T_1 (Lys 25-RNase T_1) was obtained from Calbiochem Corporation (San Diego, CA). Suprapur KCl and NaCl were purchased from Merck (Darmstadt). Guanosine 2′-monophosphate (disodium salt) and guanosine 3′-monophosphate (trisodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest-purity grade available from commercial sources. Water, doubly distilled over quartz, was further purified by Millipore system. The purity of enzyme was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Prior to luminescence measurements, the enzyme was dialyzed for at least 24 h (under nitrogen) against the chosen buffer (0.05 *M* NaOAc/acetic acid, pH = 4.5; 0.05 *M* sodium cacodylate, pH = 5–7; and 0.05 *M* TES, pH = 7–8; all containing 10 m*M* EDTA). The concentration of RNase T_1 was calculated by using an absorbance of 1.67 at 278 nm for a 1 mg/ml solution [31]. All luminescence experiments were carried out at a protein concentration of 1×10^{-6} *M*.

To obtain reproducible phosphorescence data in fluid solution, it is of paramount importance to remove thoroughly all dissolved oxygen. The procedure followed to obtain satisfactory deoxygenation was described in a previous report [32].

A conventional homemade instrument was employed for all phosphorescence intensity and spectra measurements [33]. The excitation provided by a Cermax xenon lamp (LX 150 UV, ILC Technology) was selected by a 250-mm grating monochromator (Jobin-Yvon, H25) and the emission was detected with an EMI 9635 QB photomultiplier. Phosphorescence decays in fluid solution were obtained following pulsed excitation by a frequency-doubled flash-pumped dye laser (UV 500 M, Candela) with a pulse duration of 1 μs and an energy per pulse typically of 1–10 mJ. The decay of tryptophan phosphorescence was monitored at 430 nm by an electronic shutter arrangement permitting the emission to be detected 1 ms after the excitation pulse. The decaying signal was digitized by an Applescope system (HR-14, RC Electronics) and then transferred to an Apple II computer for averaging. Subsequent analysis of decay curves in terms of a sum of exponential components was carried out by a nonlinear least-squares fitting algorithm, implemented by the program Global Analysis (Global Unlimited, LFD University of Illinois, Urbana). All decay data reported here are averages obtained from three or more independent measurements.

RESULTS

Phosphorescence Decay Kinetics in Fluid Solution

In thoroughly deoxygenated solutions the phosphorescence emission of Trp-59 is readily observable in buffer up to and above room temperature. In 50 m*M* cocadilate buffer, pH 5.5, the phosphorescence decay at 20°C is strictly exponential, with a lifetime of 32 ± 1 ms. This lifetime is larger than the values of 3.5 ms reported by Imakubo and Kai [34] and 14 ms by Vanderkooi *et al.* [35] and probably reflects different pH and deoxygenating conditions. Another parameter that affects the value of τ is the protein concentration. Above 5 μ*M* one observes a gradual but progressive reduction in τ. Since free tryptophan in solution was found to be an efficient quencher, we presume that the concentration dependence of τ is indicative of intermolecular quenching by ground-state Trp-59. For this reason all phosphorescence measurements were carried out at a protein concentration of 1 μ*M*.

Upon lowering the temperature or increasing the solvent viscosity, with the addition of glycerol, the decay slows down and becomes distinctly heterogeneous (Fig. 1). The parameters derived from single- and double-exponential fitting of decays in buffer and in 70% glycerol/buffer (w/w) at various temperatures are collected in Table I. Fitting statistics and plot of residuals (Fig. 2) indicate that a two-component model describes quite satisfactorily the data in both solvents. The short lifetime has an amplitude that ranges from 0.15 to 0.17 in buffer, and from 0.14 to 0.31 in glycerol/buffer, and a magnitude five to six times smaller than the long lifetime. In Table I we also report the steady-state phosphorescence intensity, P, normalized by the triplet lifetime and the fluorescence intensity, F. With multiple decaying components this becomes $\Sigma P_i/\tau_i F, P_i$ being the fractional phosphorescence intensity with lifetime τ_i.

