Tyrosine Quenching of Tryptophan Phosphorescence in Glyceraldehyde-3-Phosphate Dehydrogenase from *Bacillus stearothermophilus*

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ABSTRACT Tyrosine is known to quench the phosphorescence of free tryptophan derivatives in solution, but the interaction between tryptophan residues in proteins and neighboring tyrosine side chains has not yet been demonstrated. This report examines the potential role of Y283 in quenching the phosphorescence emission of W310 of glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* by comparing the phosphorescence characteristics of the wild-type enzyme to that of appositely designed mutants in which either the second tryptophan residue, W84, is replaced with phenylalanine or Y283 is replaced by valine. Phosphorescence spectra and lifetimes in polyol/buffer low-temperature glasses demonstrate that W310, in both wild-type and W84F ($Trp^{84} \rightarrow Phe$) mutant proteins, is already quenched in viscous low-temperature solutions, before the onset of major structural fluctuations in the macromolecule, an anomalous quenching that is abolished with the mutation Y283V ($Tyr^{283} \rightarrow Val$). In buffer at ambient temperature, the effect of replacing Y283 with valine on the phosphorescence of W310 is to lengthen its lifetime from 50 μs to 2.5 ms, a 50-fold enhancement that again emphasizes how W310 emission is dominated by the local interaction with Y283. Tyr quenching of W310 exhibits a strong temperature dependence, with a rate constant $k_q = 0.1\ s^{-1}$ at 140 K and $2 \times 10^4\ s^{-1}$ at 293 K. Comparison between thermal quenching profiles of the W84F mutant in solution and in the dry state, where protein flexibility is drastically reduced, shows that the activation energy of the quenching reaction is rather small, $E_a \leq 0.17$ kcal mol^{-1}, and that, on the contrary, structural fluctuations play an important role on the effectiveness of Tyr quenching. Various putative quenching mechanisms are examined, and the conclusion, based on the present results as well as on the phosphorescence characteristics of other protein systems, is that Tyr quenching occurs through the formation of an excited-state triplet exciplex.

INTRODUCTION

In the last decade, the sharp dependence of the phosphorescence lifetime of tryptophan (Trp) on the viscosity of the medium (Strambini and Gonnelli, 1985, 1995) has represented an important intrinsic monitor of the dynamical features of protein structure (Vanderkooi, 1992; Schauerte et al., 1997; Strambini, 1989). Thus Trp phosphorescence has been used to report on the flexibility of the polypeptide in terms of the type and extension of secondary and tertiary structure elements, in terms of the physical state of the sample (crystalline (Strambini and Gabellieri, 1987), dry powder (Strambini and Gabellieri, 1984), micellar (Strambini and Gonnelli, 1988), frozen (Gabellieri and Strambini, 1996)), the nature of the solvent (Gonnelli and Strambini, 1993; Strambini and Gonnelli, 1986), and external conditions of temperature and pressure (Cioni and Strambini, 1994, 1996). In addition, because the dynamical structure of the polypeptide is a very sensitive function of its conformation, Trp phosphorescence lifetimes have unveiled distinct changes in the conformation of enzymatic proteins induced by binding of substrates (Strambini and Gonnelli, 1990; Sun et al., 1997), allosteric effectors (Cioni and Strambini, 1989), or subunit aggregations that are not readily detected by the usual spectroscopic methods.

Although the room temperature phosphorescence (RTP) lifetime (τ_{RTP}) of proteins appears to be generally governed by the local flexibility about its Trp chromophores, a few examples are known of Trp residues for which τ_{RTP} is even shorter than the 0.2–0.5 ms expected for a very mobile, fully unfolded polypeptide (Gonnelli and Strambini, 1995; Gabellieri et al., 1996). For these Trps, τ_{RTP} is smaller than would be predicted from the fluidity of their environment, and, consequently, in these cases the lifetime must be dominated by efficient intramolecular quenching reactions. As a result, unless intramolecular quenching can be excluded, great caution must be exercised before τ_{RTP} can be interpreted in terms of structural flexibility. Such an assessment requires knowledge of the intrinsic quenching groups and of their quenching mechanisms.

Recent studies with the Trp derivative *N*-acetyltryptophanamide (NATA) in aqueous solutions at ambient temperature showed that among the amino acids, only cystine, cysteine, histidine, and tyrosine are effective quenchers of its phosphorescence (Gonnelli and Strambini, 1995). In proteins, except for the disulfide group, intramolecular quenching by these side chains has not been demonstrated so far. Direct evidence of disulfide quenching in proteins was obtained in low-temperature glasses where the unperturbed lifetime of 5–6 s is restored only after the reduction of the bridge (Longworth, 1971; Li et al., 1992; Smith and Maki, 1993). At ambient temperature, both sulfhydryl and

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disulfide moieties are bound to be stronger quenchers of Trp phosphorescence. Indeed, small thiol and disulfide compounds free in solution were shown to affect the lifetime of internal Trp residues, even across fairly large ($r > 0.5$ nm) and impermeable layers of the protein matrix (Vanderkooi et al., 1990). The presumed mechanism is electron exchange (Bent and Hayon, 1975; Vanderkooi et al., 1990; Li et al., 1992; Gabellieri and Strambini, 1994), an interaction process that drops exponentially with the separation and that seems to depend only weakly on the mutual orientation of the reacting centers. Based on these features, one can anticipate efficient phosphorescence quenching by Cys residues in proteins, even for immobilized Trp residues that are not in direct van der Waals contact with the Cys.

