Phosphorescence Lifetime of Tryptophan in Proteins

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ABSTRACT: This investigation enquires into the factors that are responsible for the wide range of room-temperature Trp phosphorescence lifetimes (τ) in proteins. By exploiting the enhanced sensitivity and time resolution of phosphorescence measurements, experiments were conducted to evaluate the triplet quenching potential of each amino acid side chain. From the magnitude of the Stern−Volmer rate constant it is concluded that, among the amino acids, quenching reactions at 20 °C are quite effective with His, Tyr, Trp, cysteine, and cystine, with rate enhancements of 20 and 50 times when the side chains of Tyr and His, respectively, are in the ionized form. The distance dependence of the quenching interaction, estimated from the quenching of internal Trp residues in proteins separated from the amino acid in solution by a protein spacer of various thickness, emphasizes the very short-range nature of the process. The importance of these side chains, and to some extent that of the peptide linkage, as intrinsic quenchers of Trp phosphorescence in proteins was also confirmed with short synthetic peptides prepared appositely with only one type of these residues. Finally, very short (μs) phosphorescence lifetimes of Trp residues in proteins were shown to be invariably associated with the presence of Tyr or Cys in the immediate neighborhood of the chromophore. From a survey of the amino acids that are nearest neighbors to Trp in proteins and the corresponding value of τ it was established that, in the absence of His, Tyr, Trp, and Cys, τ is ≥ 1 ms and appears to reflect mainly the local fluidity of the protein structure. Otherwise, τ can be much shorter, and for bulky His, Tyr, and Trp side chains it seems to depend dramatically on the mutual chromophore−quencher orientation. In these cases the triplet decay kinetics is shown to be a complex function of temperature, pH, and flexibility of the protein site.

The phosphorescence emission of Trp residues in proteins has been the subject of numerous investigations. Before the report of phosphorescence in fluid solution (Saviotti & Galley, 1974), all of the work on protein phosphorescence was done in low-temperature polyol/buffer glass matrices. Under these conditions Trp residues exhibit well-resolved phosphorescence spectra that often distinguish chromophores in different environments. Their decay kinetics, on the other hand, are quite uniform, and in the absence of prosthetic groups, such as hemes and coenzymes, the excited-state lifetime, τ, is invariably between 5 and 7 s. Notable exceptions are Trp residues in proximity of disulfide bridges for which τ is shorter, even if its normal value is promptly re-established upon cystine reduction (Longworth, 1971). Raising the temperature above the glass transition brings the macromolecule in a fluid environment, and the transition from a rigid to a fluid matrix is generally associated with a considerable reduction in τ. The extent of the change, however, varies dramatically from one protein to another or between different residues in the same molecule (Strambini & Gabellieri, 1990). Thus, in contrast to rigid matrices, the room-temperature phosphorescence, RTP,[1] of proteins in aqueous solutions is characterized by a wide range of τ, from a few seconds to less than milliseconds. Because until now 1 ms was the typical time resolution of phosphorescence measurements, a number of Trp residues in proteins did not display RTP.

The RTP lifetime of Trp in proteins has found, in the last decade, numerous applications as an intrinsic monitor of protein structure/flexibility (Strambini, 1987, 1989; Calhoun et al., 1988; Cioni & Strambini, 1994). Of course, the usefulness and scope of this spectroscopic approach depend critically on the correct interpretation of τ and therefore on detailed knowledge of the factors that influence the decay kinetics of the triplet state in the protein environment. Until now it seemed appropriate to interpret the large variability of τ in proteins principally in terms of the differential mobility of protein sites, i.e., in terms of the effective local viscosity. This simple model was based on two kinds of experimental observations: (1) Intramolecular quenching reactions, at least in low-temperature glasses, are totally inefficient if exception is made for the interaction with disulfides. (2) τ of the free chromophore in fluid aqueous solution appeared to be pre-eminently a function of solvent viscosity. When measured in aqueous solutions at room temperature by the triplet−triplet absorption method of flash photolysis, τ was found to be very small, between 10 and 20 μs (Bent & Hayon, 1975; Santus et al., 1980). When the solvent viscosity (η) was increased from 10^4 to over 10^9 poise, the phosphorescence lifetime of various indole derivatives increased steadily from 20−30 ms to 6 s (Strambini & Gonnelli, 1985) independently of the solvent composition. Support for a strong correlation between τ and the local viscosity has come also from a wealth of observations with Trp residues in proteins. For example, chromophores that

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[1] Abbreviations: LADH, horse liver alcohol dehydrogenase; AP, alkaline phosphatase from *Escherichia coli*; PGK, phosphoglycerol kinase from yeast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*; PFK, fructose-6-phosphate kinase from *B. stearothermophilus*; HSOD, human superoxide dismutase from erythrocytes; HSA, human serum albumin; RNase T_1, ribonuclease T_1; NATA, *N*-acetyltryptophanamide; DTT, dithiothreitol; 3-PG, 3-phosphoglycerate; RTP, room-temperature phosphorescence; PG, propylene glycol.

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Table 1: Sequence of Synthetic Peptides

peptide	sequence
P1	Lys Trp Lys
P2	pyr Glu Trp Pro Arg Pro Gln Ile Pro Pro
P3	Phe Asp Met Trp Gly Met Tyr Asp
P4	Trp Gly Gly Gly Gly Thr His Ser Glu Trp Asn Lys Pro Ser Lys Pro Lys
P5	Phe Lys Asp Pro His Gly Leu Trp Lys Gly Leu Ser His
P6	Glu Trp Gln His Arg His Thr Ala Gln Ser Ile Glu
P7	Ser Glu Trp Thr Ser Cys Ser Thr Ser Cys Gly Asn Gly Ile Gln Gln Arg Gly Arg

are known to occupy very mobile protein sites, such as residues located on the surface of the macromolecule or exposed to the aqueous phase, have invariably such a short-lived RTP that their emission could not be detected (Strambini & Gabellieri, 1990). This is the case of Trp-15 of LADH and of the single Trp of staphylococcal nuclease, subtilisin Carlsberg, and phospholipase A_2. Conversely, long-lived RTP in proteins is a prerogative of residues located at the interior of the globular structure, in regions characterized by well-ordered, rigid domains. Examples are Trp-109 of AP, Trp-314 of LADH (Saviotti & Galley, 1974), and Trp-48 of azurin (Strambini & Gabellieri, 1991). Additional, and even more compelling, evidence for a direct relationship between τ and η has been gathered in a number of experiments in which the protein flexibility was modulated. Any increase/decrease in structural flexibility induced by temperature (Gonnelli & Strambini, 1993), cosolvents, pH (Gonnelli et al., 1992), ligand binding (Cioni & Strambini, 1989; Strambini & Gonnelli, 1990), state of hydration (Strambini & Gabellieri, 1984), or addition of denaturants (Strambini & Gonnelli, 1986) was promptly revealed by a corresponding decrease/increase in phosphorescence liftime.

Re-examination of this model, however, is prompted by two recent observations: (1) τ of free Trp in buffer at ambient temperature has now been determined to be about 1 ms (Strambini & Gonnelli, 1995), that is, 100-fold larger than previously accepted. As a consequence, the viscosity dependence of τ is less steep than previously believed, and, in terms of η alone, τ in proteins should span a range between 6 s and 1 ms. However, as stated above, failure to detect RTP in some proteins implies that τ can be much smaller. In addition, lifetime determinations in proteins by flash photolysis report values of 30 and 60 μs for the mellitin monomer and tetramer, respectively (Ghiron et al., 1988), and even smaller values in other proteins (Grossweiner et al., 1976; Bazin et al., 1975). (2) The quenching effectiveness of disulfide and sulfhydril compounds, analogues of oxidized and reduced Cys side chains in proteins, appears to be temperature dependent. While at 77 K the unimolecular rate of quenching for quencher−chromophore in van der Waals' contact, k_o, was estimated to be 2×10^4 s^{-1} for the disulfide and negligible for the sulfhydril (Li et al., 1989, 1992), at 20 °C both groups are potent quenchers of Trp phosphorescence (Calhoun et al., 1988), with values of k_o estimated to be close to 10^9 s^{-1} for each compound (Vanderkooi et al., 1990). The implication is that, at room temperature, quenching by Cys side chains in proteins could be very effective in either state of oxidation of the amino acid.