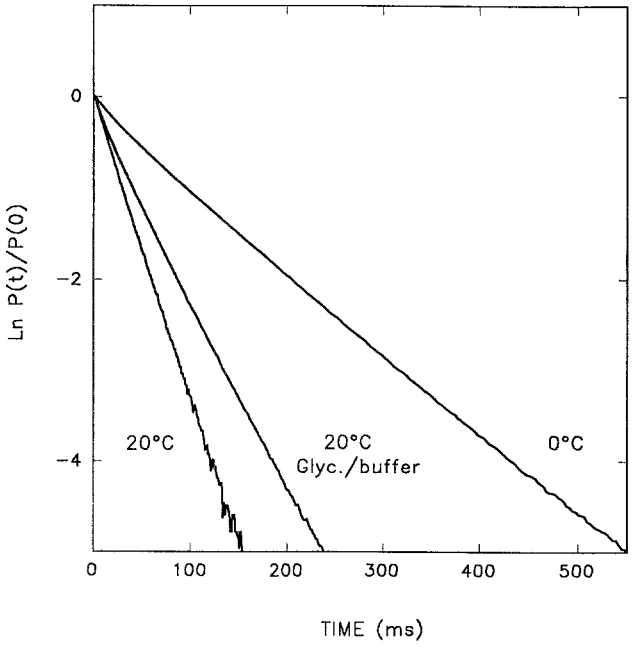

Fig. 1. Phosphorescence decay kinetics of Trp-59 of RNase T_1 in buffer (50 m*M* cacodylate, pH 5.5) and glycerol/buffer (70/30, w/w) at some selective temperatures. Nonexponentiality in the decay is noticeable at low temperatures and in viscous solvents.

Table I. Temperature Dependence of Kinetic Parameters Derived from Biexponential Fitting of Phosphorescence Decays in Buffer (50 mM Cacodylate, pH 5.5) and in Glycerol/Buffer (70/30, w/w)

T(°C)	τ_1(ms)[a]	τ_2(ms)[a]	α_2	χ^2	$\Sigma P_i/\tau_i F^{a,b}$
		Buffer			
0	25	107	.83	1.0	.98
10	11	68	.85	1.1	.99
20	31			1.2	1.02
30	25			1.1	1.05
		Glycerol/Buffer			
−30	230	1294	.69	1.0	1.03
−20	198	702	.68	1.0	.97
−10	101	351	.71	1.0	1.04
0	34	130	.72	1.0	.98
10	28	100	.77	1.0	1.02
20	12	48	.75	1.1	.99
30	7	30	.86	1.2	.95

[a] The standard error in the values of τ is typically 2-3% while the error in intensity ratios is 4–5%.

[b] The $\Sigma P_i/\tau_i F$ is relative to the value obtained at low temperature in absence of thermal quenching of the phosphorescence.

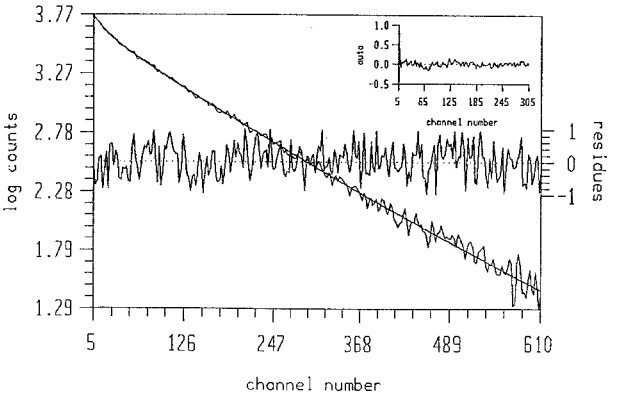

Fig. 2. A typical fitting of heterogeneous phosphorescence decays in terms of two exponential components. The data refer to 0°C, pH 5.5.