In contrast to -SH and -S-S- groups, the mechanism of quenching by Tyr and His is unknown, and therefore no prediction can be made about their potential intramolecular quenching behavior until the distance/orientation requirements of the reaction are known. Fortunately, proteins, with their variety of well-characterized and rigidly held chromophore-quencher configurations, provide interesting model systems for investigating the characteristics and the mechanism itself of intramolecular quenching. In a recent examination of the phosphorescence properties of W310 in glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* (Gabellieri et al., 1996), it was noted that at ambient temperature its emission is strongly quenched and that the τ_{RTP} of 50 μs is over 10-fold shorter than anticipated for the most mobile solvent-exposed protein sites. Inspection of the crystallographic structure (Skarzynski and Wonacott, 1988) shows that among the putative quenching side chains, only Y283 is in close contact with W310. However, because in other proteins it was noted that the proximity to Tyr is not a sufficient condition for effective quenching, it was suggested that the peculiar stacked configuration between W310 and neighboring Y283 might be responsible for the perturbed phosphorescence of W310 (Gabellieri et al., 1996). The correctness of this hypothesis is now confirmed through examination of the phosphorescence properties of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) single and double Trp mutants, where Y283 was either retained or replaced by valine. In addition, to gain further insight into the quenching mechanism by Tyr, the dependence of the quenching reaction was studied as a function of temperature and of structural flexibility. The results of these experiments, together with the analysis of data available on Trp-Tyr contact pairs in other proteins, are consistent with a quenching interaction based on triplet exciplex formation.

MATERIALS AND METHODS

Materials

Charcoal Norit A^R was purchased from Serva (Heidelberg, Germany) and was activated following the procedure described by Henis and Levitzki (1977). *N*-Acetyltryptophanamide (NATA) was obtained from Janssen Chimica (Geel, Belgium). All chemicals were of the highest purity grade available from commercial sources and were used without further purification. Double-distilled MilliQ (Millipore) water was used throughout.

Site-directed mutagenesis, production, and purification of wild-type and mutant enzymes

Production and purification of *B. stearothermophilus* W84F ($Trp^{84}\rightarrow Phe$) mutant GAPDH were carried out as previously described (Gabellieri et al., 1996). Production and purification of wild-type and W310F ($Trp^{310}\rightarrow Phe$), Y283V ($Tyr^{283}\rightarrow Val$) mutant enzymes were performed in *Escherichia coli* HB101 strain transformed by the appropriate recombinant plasmid, pBluescript II SK containing the *gap* gene under the *lac* promoter. Site-directed mutageneses were performed using the method of Kunkel et al. (1991). Cells were grown in LB medium containing ampicillin (200 mg/liter). After sonication of the transformed cells, the GAPDH was purified by ammonium sulfate fractionation (66%–92%) and chromatography on ACA 34 resin equilibrated in 50 mM Tris-HCl buffer containing 2 mM EDTA, pH 8.0 (buffer A). Fractions containing GAPDH activity were then applied to a Q-Sepharose column using a FPLC system (Pharmacia, Uppsala) previously equilibrated with buffer A. Wild-type and mutant GAPDHs were eluted at 200 mM KCl with a linear gradient from 0 to 200 mM KCl in buffer A at 5 ml/min. The molecular weight determined by mass spectrometry for the W84F, Y283V, W310F, and D32G-LP ($Asp^{32}\rightarrow Gly$, $Leu^{187}\rightarrow Ala$, $Pro^{188}\rightarrow Ser$) mutants was in agreement with the calculated molecular weight. Specific activities of the mutant enzymes were not significally affected by the mutations.

NAD^+-free enzymes (apoenzymes) were prepared by activated charcoal treatment, as described by Henis and Levitzki (1977). Before phosphorescence measurements the protein samples were dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. Enzyme concentrations were determined spectrophotometrically, using extinction coefficients at 280 nm of $\epsilon = 1.17 \times 10^5\ M^{-1}\ cm^{-1}$ for apoWT and $\epsilon = 9.42 \times 10^4\ M^{-1}\ cm^{-1}$ for apoW84F and apoW310F GAPDH.