In the present investigation we take advantage of the high sensitivity and enhanced temporal resolution achieved in phosphorescence measurements (Strambini & Gonnelli, 1995) to re-examine the factors that may affect the RTP lifetime of Trp residues in proteins. Intramolecular quenching by amino acid side chains, peptide bonds, and carboxy−amino terminal groups will be assessed from information gathered in four types of experiments: (1) determination of the Stern−Volmer bimolecular rate constant for the quenching of NATA phosphorescence by free amino acids; (2) evaluation of the distance dependence of quenching reactions by separating indole and quencher by protein spacers of various thickness; (3) investigation of the relationship between τ of Trp in short synthetic peptides and amino acid composition; and (4) correlation between τ of Trp in proteins and the nature of the neighboring side chains. The results indicate that, besides the local dynamic features of the protein structure, intramolecular quenching reactions with His, Tyr, Trp, and Cys side chains in close proximity of the indole ring can play an important role in determining RTP lifetimes in proteins.

MATERIALS AND METHODS

High-purity *N*-acetyltryptophanamide (NATA) was purchased from Sigma Chemical Co. (St. Louis, MO) and prior to use was recrystallized three times from ethanol/water. The amino acids, 99% pure by TLC, were from Sigma (St. Louis, MO) and were employed as received. Spectroscopic grade propylene glycol was from Merck (Darmstadt) and prior to use was treated with a reducing agent ($NaBH_4$) and distilled under vacuum twice. Ultrapure guanidine hydrochloride was from U.S.B. Corp. (Cleveland, OH). Water, doubly distilled over quartz, was further purified by Milli-Q Plus system (Millipore Corporation, Bedford, MA). All glassware used for sample preparation was conditioned in advance by standing for 24 h in 10% HCl suprapur (Merck, Darmstadt).

Among the synthetic peptides, P_1 (see Table 1 for the sequence) was purchased from Sigma (St. Louis, MO), and P_2 was from Bachem (Bubendorf, Switzerland). The others, P_3−P_7, were a generous gift of Dr. Ghibaudi, Mario Negri Institute (Milano, Italy). The purity of peptides was tested by reversed-phase HPLC on a C18 analytical column using an acetonitrile/water mixture.

The Cys peptide P_7, supplied in the oxidized state, was reduced by treatment with 0.1 M DTT in Tris−HCl, pH 8.5 (Janicke & Rudolph, 1989). The state of oxidation was tested by the method of Ellman (1959). Each peptide was stored in lyophilyzed form at −20 °C. Prior to use the peptide solution was dialyzed against 0.01 M sodium phosphate or 0.01 M sodium acetate buffer at the specified value of pH.

Horse liver alcohol dehydrogenase, phosphoglycerate kinase from yeast, staphyloccocal nuclease, glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*, and phospholypase A_2 were supplied by Boehringer (Mannheim, Germany). Human serum albumin, alkaline

phosphatase from *Escherichia coli*, subtilisin Carlsberg, and fructose-6-phosphate kinase from *B. stearothermophilus* were obtained from Sigma (St. Louis, MO). Ribonuclease T_1 was purchased from Calbiochem Corporation (San Diego, CA). Copper-free azurin from *Pseudomonas aeruginosa* and human superoxide dismutase from erythrocytes were a gift of Prof. Finazzi-Agrò, University of Rome (Tor Vergata, Italy). Metal-depleted HSOD was prepared by dialyzing the protein against 1 mM EDTA in 0.05 M sodium acetate, pH 3.5. To remove NAD^+ from GAPDH the enzyme was treated with activated charcoal as reported before (Strambini & Gabellieri, 1989). The apo-GAPDH obtained had an absorbance ratio A_{280}/A_{260} of 1.75−1.85.

Since the ternary complex of PGK with Mg, ATP, and 3-phosphoglycerate is more stable than the apo enzyme and its crystal structure is available at higher resolution, all phosphorescence measurements were carried out in the presence of 1 mM $MgCl_2$, 1 mM ATP, and 13 mM 3-PG.

Samples for phosphorescence measurements were prepared from freshly dialyzed peptides and proteins whose concentrations were adjusted to be between 3 and 5 μM, utilizing their molar absorptivity. Oxygen was thoroughly removed from the sample by a procedure described before (Strambini et al., 1989).

The apparatus and the procedure for the measurement of phosphorescence spectra and decays and the analytical treatment of the data were described in detail elsewhere (Strambini & Gonnelli, 1995).

Briefly, phosphorescence decays were obtained with pulsed excitation provided by a frequency-doubled flash-pumped dye laser (UV500M, Candela) tuned at 292 nm. The pulse duration was 1 μs, and the light energy per pulse was varied between 0.03 and 30 mJ. The emission was collected at a right angle from the excitation direction utilizing a double-beam configuration in a T-format. In one arm of the T a filter combination with a transmission window between 420 and 480 nm selects for Trp phosphorescence, while in the opposite arm a filter with a window between 550 and 650 nm detects mostly background light. The background emission is non-negligible (can be as large as 30% of Trp phosphorescence from a 10 μM solution) during the first 100−150 μs of the excitation pulse. It originates from impurities in the glassware, particularly (over 90%) from the sample cuvette, and depends on the scattering strength of the sample (excitation of impurities by scattered light). The spectrum of this background emission covers the entire visible range (410−700 nm), and because its lifetime is practically indistinguishable in the spectral windows 420−480 and 550−650 nm a subtraction procedure turns out to be adequate for eliminating this component from the total emission. The two photomultipliers' outputs are fed to a differential amplifier and are balanced in the presence of O_2, which quenches Trp phosphorescence. Hence, the amplifier output with deoxygenated samples represents the net phosphorescence signal. The photomultipliers are protected from the intense fluorescence light pulse by a single chopper blade that closes both slits during excitation. The time resolution of the apparatus is typically 10 μs, while, in terms of sensitivity, Trp phosphorescence ($\phi_p \approx 10^{-7}$ in buffer) can be detected above background emission down to micromolar concentrations. To this apparatus was added a UV sensitive photodiode (OSD 100-7, Centronics) to monitor the fluorescence (through a 320−400 nm transmission window, made by a 320 nm cutoff and a Schott UG5 UV-transmitting filter) collected at a right angle from the excitation. The integrated fluorescence signal (F) in each excitation pulse was used to normalize the phosphorescence intensity extrapolated to time zero, P_0. The ratio P_0/F is a convenient parameter for correcting variations in phosphorescence intensity that are due to changes in laser output, sample concentration, and fluorescence quantum yield. This ratio can decrease only if there is a reduction in intersystem-crossing quantum yield or if a fraction of chromophores exhibits a phosphorescence lifetime smaller than 10 μs, the detection limit of the apparatus.

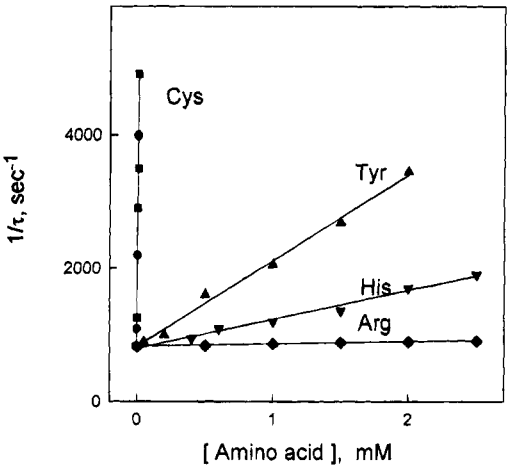

FIGURE 1: Lifetime Stern−Volmer plots for the quenching of NATA phosphorescence by amino acids in phosphate buffer (10 mM, 0.5 M NaCl, pH 7, except for His for which the pH is 8) at 20 °C.

All luminescence decays were analyzed in terms of a sum of exponential components by a nonlinear least-squares fitting algorithm (Global Unlimited, LFD, University of Illinois).

The survey of amino acid residues that are nearest neighbors to Trp in proteins and their mutual distance−orientation relationships was conducted utilizing the crystallographic structures deposited in the Brookhaven Protein Data Bank and the program Insight II (courtesy of Prof. Martinelli, University of Pisa, Italy).