Assuming that the intersystem crossing yield is not sensibly affected by the change in experimental conditions [36], the constancy of $\Sigma P_i/\tau_i F$ from -30 to $+30$°C implies that, within this temperature range, despite the considerable thermal quenching of P, all molecules are detectably phosphorescent and that the decay is representative of the whole protein sample.

For a single Trp protein, multiple triplet lifetimes are unambiguous evidence of conformational heterogeneity of the macromolecule, with conformers sufficiently stable to preserve their identity for times of the order of τ or longer. According to the triplet probe the two conformers differ in flexibility in the neighborhood of Trp-59, and from the relationship between τ and microviscosity [26], one estimates local viscosities, at 0°C in buffer, of 757 and 8909 P, respectively.

At a high temperature and low solvent viscosity the decay becomes exponential. This observation may be rationalized in two ways: either conformer interconversion occurs rapidly during the triplet lifetime or the conformer equilibrium shifts toward the dominant species. The latter hypothesis appears more likely. With increases in temperature, the amplitude of the short-lived component, both in buffer and in glycol/buffer, decreases steadily. Moreover, were τ in buffer the result of fast exchange, $1/\tau = \alpha_1/\tau_1 + \alpha_2/\tau_2$, one would expect a shorter lifetime than actually found. For instance, under rapid exchange with the data at 20°C in glycerol/buffer, one would calculate a lifetime of 20 ms. This value is lower than the 32 ms found in buffer, despite the fact that phosphorescence lifetimes are generally 60–70% longer in the viscous solvent. Hence, if glycerol reveals separate conformers even at warm temperatures, it is presumably for its increased stabilization of the short-lived species rather than for slowing down viscosity-dependent structural isomerizations.

Effect of pH, Monovalent Salts, and Substrate Analogue Binding on the Phosphorescence Lifetime

pH and concentrated NaCl or KCl have a marked influence on the stability of RNase T_1 [38,39]. Should the change in medium conditions affect principally the native fold, one would predict stabilization/destabilization to be associated with a more rigid/flexible protein structure. Both pH and NaCl (or KCl) have considerable effects on the phosphorescence decay kinetics of RNase T_1. The parameters derived from fitting phosphorescence decays at 0°C and 50 mM buffers are given in Table II. For convenience, the average lifetime, $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$, is also displayed in Fig. 3.

Between pH 4 and pH 8, τ_{av} reaches a maximum at 5.5 and decreases steeply on either side of it. Although the pH range was spanned with three buffering salts (acetate, cocadilate, and TES), measurements in overlap regions excluded specific buffer effects on the decay. The bell-shaped pH dependence of τ_{av} resembles somewhat the unfolding free energy profile [39] except that the latter has a broad maximum at 4.5, 1 pH unit lower. A change in pK of an internal amino acid residue upon unfolding can easily shift the ΔG_{max} by 1 pH unit either way.

The pH dependence of the preexponential terms (Table II) indicates that below pH 7 the conformer equilibrium is largely independent of pH, but between pH 7 and 8 the short-lived component becomes dominant and the decay at the highest pH is practically homogeneous. We recall that at alkaline pH the protein is only marginally stable, and if conformer interchange is facilitated by the lower stability, the loss of heterogeneity could be due to rapid isomerization.

Large quantities of NaCl or KCl alter τ and render the phosphorescence decay strictly exponential. Figure 3 shows that the phosphorescence lifetime decreases somewhat for salt concentrations below 0.2 M but increases by three-fold between 0.2 and 1 M, to remain roughly constant thereafter up to 2 M. It is interesting to note that the two opposite trends of τ roughly coincide with concentration ranges where one and four ions are presumably bound to the macromolecule [38]. According to microcalorimetric measurements a marked stabilization of the protein toward unfolding, i.e., an increase in melting temperature, occurs mostly in the higher concentration range, the same range where the triplet probe reports a considerable increase in rigidity of the polypeptide. On the other hand, the somewhat greater decay rate, i.e., greater flexibility, at moderate salt activities, 0–0.2 M, constrasts with an increase, even if modest, in the enthalpy of unfolding. We must point out that the discrepancy might only be apparent. There is the possibility that the loss of heterogeneity in the decay in the presence of NaCl is imputable to salt-assisted rapid isomerization of the stable conformers. In such a case what we measure is the rapid exchange lifetime, $1/\tau = \alpha_1/\tau_1 + \alpha_2/\tau_2$, which is generally smaller than $\tau_{av} = \alpha_1\tau_1 + \tau_2\tau_2$. Of course, an alternative rationalization is that even if binding of the first ion yields, on the whole, a more stable structure, the local flexibility about Trp-59 has increased.