Phosphorescence measurements

The samples were placed in 4 mm i.d. cylindrical Spectrosil cuvettes. Before phosphorescence measurements, oxygen was thoroughly removed from the sample as described by Cioni and Strambini (1989). A conventional home-made instrument was employed for all phosphorescence intensity and spectra measurements (Cioni and Strambini, 1989). Continuous excitation, provided by a Cermax xenon lamp (LX 150UV; ILC Technology, Sunnyvale, CA), was selected by a 0.25-m grating monochromator (Jobin-Yvon, H25), and the emission, dispersed by a 0.25-m grating monochromator (Jobin-Yvon, H25), was detected with an EMI 9635 QB photomultiplier. Phosphorescence decays in fluid room temperature solutions were measured on an apparatus described before (Strambini and Gonnelli, 1995). Pulsed excitation was provided by a frequency-doubled flash-pumped dye laser (UV 500 M-Candela) ($\lambda_{ex} = 292$ nm) with a pulse duration of 1 μs and a typical energy per pulse of 1–10 mJ. The emitted light was collected at 90° from the excitation light and selected by a filter combination with a transmission window between 420 and 480 nm. The photomultipliers were protected from the intense excitation and fluorescence light pulse by a high-speed chopper blade that closed the slits during laser excitation. The minimum dead time of the apparatus was ~10 μs. All of the decaying signals were digitized and averaged by a computerscope system (EGAA; RC Electronics). Subsequent analysis of decay curves in terms of discrete exponential components was carried out by a nonlinear least-squares fitting algorithm, implemented by the program Global Analysis. All reported decay data are averages of three or more independent measurements. The reproducibility of τ_P was typically better than 5%.

Dry protein samples were prepared by placing the lyophilized protein under vacuum for 10 h at a temperature of 50°C. The degree of hydration, checked by weighing the amount of water lost upon drying the sample

under vacuum for 24 h at a temperature of 80°C, was typically less than 0.5%.

RESULTS AND DISCUSSION

Phosphorescence emission of W310 in low-temperature PG/buffer glasses

GAPDH is a tetramer with two Trp residues per subunits. High-resolution phosphorescence spectra in propyleneglycol (PG)/buffer glasses (Gabellieri et al., 1996) show that the individual emission of each Trp is spectrally well resolved with the 0,0 vibronic band ($\lambda_{0,0}$) centered at 406 nm for W84 and at 411 nm for W310 (Fig. 1), the spectral assignment being confirmed by the two single Trp mutants W84F and W310F. To test the influence of Y283 on the emission of neighboring W310, the simplest way would be to compare the phosphorescence properties of the two single Trp mutants W84F (W310 in the presence of Y283) and W84F-Y283V (W310 with Y283 replaced by Val). To date, the latter mutant has not been available. Therefore the effects of Y283 on the phosphorescence of W310 were deduced from the phosphorescence properties of the Y283V and W84F mutants by subtracting from the total emission of Y283V the contribution of W84, which was separately characterized under all relevant experimental conditions by using the W310F protein.

Visual inspection of Fig. 1, as well as quantitative spectral decomposition of the wild-type (WT) phosphorescence spectrum in terms of the individual spectra of W84 and W310 (Fig. 1), shows that at 100 K the quantum yields of the two Trp residues (λ_{ex} = 295 nm) are quite similar. As the temperature of the glass is raised, there is, however, a progressive quenching of W310, until at 180 K, well below the glass transition temperature ($T_g \approx$ 190 K), its contribution is no longer detectable. Such an early quenching of W310 is abolished when Y283 is replaced by Val. Fig. 1 shows the phosphorescence spectrum of the Y283V mutant, in the region of the 0,0 vibronic band. The change in the W310 environment induced by the amino acid replacement has caused its spectrum to broaden and to blue shift by at least 2–3 nm. As a result, its phosphorescence appears less intense and the individual emissions of W310 and W84 are less resolved than in the WT protein. Nevertheless, it is evident that the contribution of W310 to the overall spectrum of the Y283V mutant is retained at higher temperature (180 K). It should be noted that a broader W310 phosphorescence spectrum reflects a more heterogeneous environment of the probe in the Y283V mutant relative to WT. This is not unexpected, as the replacement of Tyr with the smaller Val side chain will reduce the packing density and eliminate H-bonding to the phenolic hydroxyl, with both effects resulting in greater mobility and, therefore, in conformational heterogeneity of neighboring side chains.

It should be noted that a reduction in steady-state phosphorescence intensity can result from the decrease in either the intersystem crossing yield (Φ_{isc}) or the phosphorescence lifetime or both. However, because Φ_F is constant between 100 and 180 K, and previous studies have confirmed that the ratio Φ_F/Φ_{isc} for Trp is invariant with temperature (Strambini and Gabellieri, 1990), we infer that Φ_{isc} is also constant and that quenching of W310 phosphorescence is due to the lifetime reduction. Thus, in this case, the phosphorescence intensity ratio P_{W310}/P_{W84} is proportional to the lifetime ratio τ_{W310}/τ_{W84}. The decrease in lifetime of W310 in both WT and Y283V mutant proteins was estimated from phosphorescence intensities obtained by spectral decomposition, and is shown in the lower panel of Fig. 2. The results obtained with the WT are confirmed independently by the mutant D32G-LP, which in terms of Tyr and Trp residues is WT-like. Lifetime shortening is also observed directly from the multiphasic decay of the WT phosphorescence. However, one advantage of using steady-state intensities to estimate the individual lifetimes of W310 and W84 is that the assignment of decaying components is unambiguous and