RESULTS

Quenching of NATA Phosphorescence by Free Amino Acids. The phosphorescence lifetime of NATA, 10^{-5} M in phosphate buffer (5 mM plus 0.5 M NaCl, pH 7), was monitored at 20 °C in the presence of increasing concentration of each amino acid. For Cys, Tyr, and His, the experiments were conducted at pH above and below the pK of the side chain. Typical Stern−Volmer plots are shown in Figure 1. In every case the plots ($1/\tau - 1/\tau_o = k_q$[amino acid]) were linear, and the magnitude of the second-order quenching rate constant, k_q, obtained from the gradient is given in Table 2. Among the amino acids, cystine and cysteine are the most effective quenchers, k_q being comparable between reduced and oxidized forms and independent of the state of ionization fo the sulfhydril group. Compared with the results obtained by flash photolysis with the lipoate anion, a small cyclic disulfide, k_q, is 1 order of magnitude smaller (Bent & Hayon, 1975). Since the slightly larger size of the amino acid molecule cannot account for the discrepancy, it is likely that the artifacts with the absorption method that are responsible for the erroneous determination of the

Table 2: NATA Phosphorescence Quenching by Free Amino Acids[a]

amino acid	p*K*	pH	k_q[a] (M^{-1} s^{-1})
cysteine	8.2	7	5.0×10^8
cysteine		9	4.5×10^8
cystine		7	4.0×10^8
Tyr	10.1	7	1.3×10^6
Tyr		12	2.3×10^7
His	6.0	5	2.0×10^7
His		8	4.0×10^5
Trp		7	1.7×10^7
Asp	3.6	7	2.0×10^5
Met		7	1.5×10^5
Lys	10.5	7	1.3×10^5
Glu	4.2	7	5.4×10^4
Ser		7	4.8×10^4
Phe		7	4.2×10^4
Thr		7	4.3×10^4
Val		7	4.2×10^4
Leu		7	4.0×10^4
Ile		7	3.9×10^4
Pro		7	3.8×10^4
Gln		7	3.6×10^4
Asn		7	3.6×10^4
Arg	12.5	7	3.3×10^4

[a] Second-order bimolecular rate constants in buffer at 20 °C. The standard error is less than 10%.

triplet lifetime (Strambini & Gonnelli, 1995) may also be the cause of an unduly large k_q. The other amino acids are less effective quenchers. For neutral Tyr and His, the value of k_q decreases by 2−3 orders of magnitude and is at least 1 order of magnitude smaller than for self quenching by Trp molecules in the ground state (Strambini & Gonnelli, 1995). The behavior of Tyr and His is singular, however, because, as opposed to the other amino acids, k_q is strongly pH dependent in the region of the side-chain p*K*. The enhancement of k_q is 50-fold with His protonation and 20-fold with Tyr deprotonation. For the other amino acids k_q is about 10^5 M^{-1} s^{-1} or smaller and does not correlate with the nature of the side-chain functional groups. We point out that the actual value of k_q may be even smaller because with such low quenching efficiencies the danger of quenching by impurities in stock compounds is high. All that is needed is an impurity present at the level of 1 part in 10^5 to entirely dominate the decay behavior.

Distance Dependence of the Quenching Reaction with His, Tyr, Cysteine, and Cystine. For evaluating the distance dependence of the unimolecular quenching rate, $k(r)$, quencher and chromophore must be separated by spacers of various thicknesses. One way to achieve this is to utilize Trp residues inside proteins that lie at variable distances from the surface of the macromolecule and put the quencher in the aqueous phase. Under conditions where the rapid diffusion limit applies (Thomas et al., 1978), as is the case with inefficient quenching of long-lived phosphorescence (Vanderkooi et al., 1990), k_q obtained from a Stern−Volmer plot is simply related to $k(r)$ and to the distance of closest approach, r, between chromophore inside the protein and quencher in the aqueous phase.

To obtain k_q at various chromophore−quencher separations the following proteins were selected: RNase T_1, GAPDH, LADH, and apoazurin. From their crystallographic structures we noted that the edge to edge distance (r) from the indole ring and the water of hydration varied between 2 and 7 Å. Experiments were conducted at high ionic strength (1 M NaCl) to minimize electrostatic protein−quencher interactions and at pH 7, except when studying quenching by protonated His (pH 5). The maximum concentrations of the free amino acids, limited by their moderate solubility, were typically 1 mM for cystine and Tyr and 10 mM for His and cysteine. Up to these concentrations practically no quenching of phosphorescence was found for apoazurin ($r \approx 7$ Å), LADH ($r \approx 5$ Å), or RNase T_1 ($r \approx 2$ Å). Given the protein unperturbed lifetimes, τ_o, of 700, 150/750, and 32 ms, respectively, a 10% reduction in τ (the typical variability of τ determination) places an upper limit in the quenching rate constant, which for both cystine and Tyr are 1.6×10^2 M^{-1} s^{-1} (azurin and LADH) and 3.2×10^3 M^{-1} s^{-1} (Rnase T_1), respectively, and a factor of 10 less for His and cysteine. A modest amount of quenching was instead observed for Trp-84 of GAPDH ($r \approx 3$ Å) by cystine and Tyr. The values of k_q derived from a three-point Stern−Volmer plot are collected in Table 3 together with the intrinsic sensitivity limits. The table shows that only cystine ($k_q = 4 \times 10^3$ M^{-1} s^{-1}) and Tyr ($k_q = 3 \times 10^3$ M^{-1} s^{-1}) have rate constants a few times greater than the detection limit. Thus, even with GAPDH, quenching of internal residues is drastically reduced relative to NATA. In addition, we must point out that these are only upper limits of k_q as it cannot be excluded that part of the observed quenching may be due to small-molecule impurities that, given their size, are able to migrate directly to the chromophore inside the protein.

Table 3: Quenching of GAPDH Phosphorescence by Free Amino Acids[a]

buffer/pH	τ_o (ms)	quencher	k_q (M^{-1} s^{-1})	k_q'[b] (M^{-1} s^{-1})
phosphate/7	84	cysteine	$(1.6 \pm 0.5) \times 10^2$	1.2×10^2
phosphate/7	84	cystine	$(4.0 \pm 1.0) \times 10^3$	1.2×10^3
phosphate/7	84	Tyr	$(3.0 \pm 1.0) \times 10^3$	1.2×10^3
phosphate/7	84	His	$(2.0 \pm 0.8) \times 10^2$	1.2×10^2
cocadilate/5	79	His	$(0.8 \pm 0.5) \times 10^2$	1.2×10^2

[a] Second-order quenching rate constants in buffer at 20 °C. [b] These represent values of k_q commensurate with the experimental error.

The results of these experiments emphasize the short-range nature of the quenching reactions. This contrasts with the conclusion reached by Calhoun et al. (1988) in similar experiments with small sulfur derivatives (H_2S, CS_2, and ethanethiol). Indeed, for RNase T_1, LADH, and azurin the authors reported second-order quenching rate constants that, in comparison with cysteine and cystine, are 2−5 orders of magnitude larger. The discrepancy in k_q suggests that these small quenchers, as opposed to the amino acids, migrate through the protein and get in close proximity to the chromophore. Certainly, the distance dependence of the unimolecular rate derived in a subsequent study ($k(r) = k_o \exp(-r)$, $k_o \approx 10^9$ s^{-1}; Vanderkooi et al., 1990) fails to account for the poor or negligible quenching efficiency found with cystine and cysteine.

Phosphorescence Lifetime of Trp in Short Peptides. In order to test the quenching effectiveness of specific side chains, peptides were selected that contain only weakly quenching amino acids (P_1 and P_2), moderately quenching Tyr (P_3) and His (P_4−P_6), and strongly quenching Cys (P_7) in both reduced and oxidized forms. The sequences of P_1−P_7 are given in Table 1.