At pH 5.3, 2′-GMP and 3′-GMP bind to RNase T_1 with dissociation constants of 4 and 11 μM, respectively [40]. Phosphorescence decays were obtained at 98% saturating concentrations of the substrate analogues (20 and 60 mM for 2′-GMP and 3′-GMP, respectively) across

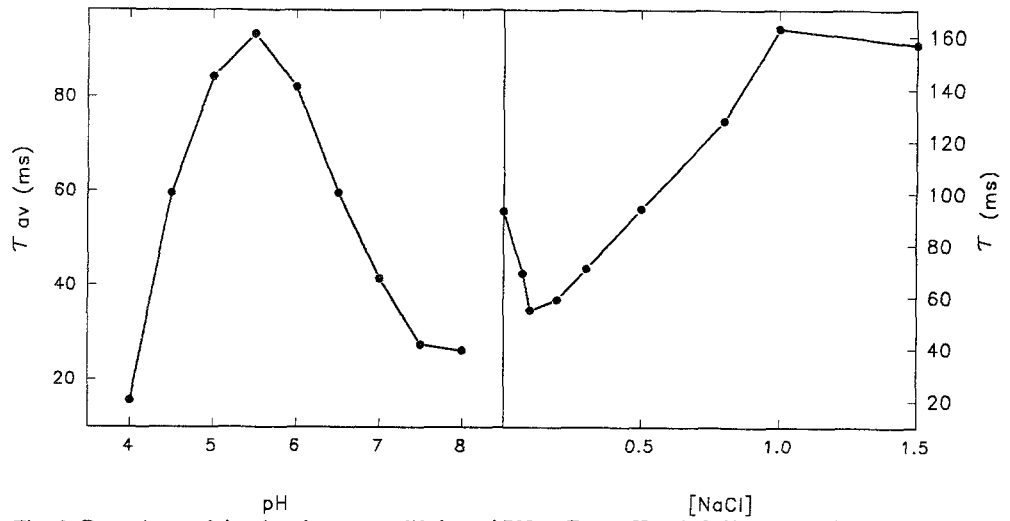

Fig. 3. Dependence of the phosphorescence lifetime of RNase T_1 on pH and NaCl concentration. $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$ refers to the data in Table II.

Table II. pH Dependence of Phosphorescence Lifetimes (ms)

pH[a]	τ_1	τ_2	α_2	χ^2
4.0	6	18	.80	1.1
4.5	15	70	.81	1.0
5.0	21	100	.80	1.1
5.5	25	107	.83	1.0
6.0	23	95	.82	1.0
6.5	18	70	.80	1.0
7.0	15	48	.80	1.1
7.5	21	180	.04	1.0
8.0	18	180	.05	1.0

[a] The buffers used are: acetate (pH 4.0–5.5), cacodylate (pH 5.5–7.0), TES (pH 7.0–8.0).