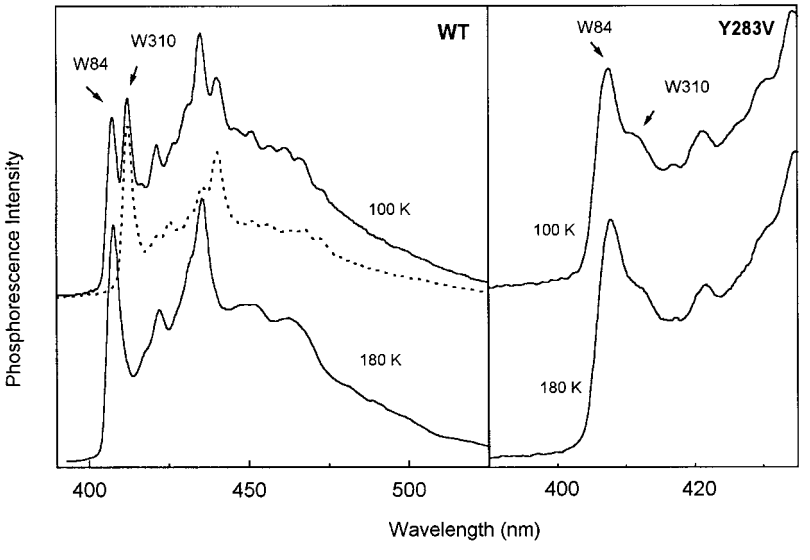

FIGURE 1 Temperature dependence of the Trp phosphorescence spectrum (λ_{ex} = 295 nm) of GAPDH WT and Y283V mutant, in a 50/50 (v/v) PG/phosphate buffer (20 mM, pH 7.5) glass. The spectrum of the W84F mutant (*dotted line*) is inserted to show the resolution of the individual vibrational bands and the contribution of W310 to the overall spectrum. The protein concentration was ~10 μM.

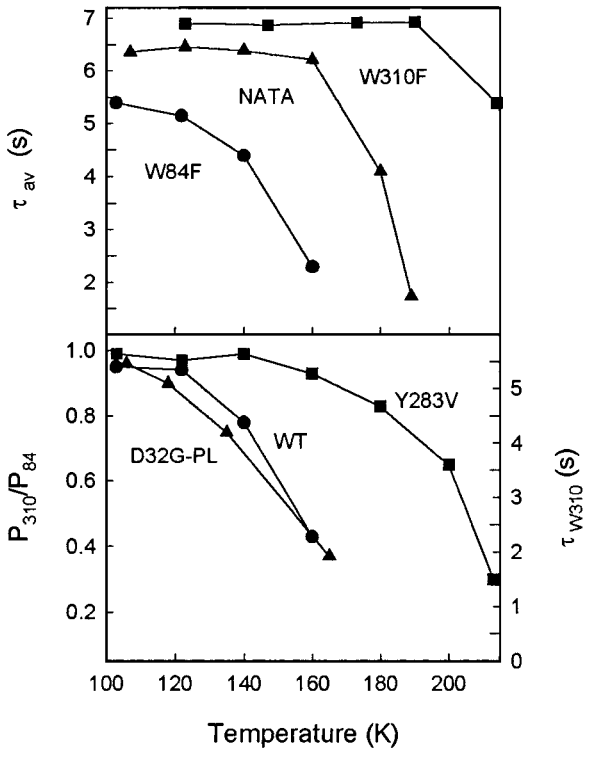

FIGURE 2 (*Top*) Temperature dependence of the phosphorescence lifetime of W84 and W310 of GAPDH single Trp mutants W310F and W84F, in PG/buffer glasses. The lifetime of free NATA in the same solvent is shown for comparison. (*Bottom*) Temperature dependence of the steady-state phosphorescence intensity (P) of W310 in GAPDH WT and mutants D32G-LP and Y283V. The intensity P_{310} is obtained by subtracting from the total emission the contribution of W84, which is exclusive below 406 nm. Indicated on the opposite axis is also the lifetime of W310 calculated from the proportionality $\tau_{W310}(T)/\tau_{W84}(T) \propto P_{W310}(T)/P_{W84}(T)$ and limiting (100 K) lifetimes of 5.5 and 6.9 s for W310 and W84, respectively.

straightforward. The results demonstrate that, depending on the temperature, τ_{W310} increases considerably upon replacement of Y283 by Val and that in this mutant, τ_{W310} begins to shorten only after softening of the glass and onset of structural flexibility ($T > 180$K). Reported in the upper panel of Fig. 2 are the phosphorescence lifetimes measured in the same conditions for W310 in the W84F mutant, for W84 in the W310F mutant, and for NATA. The results show that as the temperature is raised, the reduction of τ_{W310} in the single Trp mutant roughly coincides with τ_{W310} calculated for the WT protein. Moreover, they show that it occurs while the glass is still relatively rigid, as inferred from the constant maximum value of τ_{NATA}. Thus both intensities and lifetimes suggest that the early quenching of W310 is due to the presence of Y283, namely, to a specific interaction between the excited triplet state of indole and the phenol ring. It should be noted that Tyr-perturbed Trp phosphorescence in rigid glasses has never been reported before, not even in Trp-Tyr copolymers at 77 K (Longworth, 1971).