The florescence spectrum of each peptide is practically superimposable to that of NATA, an indication that the indole ring is fully in contact with the aqueous solvent. Phospho-

Table 4: Phosphorescence Lifetime of Synthetic Peptides in Buffer and in 6 M Gu−HCl

		buffer				Gu−HCl				
		0 °C		20 °C		0 °C			20 °C	
sample	pH	P_0/F[a]	τ (ms)	P_0/F	τ[a] (ms)	P_0/F	$\tau_1(\alpha_1)$ (ms)	τ_2 (ms)	P_0/F	τ (ms)
NATA	8		1.61		1.2		0.35			0.19
NATA	4		0.80		0.63					
P_1	8	1.0	1.10	1.0	0.61	1.0	0.25		1.0	0.16
P_2	8	1.0	0.18	1.0	0.083	0.7	0.15		0.4	0.110
P_3	8	1.0	0.13	0.7	0.046	0.3	0.088			<0.01
P_4	8	0.2	0.19	0.2	0.069	0.3	0.044 (0.52)	0.21	0.1	0.13
P_4	4	0.14	0.30	0.16	0.150					
P5	8	0.3	0.061	0.35	0.022	0.4	0.133		0.2	0.039
P6	8	0.4	0.040	0.40	0.020	0.4	0.086		0.2	0.033

[a] The precision of P_0/F is typically 15%−20%, and that of τ is better than 10%.

rescence decays were monitored in phosphate buffer (10 mM, pH 8) at 0 and 20 °C. His peptides were also examined at pH 4 (10 mM NaCl plus 10^4 M HCl). Often the initial, time-zero, phosphorescence intensity was weaker than that of equifluorescent solutions of NATA. The decrease in intensity was monitored by the ratio P_0/F, where P_0 is the phosphorescence intensity extrapolated at time zero and F is the integrated fluorescence intensity in the same excitation pulse.

Each phosphorescence decay could be adequately fitted to an exponential function. τ and the P_0/F ratio, relative to that of NATA, are reported in Table 4. Incorporation of Trp into short peptides invariably reduces its triplet lifetime. The extent of the change is modest with Lys-Trp-Lys but substantial with the longer P_2, for which τ decreases 7-fold at 0 °C and 15-fold at 20 °C. This implies that either the peptide linkage or one or more amino acids among Glu, Gln, Arg, Pro, and Ile can deactivate the triplet state. Relative to P_2, the insertion of Tyr in P_3 does not drastically reduce τ. A greater influence on τ is instead exerted by His in P_5 and P_6, for which τ, relative to NATA, is 26 and 40 times smaller, respectively, at 0 and 20 °C. In the protonated form, His peptides P_5 and P_6 are not detectably phosphorescent.

Two factors may cause the loss in intensity (decrease in P_0/F ratio): one is the reduction in the rate of intersystem crossing, k_{isc} ($P_0/F = k_{isc}k_p/k_f$, where k_p and k_f are the radiative rates from the triplet and singlet states, respectively), and the other is the shortening of τ to beyond the resolution of the apparatus (10 μs). For aromatic hydrocarbons k_{isc} is only moderately affected by environmental conditions, while for some Trp in proteins it was found to be unaltered as the temperature was varied between 200 and 300 K (Strambini & Gabellieri, 1990). For this reason we shall consider k_{isc} to be practically constant and attribute the loss in P_0/F to very short triplet lifetimes. Hence, we conclude that His protonation leads to a drastic reduction in τ of P_5 and P_6, an effect that is consistent with the increase in the Stern−Volmer rate constant.

Among His peptides, P_4 is singular in two respects: it has two Trp residues instead of one, and τ is relatively large. Moreover, τ increases upon His protonation. In terms of the amino acid composition a large τ is not simply accounted for because in addition to quenching by His, deactivation of the excited state can occur through excimer formation with the other indole ring in the ground state (Strambini & Gonnelli, 1995). Also, it is unlikely that the excitation remain localized in the originally excited chromophore because rapid exchange between the chromophores should be permitted through triplet−triplet energy transfer (Gabellieri & Strambini, 1994).

Peptide P_7 contains a disulfide bridge or two sulfhydril groups. As expected from the large value of k_q for these side chains, no phosphorescence could be detected from P_7, again an indication that in either case $\tau < 10$ μs.

Besides these examples of undetectable RTP inspection of Table 4 reveals that for Tyr and His peptides the P_0/F ratio is less than unity. Thus, we conclude that a fraction of peptide molecules equal to $1 - (P_0/F)$ is too short-lived for detection, and we infer that the phosphorescence decay of these peptides is heterogeneous.

The differential quenching ability of His in peptides P_4 to P_6 and the occurrence of heterogeneous decays are probably both manifestations of stable preferential configurations of the polymer chain in which the chromophore is either shielded from or brought in close proximity to the quenching moiety. To minimize the stabilization of particular conformations, experiments were also conducted in 6 M Gu−HCl, where the inhibition of secondary structure and the screening of electrostatic interactions should favor random, rapidly interchanging configurations of the polymer.

Control experiments demonstrated that Gu−HCl exhibits a non-negligible (about 30%−40% of the typical intensity of NATA) emission in the spectral window of Trp phosphorescence with $\tau = 40$ μs, practically the same at both 0 and 20 °C. Curiously, this component is lacking in the presence of NATA or peptides like P_1, P_2, and P_7 whose τ differ considerably from 40 μs. From this we infer that the solvent emission is effectively quenched by the indole nucleus. Consequently, no correction to the decay of the peptides in GuHCl was made for the solvent contribution. The phosphorescence lifetimes in Gu−HCl solutions are reported in Table 4. For NATA at 0 °C, τ is about four times less than in dilute buffer. Since the actual decrease of τ depended on the commercial source of the denaturant, we presume that the reduction is partly due to trace impurities. For P_1 to P_4, τ is smaller in Gu−HCl, and the decrease seems to be more than accountable by impurity quenching. The same applies to the larger portion of "statically" quenched ($\tau < 10$ μs) component in P_3. In contrast, τ of short-lived P_5 and P_6 is practically doubled in the presence of denaturant. P_4 is again anomalous with at least two lifetime components at 0 °C but has a single, larger than average τ at 20 °C. At the higher temperature its P_0/F ratio is smaller, and it is likely that the apparent lengthening of τ is due to the selective quenching of short-lived conformers. Overall, by decreasing the conformational

Table 5: Phosphorescence of Synthetic Peptides in Viscous Solutions[a]

peptide	pH	P_0/F	τ_1 (ms)	τ_2 (ms)	α_1	χ^2
			40 °C			
P_1	8	1.0	27.0		1.0	1.2
P_2	8	1.0	26.2		1.0	1.1
P_3	8	1.0	25.7		1.0	1.7
P_4	8	0.9	28.1	3.1	0.58	2.1
P_4	4	0.8	26.8	0.8	0.41	2.7
P_5	8	0.9	8.0		1.0	1.3
P_5	4		3.6	0.7	0.22	1.0
P_6	8	0.8	7.7		1.0	1.5
P_6	4		3.5	0.6	0.11	1.0
P_7(ox)	8	0.4	24.4	2.6	0.27	1.1
P_7(red)	8	0.6	27.0	6.2	0.48	1.0
			−20 °C			
P_1	8	1.0	5.6		1.0	1.3
P_2	8	1.0	3.9		1.0	1.1
P_3	8	1.0	1.9		1.0	1.6
P_4	8	0.9	3.8	0.14	0.36	1.5
P_4	4	0.8	6.0	0.52	0.48	1.2
P_5	8	0.9	1.1		1.0	1.4
P_5	4		0.13		1.0	1.2
P_6	8	0.8	0.9		1.0	1.6
P_6	4		0.10		1.0	1.3
P_7(ox)	8	0.2	1.7	0.3	0.31	1.1
P_7(red)	8	0.2	6.6	1.7	0.71	1.0
			0 °C			
P_1	8	1.0	1.5		1.0	1.5
P_2	8	1.0	1.0		1.0	1.3
P_3	8	1.0	0.39		1.0	1.7
P_4	8	0.9	0.83	0.24	0.53	1.1
P_4	4	0.5	1.52	0.04	0.66	1.3
P_5	8	0.8	0.35		1.0	1.6
P_5	4					
P_6	8	0.8				
P_6	4					
P_7(ox)	8	<0.1				
P_7(red)	8	<0.1				
			20 °C			
P_1	8	1.0	0.71		1.0	1.1
P_2	8	1.0	0.43		1.0	1.3
P_3	8	0.8	0.15		1.0	1.1
P_4	8	0.7	0.29	0.09	0.46	1.0
P_4	4	0.3	0.57		1.0	1.1
P_5	8	0.8	0.17		1.0	1.0
P_6	8	0.8	0.11		1.0	1.3

[a] Lifetimes (τ, ms) and amplitudes (α) obtained from a biexponential fitting of phosphorescence decays in PG/buffer at some selected temperatures.

constraints, the decay kinetics of His peptides in GuHCl become more uniform and the quenching effectiveness of Tyr becomes greater. Remarkable is the effect of temperature on the latter.