Table III. Temperature Dependence of the Phosphorescence Lifetimes (ms) of RNase T1 Complexed to 2'GMP and 3'GMP[a]

T (°C)	RNase T1 τ_{av}(ms)	RNase T1:2'GMP τ(ms)	RNase T1:3'GMP τ(ms)
0	93	60	80
10	59.5	35	48
20	31	23	28
30	25	14	18

[a] The average lifetime of the free protein (τ_{av} is included for comparison.

the temperature range from 0 to 30°C. As with salts, all decays are well approximated to a single exponential law. The triplet lifetime is reported in Table III, where the average lifetime of the free protein is included for comparison. Complex formation with the substrate analogues reduces τ, the change being greater with the more tightly bound 2'-GMP. Additional experiments at various ligand concentrations and in glassy solutions were carried out to test whether the reduction in τ could possibly be attributed to quenching reactions by free or bound ligand or trace impurities in commercial nucleotides. First, we note that binding is required for the change in τ, as the difference in τ was found to be roughly proportional to the fraction of complexed protein. Further, as τ remains constant at ligand concentrations three- to fourfold in excess of 98% saturation, quenching by excess free ligand or impurities is ruled out. Finally, a direct triplet quenching interaction by bound nucleotides was excluded because in low-temperature (190 K) propylene glycol/buffer (50/50, v/v) glasses, where protein motions are negligible, τ is unaffected by complexation.

DISCUSSION

High-resolution X-ray structures of Lys-25 RNase T_1 and of its complexes with 2'-GMP and 3'-GMP [1–6] show the indole side-chain of Trp-59 wrapped within the central β sheet tucked away from the solvent interface except for a conserved five-water molecule chain linking the ring nitrogen to bulk water. This residue is characterized by one of the smallest thermal factors [1,41] of the entire polypeptide, an indication that the aromatic ring lies in a rather immobile pocket of the macromolecule. The observation of long-lived Trp phosphorescence at ambient temperature confirms a rigid chromophore's environment. According to the phospho-

rescence probe, however, the solution structure of the free protein is more complex in that one can distinguish at least two stable conformations of the macromolecule. Both the slow isomerization rates and the extent to which they differ in the flexibility of the internal β sheet support the notion that these are quite distinct, stable macrostates of the protein. Whether, by analogy to myoglobin [42], they also have diverse functional properties will be interesting to determine. As for the actual number of states or their structural differences, information is scanty. We note simply that the equilibrium is clearly affected by pH, temperature, and solvent composition. As glycerol tends to stabilize conformations of proteins with less surface area [43], we presume that the short-lived, more flexible component, which is better represented in the presence of glycerol, is also more compact.

Heterogeneity in the solution structure of RNase T_1 has been inferred from other spectroscopic evidence. The fluorescence of Trp-59 displays heterogeneity both in its decay kinetics [12,13] and in the acrylamide bimolecular quenching rate [12]. Optically detected magnetic reasonance "hole burning" experiments in low-temperature glasses [44] reported inhomogeneous broadening of the phosphorescence spectrum due to distinct Trp-59 environments. More recently, multiple crosspeak patterns in the H-reasonance of Pro-55 and of one unidentified His provide evidence of multiple local conformations which are slowly exchanging in the NMR time scale [7].

Although structural heterogeneity in RNase T_1 was inferred from the decay of both the excited singlet and the triplet state of Trp-59, it is important to recognize that no correspondence is expected or found among the conformers resolved by prompt and delayed luminescence. Fluorescence is not heterogeneous at pH 5.5. Further, temperature has an opposite effect on the decay behavior of prompt and delayed emission. At 0°C and pH 5.5, the phosphorescence is heterogeneous, but at 20°C there is a sole dominant component. In contrast, the fluorescence decay becomes heterogeneous only above 30°C [15].