Last, with regard to the protein environment about W310 and W84, we note that according to their unquenched lifetime (in the Y283V mutant) in soft glasses, they exhibit considerably different local flexibilities. Above 180 K, where the glass is sufficiently fluid to confer substantial flexibility to the protein structure, τ_{W310} (in the absence of Y283) is greater than τ_{NATA} but smaller than τ_{W84}. This indicates that the W310 site is more rigid than the outside solvent, but is much more flexible than the region of the macromolecule hosting W84. This conclusion is also confirmed from the magnitude of the lifetimes in buffer at ambient temperature.

Phosphorescence emission of W310 in buffer

In PG/buffer glasses, τ_{W310} of WT and W84F mutant drops sharply on approaching the glass transition temperature, and the decay kinetics of W310 are no longer distinguishable from the short-lived emission from impurities in the organic solvent. Consequently, in fluid solutions the emission of W310 can only be examined in pure buffer. The phosphorescence decay of W310 in W84F GAPDH in phosphate buffer (20 mM, pH 7.5) is monophasic, with τ_{W310} equal to 150 ± 12 μs at 0°C (Fig. 3) and 50 ± 5 μs at 20°C (decay curve not shown). A similarly short-lived component is also present in the distinctly multiphasic decay of the WT protein and is assigned to W310 (data not shown). In the latter, the rest of the emission has a lifetime of 350 and 95 ms at 0°C and 20°C, respectively, and is assigned to W84 (by analogy to the phosphorescence lifetime of W84 in the W310F mutant). When Y283 is replaced with Val, the long-lived component is unaffected, but the microsecond lifetime shifts into the millisecond range, with τ_{W310} in the Y283V mutant becoming 4.5 ms at 0°C (Fig. 3) and 2.5 ms at 20°C (data not shown). Two conclusions can be drawn from these findings: 1) Even in buffer at ambient tempera-

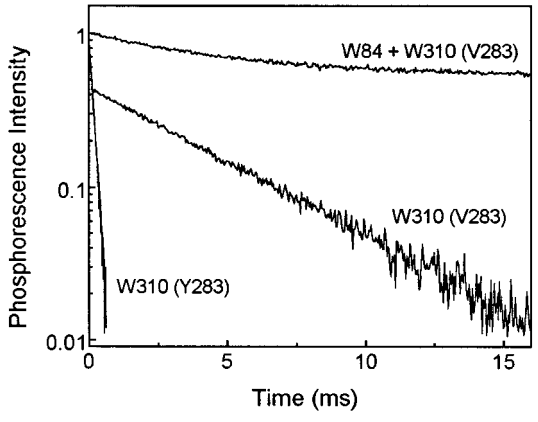

FIGURE 3 Example of primary phosphorescence decay data ($\lambda_{ex} = 292$ nm) in phosphate buffer (20 mM, pH 7.5) at 0°C, of GAPDH mutants Y283V (indicated as W84 + W310(V283)) and W84F (indicated as W310(Y283)). The decay of W310 when Y283 is replaced by Val (indicated as W310(V283)) is derived by subtracting from the top trace the decay of W84 (from the mutant W310F), weighed according to the amplitude of the long-lived component in the former. The decay signals are the averages of 10–15 sweeps. The protein concentration was ~3.5 μM.

ture, τ_{W310} in the WT protein appears to be dominated by the interaction with Y283, because its substitution with an inert side chain results in lengthening of the lifetime by over 50-fold. 2) The magnitude of 2.5 ms for τ_{W310} in the Y283V mutant, although longer than the ~0.5 ms typically found for Trp residues exposed to the aqueous phase, is rather small for a buried protein site, an indication that the protein matrix around W310 is quite flexible. Such a high internal mobility might be associated to the location of W310 at the hinge between the catalytic and coenzyme domains. This area is the center of rotation of the two domains during the apo to holo transition. It should be noted that the perturbation of protein structure resulting from the replacement of Tyr with Val, as evidenced by the broadening of the low-temperature phosphorescence spectrum, is expected to increase the local fluidity.