Phosphorescence Lifetime of Peptides P_1 to P_7 in Viscous PG/Buffer. To understand the role of temperature and of segmental flexibility in intramolecular quenching reactions, the phosphorescence decay kinetics of peptides P_1−P_7 were compared to that of NATA over a wide range of temperature and solvent viscosity. With a 50/50 (w/w) PG/buffer solution a temperature variation between 160 and 293 K corresponds to a change in solvent viscosity from 10^{13} to 0.8 poise (Strambini & Gonnelli, 1995). Experiments were carried out both at pH 8 (10 mM phosphate) and at pH 4 (10 mM NaCl plus HCl). Control runs pointed out that with this solvent mixture the time resolution of decay measurements was not set by the apparatus but by the emission associated with PG, which is intense up to 150 μs at −40 °C and up to 20 μs at 20 °C.

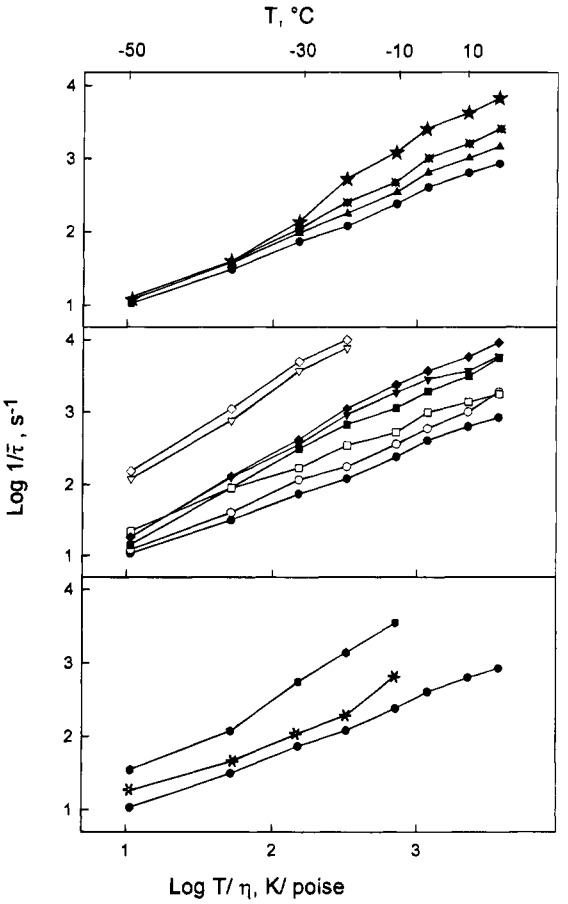

FIGURE 2: Comparison between the average phosphorescence lifetimes ($\tau = \sum\alpha_i\tau_i$) of NATA and of peptides P_1−P_7 in 50/50 PG/phosphate buffer at temperatures ranging between −50 and 20 °C. The top panel refers to NATA (●), P_1 (▲), P_2 (octagram), and P_3 (★) at pH 8. The middle panel refers to NATA (●), P_4 (■), P_5 (▼), and P_6 (◆) at pH 8 and at pH 4, closed and open symbols, respectively. The lower panel refers to NATA (●) and to Cys containing P_7 reduced (asterisk) and oxidized (solid hexagon) at pH 8. From the nonlinearity of log $1/\tau$ vs log T/η plots and gradients that exceed 1 it is evident that in many circumstances the quenching reaction is under rate control.

In rigid glasses, at 160 K, the decay of each peptide is well approximated to an exponential function and the lifetime is between 6.2 and 6.5 s. As the temperature is raised above the glass transition, 190−200 K, τ decreases rapidly and the decay of P_3−P_7 becomes distinctly heterogeneous. At even higher temperatures, above 230 K, the heterogeneity of P_3, P_5, and P_6 narrows down, and eventually, above 253 K, the decay is again well described by a single exponential function. Often, heterogeneous decays are adequately fitted with two discrete components, but in some cases both χ^2 and the distribution of residuals show that three or more components are needed. Presently, we limit ourselves to consider the main features of the decay, such as whether or not it is homogeneous, and the average lifetime, leaving the actual distribution in lifetimes to a separate, more detailed discussion to be presented elsewhere.

The lifetimes and relative amplitudes derived from a fit with one or two discrete components are collected in Table 5 for selected temperatures between −40 and 20 °C. Since only His peptides were affected significantly by lowering the pH to 4, the data at acid pH is reported only for them. For visual inspection the average lifetime, $\tau = \sum\alpha_i\tau_i$, of P_1−P_7 is compared to that of NATA in Figure 2 where $1/\tau$ is

plotted against T/η. These data refer to one experiment. Differences in lifetimes in successive experiments did not exceed 10%.

Above 240 K, the phosphorescence of peptides is shoerter-lived than that of NATA and the difference is generally amplified as the temperature is raised from −30 to 20 °C. Peptides P_1 and P_2 lack strongly quenching amino acids and, as expected, behave more similarly to NATA. At the other extreme, Cys-containing P_7, reduced and oxidized, is, as expected, most readily quenched. Even below −40 °C, τ is considerably shorter than that of NATA and the intensity ratio P_0/F is substantially less than unity. This fraction of "static" quenching is about 50% at −40 °C, but on approaching 0 °C most of the P_7 emission is quenched. Reduction of the disulfide bridge decreases considerably the quenching effectiveness of Cys, expecially at low temperature where the difference in τ and P_0/F with the oxidized form is greater. Tyr (P_3)- and His (P_4, P_5, and P_6)-containing peptides behave intermediately. At −50 °C, their lifetimes (Figure 2) approach that of NATA and the intensity ratio is close to unity. As the temperature is raised, specific quenching sets in to decrease the P_0/F ratio and τ. Among His peptides the phosphorescence decay of P_4 stands out for being long-lived and heterogeneous throughout.

His protonation at pH 4 leads to pronounced phosphorescence quenching of His peptides. There is a general decrease of P_0/F, but most remarkable is the reduction in τ of P_5 and P_6, whose emission is no longer detectable above −20 °C. P_4, on the other hand, is once again peculiar in that its emission has an average lifetime even larger than at pH 8 ($T > -30$ °C). Above 10 °C, the short-lived component in the decay vanishes and the observed decay is homogeneous.

Phosphorescence Lifetime of Trp in "Critical" Protein Environments. The ability to detect submillisecond lifetimes has opened the way to the study of Trp residues in proteins for which, because of their peculiar environments, no RTP had been reported before. In order to correlate τ with the nature of the Trp environment only proteins with available crystal structures were considered. One exception is HSA, for which it was deemed interesting to compare the phosphorescence decay with the triplet decay kinetics obtained by triplet−triplet absorption (Hicks et al., 1978). The selected sample of short-lived residues are (1) solvent-exposed Trp-15 of LADH, Trp-268 and Trp-220 of AP, Trp-308 of PGK, Trp-140 of staphylococcal nuclease, Trp-32 of HSOD, Trp-3 of phospholipase A_2, and Trp-113 of subtilisin Carlsberg and (2) internal Trp-310 of GAPDH and Trp-214 of HSA. In each case their lifetimes in low-temperature rigid glasses are about 6 s, and, in past experiments, τ vs temperature profiles showed that on approaching room temperature τ decreased to below the millisecond resolution of the apparatus.

The multitryptophan proteins examined here have two Trp residues per subunit, except for AP which has three. In each case there is a good fraction of the intensity with a lifetime 2−3 orders of magnitude larger with respect to the short-lived components emerged in this study (see Figure 3). The slowly decaying components were previously assigned to specific Trp residues, namely, Trp-109 for AP (Strambini, 1987), Trp-314 for LADH (Saviotti & Galley, 1974), Trp-333 for PGK (Cioni et al., 1993), and Trp-310 for GAPDH (Strambini & Gabellieri, 1989). Recently, aided by site-specific mutagenesis, the correct assignment of GAPDH was found to be Trp-84 and not Trp-310 (E. Gabellieri and G. B. Strambini, unpublished data). The phosphorescence intensities obtained with a time resolution of 10 μs were analyzed after substraction of the long-lived component, and the decay parameters obtained from fitting the modified intensities were ascribed to the other Trp residue in the subunit. With AP, the residual emission was tentatively assigned to Trp-220 because Trp-268, being in contact with a disulfide bridge and one Tyr, is presumably quenched. A rigorous test of the correctitude of this procedure would require single-Trp mutants. In the case of GAPDH, a mutant with only Trp-310 is available and its RTP lifetime was found to be very similar to the short-lived component monitored with the wild-type protein.