In recent years, both emissions have become increasingly popular for studying conformational substates in proteins, and it may therefore be instructive to consider the main differences between the two approaches in terms of probing protein structure. First, we point out that fluorescence and phosphorescence lifetimes are sensitive to different aspects of the protein environment. In the absence of specific intramolecular interactions with lower-energy chromophores or redox centers carried by prosthetic groups, the fluorescence lifetime (τ_F) of Trp in proteins is governed by the polarity and chemical nature of the groups in its solvation shell [15,45,46]. Groups such as carbonyls, protonated amines and imidazole, –SH, and –S–S– are most effective in deactivating the fluorescent state. If exception is made for the perturbation induced by disulfide bridges [47,48], the phosphorescence lifetime (τ_P), rather than by solvent composition, is influenced predominantly by local viscosity [26]. Thus, in proteins, τ_P will report essentially on the flexibility of the embedding structure. It is evident that, whereas τ_F will be affected by even minor structural changes, such as side-chain rotations, which bring quenching groups into proximity with the indole ring, differences in τ_P will, in general, monitor more extensive structural rearrangements of the polypeptide because changes in flexibility, especially of core regions, entail different bonding patterns. The other fundamental difference between prompt and delayed emission concerns the time scale of observation. On the millisecond-to-second time scale of phosphorescence, as compared to the nanosecond time scale of fluorescence, only conformers separated by large activation free energies (>60 kJ mol^{-1}) will be sufficiently long-lived to be detected as separate entities.

Recent experimental and theoretical work (Ref. 42 and references therein) on the conformational energy landscape and motions in proteins has established that conformational substates can be roughly classified into a hierarchy with several tiers. At the highest levels, macrostates are few, interconvert slowly, and, in the case of myoglobin, were shown to possess distinct functional properties. Lowering the temperature/increasing the solvent viscosity results in the freezing-out of substates of ever-lower tiers in a stepwise manner, namely, in the appearance of a wider distribution of molecular subspecies with distinct structural and functional properties. Within this framework it is clear that τ_P can, at best, distinguish among macrostates of the protein near the top of the hierarchy, whereas τ_F will resolve substates of far lower tiers. If fluorescence and phosphorescence techniques are complementary in this respect, there are, however, complexities underlying fluorescence decays which can make interpretation of lifetimes in terms of protein structure a difficult task. For example, complications arise when nonexponential decays are due to spectral red shifts or dynamic quenching [15]. Perhaps partly as a result of this, in RNase T_1 and in other proteins, it was found that fluorescence decays display a greater heterogeneity at high temperatures and/or denaturant concentrations [15], conditions that, by favoring transitions among substates, should instead lead to more homogeneous average structures. In contrast, the observation that the triplet probe resolves distinct macrostates of RNase T_1 only upon lowering the temperature fits

nicely with the picture of a hierarchical structure of substrates. The significance of the phosphorescence lifetime as a structural parameter is again emphasized in RNase T_1 by the close correspondence between the internal flexibility of the macromolecule, as reported by the triplet probe, and other indirect measures of its dynamic properties. Mention was already made of the large value of τ_P for a site with quite small thermal factors in the crystal structure. Of even greater relevance is the finding that the internal flexibility derived by τ_P mimicks rather closely the stability profile toward unfolding as a function both of pH and of salt concentration.

A time-dependent fluorescence anisotropy study with the substrate analogues 2′-GMP and 3′-GMP derived rotational correlation times for independent indole motions, which were interpreted in terms of more hindered rotation of the indole ring upon complex formation [14]. From the temperature dependence of the rotational constant, activation enthalpy barriers of about 20 kJ mol^{-1} were derived for the free protein and 2′-GMP complex and 7 kJ mol^{-1} for the 3′-GMP complex. Although there may not be a simple correlation between rotational freedom and triplet lifetime, these findings are at variance with both the increased flexibility inferred from the reduction in τ and the activation enthalpies derived from the temperature dependence of $1/\tau$ (Fig. 4). The latter are about 50 kJ mol^{-1}, roughly the same for all three