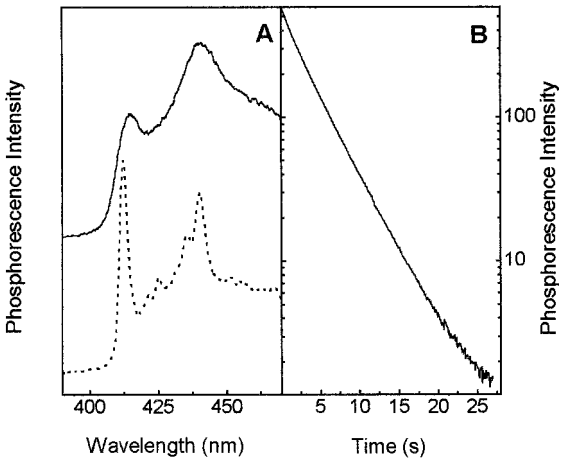

FIGURE 4 (*A*) Phosphorescence spectrum (λ_{ex} = 295 nm, T = 140 K) and (*B*) decay (λ_{em} = 440 nm, T = 252 K) of the GAPDH mutant W84F in the dry powder state. The spectrum of the protein in dilute PG/buffer solutions (*dotted curve*), at the same temperature of 140 K, is included to manifest the degree of spectral broadening in the dry state.

Phosphorescence emission of W310 in dry protein powders

According to the above findings, τ_{W310}, both in low-temperature glasses and at ambient temperature in buffer, is dominated by a local interaction with Y283. The rate of this quenching reaction, $k_q = (1/\tau - 1/\tau_0)$, where τ_0 is the unpertubed lifetime measured in the Val mutant (e.g., 5.5 s at 140 K), is vanishingly small below 140 K ($k_q < 0.1\ s^{-1}$), but reaches up to $2 \times 10^4\ s^{-1}$ at 293 K, an increase of over five orders of magnitude. The sharp temperature dependence of k_q may be due to a quenching mechanism characterized by a large activation barrier ($E_a \approx 7.4$ kcal mol^{-1}), and/or to a dependence of the quenching reaction on the fluidity of the protein matrix (upon which might depend the attainment of an optimally quenching Tyr-Trp configuration), which is drastically enhanced across this temperature range. To distinguish between purely thermal activation from sterically hindered diffusional effects, the phosphorescence properties of the protein were studied in the dry state by completely dehydrating the sample. Indeed, the dry state generally exhibits exceptional structural rigidity (Strambini and Gabellieri, 1984; Gregory, 1995), thus largely inhibiting structural fluctuations.

The phosphorescence emission of dry samples of the W84F mutant were examined over the 130–303 K temperature range. The phosphorescence spectrum, as compared to dilute aqueous solutions, is distinctly less resolved (Fig. 4 *A*), the broad width of the 0,0 vibronic band attesting to marked heterogeneity in the environment of W310. This perturbation of protein structure in the dry state has already been observed with other proteins (Strambini and Gabellieri, 1984). Structural heterogeneity is also reflected in the intensity decay kinetics. Above 250 K, the decay is markedly nonexponential (Fig. 4 *B*), and at least three lifetime components are required to satisfactorily fit the data.

The lifetime values (τ_i) and the corresponding amplitudes (α_i) at some representative temperatures are collected in Table 1. The results show unequivocally that the average lifetime in the dry state, $\tau_{av} = \Sigma\alpha_i\tau_i$, is larger than in solution and that the difference increases dramatically with temperature. For instance, 20°C τ_{W310} increases by 2×10^4-fold from 50 μs to 1 s. Hence the great efficiency of Tyr quenching in fluid solutions apparently requires a structurally flexible protein environment.

An upper limit for the temperature dependence of k_q can be estimated by assuming that in the dry state $1/\tau_{av}(T) = 1/\tau_0 + k_q(T)$, where $\tau_0 = 5.5$ s is the low temperature limit. The Arrhenius plot of log $k_q(T)$ against $1/T$, shown in Fig. 5, is characterized by two linear regions intersecting at 255 K. Below this temperature the activation energy derived from the gradient is quite small, ~0.17 kcal mol^{-1}, implying that the intrinsic quenching reaction is largely temperature independent. Above 255 K the activation energy value is 7.0 kcal mol^{-1}. Therefore, at warmer temperatures, other factors or mechanisms contribute to the effectiveness of the quenching reaction. It should be noted, however, that at these higher temperatures, protein motions are not totally blocked in dry powders (Gregory, 1995). This is also confirmed by the unperturbed τ_{av} of W84, which, between 250 and 300 K, progressively decreases from the low temperature limit of 5.8 s to 1.04 s. Thus it is quite plausible that this

TABLE 1 Phosphorescence lifetimes (τ_i) and corresponding amplitudes (α_i) of W84F GAPDH powders at some representative temperatures

T (K)	τ_1 (s)	τ_2 (s)	τ_3 (s)	α_1	α_2	τ_{av} (s)
152		3.05	6.10		0.78	3.72
211		2.95	5.51		0.75	3.59
231		1.21	4.3		0.33	3.28
252		1.3	3.98		0.42	2.85
272	0.60	1.03	3.04	0.20	0.32	1.91
293	0.45	1.7	3.54	0.61	0.34	1.06
303	0.29	1.27	3.0	0.68	0.27	0.69