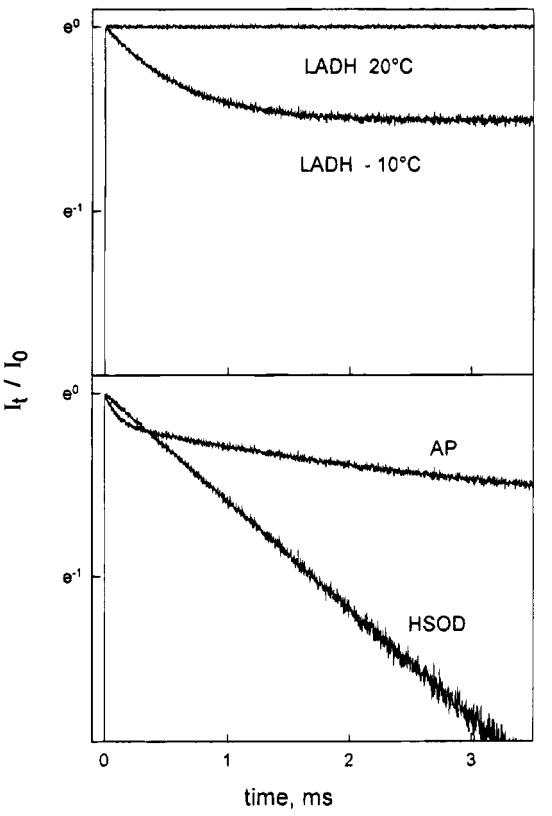

FIGURE 3: Decay of Trp phosphorescence of sample proteins in phosphate buffer, pH 8, at 20 °C. In the upper panel the emission from LADH at −10 °C in PG/buffer (50/50, pH 8) emphasizes the short-lived contribution of Trp-15, which is not detectable at ambient temperature.

The phosphorescence decay of each protein was monitored in phosphate buffer, 100 mM, pH 8, at 20 °C, and at a protein concentration ranging between 5 and 10 μM. A sample of raw data is given in Figure 3, while the lifetime components derived from fitting the decays with a discrete number of exponential components are given in Table 6. Among the proteins of Table 6, subtilisin Carlsberg, phospholipase A_2, and Trp-15 of LADH were not detectably phosphorescent at room temperature. However, it was sufficient to add 50% propylene glycol to the buffer solution and lower the temperature to −10 °C to clearly observe the delayed emission and record the lifetime. The emergence of a short-lived component in the decay of LADH at −10 °C, which is absent at 20 °C, is clearly evident in Figure 3. Thus, quenching of these residues appears to be a dynamic rather than a static process, and the lack of RTP is interpreted in terms of a lifetime shorter than 10 μs.

Table 6: RTP Lifetime of a Selection of Single- and Multitryptophan Proteins

protein	Trp no.	T (°C)	τ_1 (ms)	τ_2 (ms)	α_1	χ^2	solvent	pH
LADH	314	20	150	700	0.67	1.0	TES	8
LADH	15	20	<0.01				phosphate	8
LADH	15	−10	0.53		1.0	1.0	PG/phosphate	8
AP	109	20	2060		1.0	1.0	Tris	8
AP	268/220	20	0.095	2.9	0.15	1.2	phosphate	8
PGK	333	20	51	151	0.83	1.3	Tris	7.5
PGK	308	20	0.65		1.00	1.2	phosphate	8
GAPDH	84	20	23	91	0.12	1.1	phosphate	7.2
GAPDH	310	20	0.18	1.1	0.76	1.4	phosphate	8
PFK	179	20	235		1.0	1.0	Tris	8
HSOD	32	20	1.7		1.0	0.9	phosphate	8
S. nuclease	140	20	0.65		1.0	1.1	phosphate	8
subtilisin Carlsberg	113	20	<0.01				phosphate	8
subtilisin Carlsberg	113	−10	0.08		1.0	1.0	PG/phosphate	8
phospholipase A_2	3	20	<0.01				phosphate	8
phospholipase A_2	3	−10	1.0	5.6	0.78	1.1	PG/phosphate	8
HSA	214	20	0.183	0.59	0.69	1.0	phosphate	8

Although the residues mentioned above are in contact with the solvent, exposure to the aqueous phase does not necessarily imply very rapid decay kinetics. Indeed, for Trp-140 of *S.* nuclease, Trp-32 of HSOD, Trp-220 of AP, and Trp-308 of PGK, which are also in contact with the solvent, τ is not too dissimilar from that of NATA. Conversely, there are internal residues which are relatively short-lived. For HSA, about 70% of the emission has a τ of 0.18 ms while for the remainder τ is 0.59 ms. By comparison, the triplet−triplet absorption method found for HSA a single lifetime of 0.5 ms (Hicks et al., 1978). Another example is Trp-310 of GAPDH. Although it lies roughly at the center of a 40 kDa subunit that is rich in ordered secondary structure (large β-sheet and α-helical rods) and thus presumably rigid, the decay is surprisingly short-lived with a predominant component of 0.15 ms. PFK, instead, provides an example of an internal residue with a long RTP lifetime.

DISCUSSION

Intrinsic Quenchers of Protein Phosphorescence. In aqueous solution at room temperature a number of Trp residues in peptides and proteins were found to possess phosphorescence lifetimes that are substantially smaller than the 1 ms lifetime of free NATA. Certainly the fluidity of protein sites does not exceed that of bulk water, and consequently $\tau < 1$ ms is not justified in terms of the dependence of τ on the local viscosity (Strambini & Gonnelli, 1995). At least in these cases we must conclude that τ is dominated by intramolecular quenching reactions. In the absence of prosthetic groups, putative quenching centers must be sought among the side chains and possibly the peptide linkage of the backbone.

A measure of the quenching potential of the amino acid side chains can be gained from the magnitude of the Stern−Volmer rate constant, k_q, that describes the interaction between amino acid and free NATA in solution. According to k_q (Table 2), the side chains may be grouped into three classes: (1) strongly quenching ($k_q \approx 5 \times 10^8$ M^{-1} s^{-1}), such as cystine and cysteine; (2) intermediately quenching ($k_q = 5 \times 10^5 - 2 \times 10^7$ M^{-1} s^{-1}), such as Tyr, His, and Trp; and (3) weakly quenching or nonquenching ($k_q \leq 10^5$ M^{-1} s^{-1}), the remainder. As already pointed out, the possibility of quenching by trace impurities in stock compounds renders very arduous an accurate determination of small k_{qs} and hence the distinction between poorly quenching and nonquenching side chains of class 3 above. Impurity quenching might indeed explain the large differences in k_q among amino acids with the same functional groups. As will be said below, from the correlation between τ and the nature of the amino acid side chains that come into contact with Trp residues in peptides and proteins, it is apparent that amino acids belonging to the third class are not effective quenchers of Trp phosphorescence. In conclusion, k_q emphasizes that at 20 °C, as opposed to temperatures below −100 °C where only disulfide bridges are able to deactivate the triplet state, there are four amino acids which are potential quenchers of Trp phosphorescence in proteins. Among these, His and Tyr increase in effectiveness by 50- and 20-fold, respectively, when their side chain is in the ionized state. Interestingly, such pH dependence of k_q can be exploited for choosing pH conditions that minimize the quenching action of these side chains, or, alternatively, it can be utilized as a diagnostic tool for the presence of Tyr or His quenching.

The information gathered from intermolecular quenching reactions between freely diffusing molecules sets some necessary conditions for intramolecular quenching in proteins. These may be insufficient, however, because predictions of intramolecular quenching based solely on the magnitude of k_q, even after due consideration of the large effective quencher concentration, fail to account for the possible effect of through-bond quenching pathways. Furthermore, since in a protein the quencher and the chromophore have relatively fixed positions, a proper assessment of the potential quenching rate requires knowledge of how the process depends on their separation and possibly their mutual orientation.