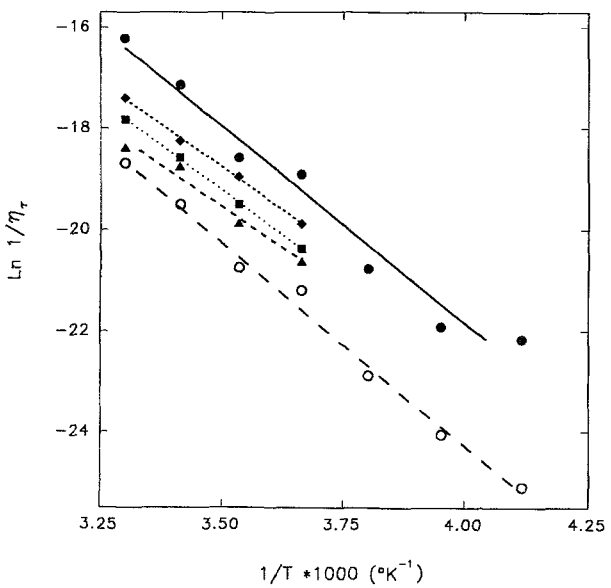

Fig. 4. Arrhenius plot of the effective viscosity, η_τ, at the site of Trp-59 derived from phosphorescence lifetimes collected in Tables I and II ($\eta_\tau \approx 3.8 \times 10^5\ \tau^{1.69}$ [26]). RNase T_1 in buffer (■) and in glycerol/buffer (○ and ● for τ_1 and τ_2, respectively) and its complexes with 2′-GMP (△) and 3′-GMP (▲). Activation enthalpies are 13.7 (■), 12.8 (△), 13.0 (▲), 18.1 (○), and 14.9 (●) kcal mol^{-1}.

protein forms. We note that the anisotropies in question are the lowest among a set of previous [22,23] and subsequent [19] reports. Further, as pointed out by the authors, a significant contribution to the total fluorescence, especially in the complexes, may come from Tyr-42 or Tyr-45 and/or the guanine base due to their participation in a stacked configuration [2–4]. Also, a comparison of crystallographic structures between free and inhibitor bound forms (even if at different pH's) shows that structural rearrangements are confined to the flexible loop at the binding site of guanine and phosphate and that thermal factors of Trp-59 are essentially invariant [1,2]. Similar conclusions were drawn from NMR spectra of the protein in solution [7].

Structural Fluctuations and Acrylamide Quenching of Trp Fluorescence

A popular method that is believed to probe the frequency and amplitude of structural fluctuations in proteins is quenching the fluorescence of internal Trp residues with acrylamide [21]. Concern, however, has been expressed over the interpretation of reduced quenching rate constants in terms of hindered diffusion through the protein matrix, since it is based on the assumption that acrylamide penetrates the globular structure and collides with the chromophore. The validity of the penetration model is questioned because (i) there is the possibility that the quenching interaction may be of a longer range and not require van der Waals contact with the indole ring [12,53] and (ii) given the high quencher concentrations involved and the propensity of acrylamide to bind to proteins, the concentration inside the macromolecule might be greater than in bulk, and some kind of quasi-static quenching may become important [49,54,55]. Also, another concern of binding or preferential partitioning of acrylamide is the extent to which the native fold may be perturbed by it.

According to the penetration model, fluctuations in the polypeptide structure are responsible for creating transient holes that allow quencher migration to the indole ring. Hence, the bimolecular quenching rate constant, k_q, provides a measure of the resistance, or effective viscosity to diffusion (η_d), along pathways that lead from the solvent to the chromophore. Although protein motions relevant for determining k_q and τ_P may not, in general, refer to the same region of the macromolecule, if collisions between acrylamide and the indole ring are required for quenching, then the solute must diffuse through the inner core of the chromophore's environment, and one expects $\eta_d \geq \eta_\tau$. Further, since only high-amplitude fluctuations are effective for quencher migra-

tion, one would predict $\Delta H^*(d) \geq \Delta H^*(\tau)$, where ΔH^* is the activation enthalpy for the underlying protein motions. Comparing fluorescence quenching and phosphorescence data on RNase T_1 at pH 5.5, we deduce that $\eta_d \approx 40$ cP (from the reduction of kq relative to free Trp in buffer [21]), as opposed to $\eta_\tau \approx 1.1 \times 10^5$ cP and $\Delta H^*(d) = 8$ kcal mol^{-1} [13] versus 13.7 kcal mol^{-1} for $\Delta H^*(\tau)$ (Fig. 4). The large discrepancy between both effective viscosities and activation enthalpies suggests that protein motions in the neighborhood of Trp-59 are much more hindered than might be presumed from acrylamide quenching.