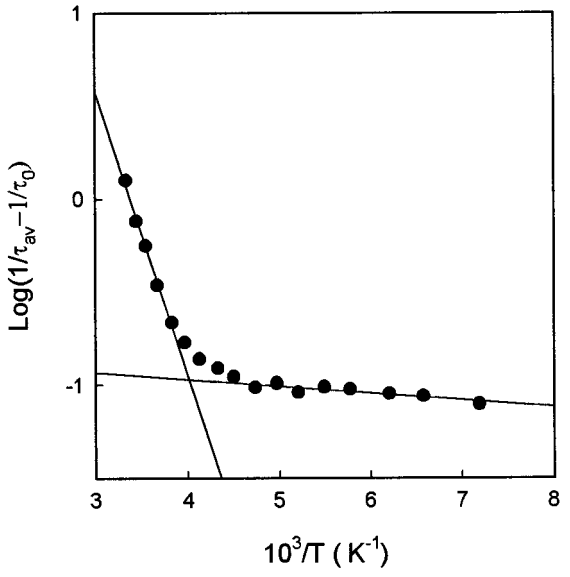

FIGURE 5 Arrhenius plot of the temperature dependence of $k_q(T) = (1/\tau_{av} - 1/\tau_0)$, with $\tau_0 = 5.5$ s, obtained from the average phosphorescence lifetime of W84F GAPDH in the dry powder state.

thermally activated, high-temperature $k_q(T)$ component again reflects the overcoming of steric constraints to achieve a more effective Trp-Tyr quenching arrangement. Both this thermal component of k_q in dry powders and the large difference of k_q between solution and dry state suggest that the effectiveness of Y283 quenching of W310 phosphorescence in GAPDH is critically dependent on the flexibility of the polypeptide structure. This characteristic points to a quenching mechanism in which the rate is a steep function not only of the proximity, but also of the mutual orientation of the reacting centers.

Naturally, knowledge of the quenching mechanism is fundamental for making reliable predictions about potential perturbations of Trp phosphorescence in proteins by surrounding Tyr residues. Among the interactions between the excited triplet-state of Trp and the phenol ring of Tyr that may lead to quenching of phosphorescence, the most important are (Birks, 1970) 1) electron exchange or transfer, 2) proton transfer to the indole ring, and 3) triplet exciplex formation. An electron exchange/transfer mechanism has been postulated for quenching by a number of reducing/oxidizing compounds (organic and inorganic), including -SH and -S-S- groups. Generally, the quenching rate constant is considerable, even in low-temperature glasses (Strambini and Gabellieri, 1991), and it falls off exponentially with donor-acceptor separation (critical distances up to 1.2 nm) (Vanderkooi et al., 1990; Strambini and Gabellieri, 1991). This mechanism does not require molecular contact, and judging from the experimental evidence available, the rate is not expected to be strongly dependent on the mutual orientation of reacting centers. None of these criteria conform to the characteristics displayed by Tyr quenching. The latter appears to be highly inefficient at low temperature (see present results with W310 of GAPDH and older reports on Tyr-Trp copolymers; Longworth, 1971), requires close proximity if not direct molecular contact, and, as deduced from its dependence on protein motions, is critically dependent on the geometry of the contact complex. On these grounds, an electron exchange mechanism can be safely ruled out.

A direct role of proton transfer to the indole ring in quenching Trp phosphorescence was inferred from a proton bimolecular quenching rate constant for NATA, in H_2O at 20°C, of 10^7 M^{-1} s^{-1}, and also from the at least 50-fold enhancement of the bimolecular quenching rate constant of His in its protonated state (Strambini and Gonnelli, 1995). Quenching of NATA by Tyr, however, increases (17-fold) on going to alkaline pH, above its pK_a of 10.4 (Strambini and Gonnelli, 1995). It follows that the Tyr^- anion is a better quencher than neutral Tyr, and therefore, at least for the ionized state, a mechanism other than proton transfer must be operating. Indeed, the characteristics exhibited by Tyr quenching, namely, the strict requirement for a particular configuration of indole and phenol rings and the greater effectiveness of the Tyr anion, are more consistent with triplet exciplex formation, that is, a complex formed between indole in the excited triplet state and Tyr in the ground state. First, the binding energy of such complexes depends critically on the mutual orientation of the aromatic rings and is greatest for a stacked assembly, a sandwich-like complex between the indole and phenol moieties oriented such that their static electric dipole moments lie at 180°C (Birks, 1970) (Fig. 6 *a*). Furthermore, much of the binding energy of these complexes is predicted to be contributed by a charge transfer state in which indole and phenol act as electron acceptor and donor, respectively. The charge trans-