Short peptides, having labile secondary structures, should behave much like flexible polymer chains and hence should offer a simplified picture of intramolecular quenching that is practically free of restraints imposed by distance and orientation. The lifetime of Trp in synthetic peptides P_1−P_7 by and large validates the predictions based on k_q, although an interaction with the peptide linkage together with non totally random configurations of the polymer chain may compound the decay behavior. The smallest peptide, Lys-Trp-Lys, has a τ similar to that of NATA. This lack of intramolecular quenching establishes that the side chain of Lys, the carboxy and amino terminal groups, and the nearest peptide linkage have little or no influence on the decay properties of the triplet state. P_2, on the other hand, is a

Table 7: RTP Lifetimes of Trp Residues and Protein Environment[a]

protein	Trp no.	τ_1, τ_2 (ms)	solvent exposure	side chains in proximity of indole ring
AP	109	2060	no	Asp Glu Glu Ser Ser **Tyr** Met Thr Ala Ala Ala Leu Leu Ile
LADH	314	150, 700	no	Lys Ser Met Met Thr **Trp** Val Val Leu Leu
azurin	48	600	no	Asn Thr Phe Phe Phe Phe Val Val Val Leu Leu Leu Ile Ile Ile
PFK	179	236	no	Arg Asp Lys Asn Met Ser Phe Phe Phe Gly Ala Val Ile
PGK	333	51, 152	no	Asn Thr Thr Pro Pro Gly Val Leu Leu Leu Leu Ile
GAPDH	84	23, 91	no	Lys Asn Asn **His** Gly Ala Ala Ala Val Val Leu Ile
RNase T_1	59	32	no	Glu Glu **Tyr Tyr** Pro Pro Gly Phe Ala Ala Val Val Leu
Trp synthase	177	14, 24[b]	no	Asp **His Tyr Tyr** Ser Ala Ala Leu Leu Leu Leu Ile
GAPDH	310	0.18, 1.1	no	Asp Arg **Tyr** Thr Ser Ser Ser Pro Val Val Leu Ile
AP	268	<0.01[c]	no	Arg Arg **Tyr Cys Cys** Pro Thr Gly Ala Val Ile
AP	220	0.09, 2.9[c]	yes	Glu Arg Glu Lys Ala
HSOD	32	1.7	yes	Glu Lys Lys Lys Asn Ser Ala Ala Leu Ile
S. nuclease	140	0.15, 0.74	yes	Glu Glu Lys Asn Phe Gly Val Val
PGK	308	0.65	yes	Lys Asp Met Phe Gly Ala Ile
phospholipase A_2	3	<0.01	yes	Glu **Tyr Tyr** Asn Asn Leu
LADH	15	<0.01	yes	Arg Arg Glu Glu Lys Ser **Cys** Pro Val Leu Ile
subtilisin Carlsberg	113	<0.01	yes	Asp Glu **Tyr** Ser Met Gly Gly Phe Ala Ala Ala

[a] The amino acids listed have at least one atom of the side chain within 5 Å, center to center, of one atom ot the indole ring. [b] Strambini et al. (1992). [c] Tentative assignment of lifetime components.

nine-residue peptide composed exclusively of amino acids of class 3, and its τ is substantially shorter than that of NATA (Table 4). Thus, either one or more side chains among Glu, Pro, Arg, Gln, and Ile or peptide linkages along the backbone can to some extent deactivate the chromophore. A survey of the side chains that are in contact with long-lived Trp residues in proteins (Table 7) shows that not only the above amino acids but the entire set of class 3 side chains occur in the neighborhood of one or more of these residues. This suggests that they have no propensity for quenching RTP and that the smaller τ of P_2 is probably due to a modest quenching reaction with the peptide linkage.

Peptides P_3–P_7 confirm the quenching effectiveness of His, Tyr, and Cys. They exhibit short-lived RTP, τ decreasing to beyond the detection limit when Cys is present in either oxidation state or when His is protonated (except for P_4). Of course, τ, is not simply related to the k_q of its constituent amino acids nor is it the same among His peptides P_4–P_6. Preferential conformations of the polypeptide are expected to modulate the chromophore–quencher encounter probability and in turn the overall quenching efficiency. Electrostatic steering forces could indeed be the cause of poor quenching by His in P_4 and might also explain why His protonation has dramatic effects on the lifetime of P_5 and P_6 (consistent with the increase in k_q) but not on P_4. From the sequence we note that P_4 is the sole peptide with two Trp residues, one of which is sandwiched between His on one side and three positively charged Lys on the other. Upon His protonation the positive charges of the Lys will set up on electrostatic repulsion that will effectively keep the quencher at bay of the indole ring and thus diminish its quenching efficiency. On the other hand, both P_5 and P_6 have two His, one of which is next to Trp in the sequence. Furthermore, in each case there is an electrostatic attraction between oppositely charged residues on either ends of the chain that will tend to keep His and Trp sandwiched in and, therefore, in close proximity. These speculations find support in the results obtained in 6 M GuHCl where to an increased randomness of the polypeptide configuration there corresponds a more uniform decay behavior of P_4–P_6. Finally, conformational constraints appear also to account for the relatively poor quenching efficiency of Tyr in P_3, as large enhancements on the decay rate are brought about in the presence of Gu–HCl.

Intramolecular Quenching in Proteins: Distance and Orientation Dependence. Because in proteins the position between putative quenchers and chromophore is relatively fixed, knowledge of the distance/orientation dependence of quenching reactions is paramount for predicting the effect of His, Tyr, Trp, and Cys residues on the RTP lifetime. To date, the quenching mechanisms are unknown, except perhaps for the reaction with disulfide bridges where the formation of a disulfide anion adduct (Bent & Hayon, 1975) lends support to an electron transfer process (Li et al., 1989). Energy transfer from the triplet state of Trp to any of these moieties is ruled out by the requisite of emission/absorption spectral overlap. What remains is the formation of a nonluminescent complex with the triplet excited state. While electron transfer is operative up to distances of 15–20 Å (Strambini & Gabellieri, 1991), exciplex formation requires contact between the reactants. In either case, it is felt that orientation should influence the rate, but experimental data on this matter is scanty.

An estimate of the distance dependence of the quenching reaction with His, Tyr, and Cys, obtained utilizing protein spacers of thickness varying between 2 and 7 Å, established that for each amino acid the reaction is short-range and, except perhaps for the phenol and disulfide moiety, probably requires direct contact between the side chain and the indole ring. On these grounds, intramolecular quenching in proteins in expected to be negligible unless the side chains are the nearest neighbor of the indole ring or unless they are transiently brought into contact with it by fluctuations in molecular structure. However, there is an important exception to this rule that we wish to point out. This is represented by Trp self-quenching, not by excimer formation, but through triplet–triplet energy migration to other, shorter-lived Trp residues in the same macromolecules. The rate of this process, $k(r)$, was found to obey an exponential dependence on the donor–acceptor separation with $k(r) = 1.5 \times 10^4 \exp(-0.9r)$, where r is the separation in Å beyond van der Waals' contact [data from Gabellieri and Strambini (1994) corrected for the assignment of GAPDH RTP to Trp-84 instead of Trp-310]. For a Trp with $\tau = 0.1$ s the critical

distance, r_c, for which $k(r_c) = 1/\tau$, is about 8 Å. This kind of quenching may not be so unusual in multitryptophan proteins where residues in internal rigid sites, with potentially long τ, could be quenched by energy migration to residues which are short-lived because they lie either in flexible sites on the protein surface or next to quenching side chains. Thermally activated triplet energy transfer from Trp-104 to Trp-60, which is quenched by a proximal disulfide residue, was indeed proposed to explain the sharp decrease in phosphorescence yield of human α-lactalbumin above 30 K (Smith & Maki, 1993). Of course, unless this possibility can be ruled out, attempts to assign phosphorescence decay components to individual residues or correlate τ with the rigidity of the local structure (Bergenhem et al., 1994) are totally unjustified.