Support for the penetration model has also been sought from the relative insensitivity of k_q to bulk viscosity. Eftink and co-workers [13,50] report that in a 50% glycerol solution, where the viscosity is roughly 12-fold greater than in buffer, k_q decreases by a factor of only 3.8, instead of 12, if diffusion is limited by the solvent viscosity (for an unfolding model $k_q = k_d \cdot k_{un}$, where k_d is the diffusion rate constant in the solvent and k_{un} is the equilibrium unfolding constant). Our experience with solvent viscosity effects on the internal flexibility of proteins (paper in preparation) tells us that there is no predictable pattern and that changes in flexibility depend, among other things, on the nature of the viscogenic cosolvent. At 20°C a 70% glycerol/water solution has a viscosity 20 times greater than that of water, but according to the τ_P of RNase T_1, there is only a 50% increase in the local protein viscosity η_τ. From this finding we conclude once again that, should the rigidity of the environment of Trp-59 limit the encounter rate with acrylamide, glycerol effects on k_q would be barely detectable. The greater influence of bulk viscosity on k_q suggests that acrylamide migration perhaps samples peripheral and more flexible regions of the macromolecule which are subject to a greater coupling with solvent motions.

If the above considerations question the direct applicability of the penetration model for acrylamide quenching of RNase T_1 fluorescence, we point out that the alternative, transient unfolding model [25], in which the chromophore is briefly exposed to the aqueous phase where the quenching reaction is supposed to occur, appears even less realistic. Exposure of the indole ring to the aqueous phase would have dramatic effects on the phosphorescence lifetime, as, in water, τ_P is about 16 μs [51,52]. With a rough, orders of magnitude calculation, we demonstrate that the unfolding mechanism is not consistent with the measured value of τ_P. Assuming only two states of the protein, native and partially unfolded, in order to account for the decrease in k_q by a factor of 40 relative to Trp in buffer [13,50], one requires that the unfolded fraction of protein be at least 1/40. Because the conformational transition must be rapid, on the nanosecond time scale, the phosphorescence lifetime should be calculated as for fast exchange, $1/\tau = \alpha_n/\tau_n + \alpha_u/\tau_u$, where α and τ are the fractions of macromolecules and their lifetime in the native, n, and unfolded, u, states, respectively. Even with conservative estimates, i.e., $\tau_n = 1$ s, $\tau_u = 20$ μs, and $\alpha_u = 0.025$, one obtains $\tau = 0.8$ ms, as opposed to the 32 ms actually found. A theoretical model for acrylamide quenching based on such a gated mechanism was recently shown to be unsuitable to fit quenching data on RNase T_1 [17].

In conclusion, the phosphorescence of Trp-59 points out inconsistencies with both penetration and rapid unfolding models. Probably a more realistic picture of acrylamide quenching is one where solute permeation on the nanosecond time scale is limited to peripheral mobile regions of the globular structure. During the fluorescence lifetime the distance of closest approach to the indole ring may be sufficiently close (1–2 Å) for the quenching reaction to be competitive with the intrinsic decay rate. In general, even barring artifacts due to acrylamide partitioning/binding, it may be hard to know to what extent a reduced value of k_q is determined by hindered diffusion, as opposed to the distance of closest approach. A similar conclusion was reached for the inefficient quenching of phosphorescence of buried Trp-109 in alkaline phosphatase by O_2 and NO [24], small neutral molecules that were thought to permeate even tight protein cores freely.

ACKNOWLEDGMENTS

The authors are grateful to Prof. C. N. Pace and Prof. U. Hahn for their generous gift of RNase T_1, to Ms. Neri Claudia for typing the manuscript, and to Mr. Favati Lamberto for his technical assistance.

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