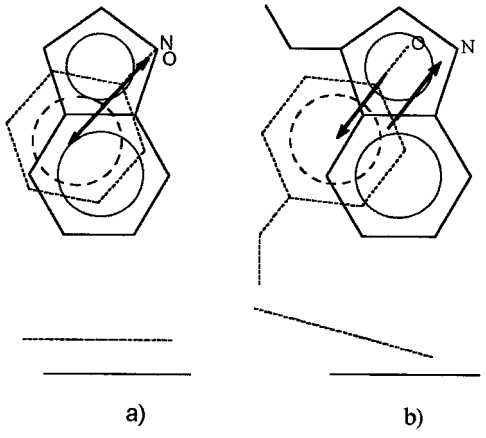

FIGURE 6 (*a*) Lowest energy configuration predicted for the formation of the triplet state exciplex between indole and phenol groups, based on the theoretical estimate of the dipole moment of the lowest excited triplet state (^3L_a) of indole (Hahn and Callis, 1997). (*b*) The stacked arrangement between the aromatic rings of W310 and Y283 of GAPDH obtained from the crystallographic structure (Skarzynski and Wonacott, 1988). The 90° out-of-plane projection of the aromatic rings, indicated below by straight lines, shows that in the protein the planes are not parallel, but make an angle of ~16°. Heavy arrows show the direction of the permanent dipole moments.

fer nature of the complex predicts an increase in k_q as the ionization potential of the donor is lowered. Because the ionization potential of tyrosinate ion is smaller than for protonated Tyr, this feature would nicely account for the greater quenching effectiveness of the anion.

Quenching by exciplex formation poses rather severe restrictions on the effectiveness of Tyr-Trp interactions in proteins, because the appropriate stacked configuration is statistically improbable and the conformational freedom required to allow even transient efficacious encounters is generally insufficient in internal protein sites. These restrictions do seem to account for the generally poor quenching effectiveness of proximal Tyr residues in proteins. Besides GAPDH, among the proteins for which the crystallographic structure is available and Trp phosphorescence has been characterized, Trp-Tyr contact pairs are found with W109 of alkaline phosphatase from *E. coli*, W177 of Trp synthase from *Salmonella typhimurium*, W59 of RNase T1, W3 of phospholipase A_2, and W113 of subtilisin Carlsberg. Except for GAPDH, in all other cases the approach between indole and phenol rings is either edge to edge or edge to plane, but never close to the required configuration for exciplex formation. Hence, unless the local structure is flexible enough to allow rapid rotational-translational motions of one aromatic ring relative to the other, Tyr quenching by exciplex formation is anticipated to be negligible. The indole rings of W109, W177, and W59 are buried more or less deeply within the globular structure, whereas those of W3 and W113 are on the surface of the macromolecule, partly or fully exposed to the solvent. Because only the latter residues are expected to enjoy substantial motional freedom, Tyr quenching is predicted to be efficient only for phospholipase A_2 and subtilisin Carlsberg. Indeed, in buffer at 20°C, τ_{W3} and τ_{W113} are both very short, below the 10-μs detection limit of the apparatus (Strambini and Gonnelli, 1995). Because in the vicinity of these residues there is no other quenching group but Tyr, we must conclude that in these proteins Tyr quenching is very effective. By contrast, the large value of τ_{RTP} measured with the internal residues W109 of alkaline phosphatase, W177 of Trp synthase, and W59 of RNase T1 (Gonnelli and Strambini, 1995) confirms that both the local protein structure is relatively rigid and Tyr quenching is inefficient. For W109 of alkaline phosphatase, a τ_{RTP} of ~2 s implies that $k_q < 0.5\ s^{-1}$, i.e., quenching is negligible.

To date, W310 of GAPDH is the only internal residue found to exhibit remarkable quenching of its phosphorescence by a proximal Tyr. According to the mechanism of exciplex formation, this requires that either the stacked configuration of indole and phenol rings indicated in Fig. 6 *a* be statistically probable, or that this internal site be unusually flexible. With W310, both conditions appear to be satisfied to a considerable extent. The unperturbed τ_{W310} exhibited by the Y283V mutant suggests that this internal site is rather flexible, whereas the crystallographic structure (Skarzynski and Wonacott, 1988) shows that the position of the aromatic rings is close to the configuration needed for exciplex formation (Fig. 6). As indicated in Fig. 6 *b*, the deviation from optimal geometry is represented by a ~0.1-nm lateral translation of one ring relative to the other and an inclination of their planes of ~16° away from parallel. Because of the small deviation, even low-energy, small-amplitude thermal fluctuations of the structure are sufficient for the realization of a strong exciplex. This would explain why, in the case of W310, quenching is effective, even for a buried Trp-Tyr pair and why it sets in at relatively low temperatures (140–170 K; Fig. 2). In comparison, the phosphorescence of phospholipase A_2 and subtilisin Carlsberg, proteins for which the aromatic rings arrangement is not favorable for exciplex formation, is quenched by Tyr in fluid solutions but not in glasses. If these correlations lend support to the exciplex mechanism, the remarkable dependence of k_q on the flexibility of GAPDH, despite the nearly optimal configuration of the aromatic rings for exciplex formation, suggests that the geometrical requirements of Tyr quenching are so stringent that chromophores buried in internal, rigid regions of the macromolecule are statistically unlikely to meet them. In other words, for internal Trp residues, Tyr quenching is expected to be a rare event.

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