The short-range nature of the quenching interaction between the triplet state and the side chains of His, Tyr, and Cys is also consistent with the observed RTP lifetime in a number of proteins. Table 7 lists a set of proteins with one to three Trp per subunit whose phosphorescence emission has in all cases, except for AP, been completely resolved and assigned to individual residues of the macromolecule. The table reports τ of each residue as well as the side chains that, according to crystallographic data, are nearest neighbors if not in van der Waals' contact with at least one atom of the indole ring. Among these proteins τ ranges between 2 s and <10 μs. Inspection of the side chains that are nearest neighbors of indole points out that whenever $\tau \ll 1$ ms, i.e., is too short to simply reflect a mobile environment and consequently should be dominated by some intramolecular quenching reaction, there invariably is a Tyr or a Cys side chain in contact with the chromophore. Examples are Trp-15 of LADH ($\tau < 10$ μs), Trp-3 of phospholipase A_2 ($\tau < 10$ μs), Trp-113 of subtilisin Carlsberg ($\tau < 10$ μs), and the internal residue Trp-310 of GAPDH ($\tau \approx 0.18$ ms). Next to Trp-15 there is Cys-132, next Trp-3 there are Tyr-75 and Tyr-73, next to Trp-113 there is Tyr-91, and next to Trp-310 there is Tyr-283. The converse is not always true; namely, the presence of a quenching side chain in proximity of indole does not necessarily imply a short RTP lifetime. Clear examples of this are found in Trp-109 of AP ($\tau \approx 2$s) and Trp-314 of LADH ($\tau = 150/700$ ms). In the former the phenol oxygen of Tyr-84 is 4.5 Å from the nearest indole atom, while in the latter the aromatic ring of Trp-314 of the adjacent subunit is practically in van der Waals' contact. What instead seems to be the rule is that the absence of quenching side chains in the immediate environment of Trp is associated with relatively long, presumably unperturbed RTP lifetimes in that the magnitude of τ is commensurate with the chromophore's site expected flexibility. Thus, for Trp-32 of HSOD ($\tau = 1.7$ ms), Trp-140 of *S* nuclease ($\tau = 0.15/0.74$), Trp-308 of PGK ($\tau = 0.65$ ms), and presumably Trp-220 of AP ($\tau = 0.09/2.9$ ms), where the aromatic ring is highly exposed to the solvent and experiences a local viscosity near that of bulk water, τ is not much different from that of free NATA. Alternatively, residues buried in the macromolecule's interior and, therefore, bound to experience a more rigid local structure display much larger RTP lifetimes. Examples are Trp-48 of azurin ($\tau = 600$ ms), Trp-179 of PFK ($\tau = 236$ ms), and Trp-84 of GAPDH ($\tau = 23/91$ ms).

Besides chromophore−quencher separation, a close inspection of Table 7 suggests that the mutual orientation is also likely to play a non-negligible role in determining the quenching rate of Tyr and Trp (as might be expected if the process involves the formation of an excited state complex). Although there is a Tyr practically in contact with Trp-109 of AP, Trp-59 of RNase T_1, and Trp-177 of Trp synthase, the RTP lifetimes of these residues are relatively large and, therefore, are little perturbed. By contrast τ of Trp-310 of GAPDH is very small, suggesting perhaps that the stacking configuration between indole and phenol rings in the latter is highly more favorable for the reaction than the edge to edge or edge to plane approach found in all the other cases. Indirect evidence for orientational restrictions in the quenching reaction with Tyr is also found in the remarkable quenching effectiveness of Tyr toward solvent-exposed, flexible Trp sites ($\tau < 10$ μs for subtilisin Carlsberg and phospholipase A_2). This correlation between motional freedom and quenching rate may indeed be based on the possibility of achieving statistically improbable but highly favorable indole−phenol orientations. Another example of the dramatic orientation effects is found for Trp self-quenching in LADH. Self-quenching is a general phenomenon with aromatic molecules ($k_q = 1.7 \times 10^7$ M^{-1} s^{-1} for Trp) and is due to excimer formation, a complex between a molecule in the excited state and a molecule in the ground state. As mentioned above Trp-314 is at the subunit interface, edge to edge with the same residue of the opposite subunit. The two indole rings lie roughly on the same plane with the edges practically in contact (3.5 Å between the centers of nearest carbon atoms). Despite the proximity, τ is remarkably large (from 700 ms up to 1.4 s in some ternary complexes; Strambini & Gonnelli, 1990), implying that for this geometrical arrangement the excimer interaction is vanishingly small.

Temperature Dependence of Quenching Reactions. The above discussion has illustrated that intramolecular quenching reactions are responsible for the wide range of RTP lifetimes among the proteins examined. Such variability in τ at ambient temperature contrasts with the remarkable uniformity in decay behavior in low-temperature glasses and clearly demonstrates that, below −130 °C, quenching by Tyr and cysteine is negligible. The same conclusion seems to apply also to His quenching as τ of peptides P_4−P_6 is again 6 s in glasses. Hence, it would seem that in every case the first-order quenching reaction is highly temperature dependent.

Between 30 and 100 K, temperature effects were reported on both the rate of indole quenching by disulfide derivatives and the rate of Trp quenching by cystine residues in proteins (Li et al., 1992). By employing model compounds the authors estimated a value of k_o, the rate constant at van der Waals' contact, of 2×10^4 s^{-1} at 77 K and an activation energy of 2−3 kJ mol^{-1}. They also verified that for the sulfhydril group k_o at 77 K is undetectably small (Li et al., 1989). Direct measurements of k_o at 20 °C are not available, but an order of magnitude estimate can be derived from the Stern−Volmer quenching rate constant (Table 2), as k_q can be described as the product of the diffusion rate constant k_d and the quenching efficiency of the encounter complex (eq 1; Birks, 1970)

$$k_q = k_d k_o/(k_o + k_{-d}) \quad (1)$$

where k_{-d} is the back-diffusion rate constant responsible for the breakdown of the complex. For the diffusion of small

molecules in water $k_d \approx 10^{10}$ M^{-1} s^{-1} so that values of $k_q \ll 10^{10}$ M^{-1} s^{-1} imply quenching efficiencies $\ll 1$. In such cases $k_q = Kk_o$, and since for weak complexes $K = k_d/k_{-d} \approx 1$, we have $k_q \approx k_o$. Each amino acid of Table 2 satisfies this condition, and hence the magnitude of its k_q may be taken as an approximate estimate of k_o. Thus, k_o at 20 °C is larger than 10^8 s^{-1} for cystine and cysteine and is only 2−3 orders of magnitude smaller for Tyr and His, values that confirm once more the strong temperature dependence of these reactions. We also note that for disulfide quenching the activation barrier above 100 K must be larger than 2.3 kJ mol^{-1}, as that would predict $k_0 = 3 \times 10^5$ s^{-1} at 20 °C.

A direct manifestation of large increases in k_o with temperature may also be found in the τ vs temperature profiles of some synthetic peptides in PG/buffer. Above the glass transition temperature of the solvent, the log $1/\tau$ vs log T/η plots are not linear, and for His peptides P_5 and P_6 (pH 4) and Cys peptides P_7 the gradient exceeds 1. This means that the rate of quenching is under rate control ($k_o < k_{-d}$, $k_q \approx k_o$) rather than diffusion control ($k_q \approx k_d$) and that the decrease in τ with temperature reflects mostly the variation in k_o.

In summary, the enhancement in time resolution and sensitivity of phosphorescence measurements has opened the possibility to monitor RTP from practically every protein. The results of the present and previous (Strambini & Gonnelli, 1995) investigations demonstrate that two are the important factors that concur in determining the RTP lifetime of Trp residues in proteins. One is the effective local viscosity of the protein/solvent matrix, the other is the extent of intramolecular quenching by His, Tyr, Trp, and Cys side chains that lie in the immediate neighborhood of the indole ring. When these quenchers are beyond the "solvation" shell, τ is unperturbed and its magnitude provides a direct measure of the flexibility of the Trp environment; it is expected to span a range from a few seconds to ≈0.5 ms as the triplet probe moves from rigid, glass-like cores of the globular structure to sites in the periphery that are as fluid as the aqueous solvent. In all other cases, τ depends in a complex way on several factors: the type of quenching side chain that the chromophore comes into contact with, the temperature, the pH, and the geometrical constraints imposed on the indole−quencher encounter complex formed. Because of quenching reactions, the range of τ extends considerably to the lower end and may reach microseconds or less. Now, the relationship between τ and structural flexibility is bound to be complex and peculiar of each protein site. In general, a mobile environment will enhance both the encounter frequency with quenchers not already in contact and favor the attainment of optimal quenching configurations, and therefore it is expected that, in many cases, τ will again strongly correlate with the flexibility of the Trp environment.

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