Characterization of Tryptophan and Coenzyme Luminescence in Tryptophan Synthase from *Salmonella typhimurium*†

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ABSTRACT: Tryptophan synthase from *Salmonella typhimurium* is a bifunctional $\alpha_2\beta_2$ complex that catalyzes the formation of L-tryptophan. We have characterized over the temperature range from 160 to 293 K the fluorescence and phosphorescence properties of the single tryptophan present at position 177 of the β-subunit and of the pyridoxal 5′-phosphate bound through a Schiff's base in the β-active site. The comparison between the fluorescence of the pyridoxal phosphate bound either to the protein or to valine free in solution indicates substantial protection for the coenzyme against thermal quenching and a greater intensity of the ketoenamine tautomer band. Trp-177 is highly luminescent, and its proximity to the pyridoxal moiety leads to an over 50% quenching of its fluorescence with both reduced and native coenzyme. The Trp phosphorescence spectrum possesses a narrow, well-defined, 0–0 vibrational band centered at 418.5 nm, a wavelength that indicates strong polar interactions with neighboring charges. The observation of delayed fluorescence in the native complex implies that the excited triplet state is involved in a process of triplet–singlet energy transfer to the ketoenamine tautomer. The rate of energy transfer, heterogeneous in low-temperature glasses with rate constants of 2.26 and 0.07 s^{-1}, becomes homogeneous in fluid solutions as the coenzyme tautomer interconversion is likely faster than the phosphorescence decay. In both apo- and holo-$\alpha_2\beta_2$, the phosphorescence from Trp-177 is long-lived even at ambient temperature. However, the lifetimes for the holoenzyme are significantly longer than those of the apoenzyme, indicating a tightening of the N-domain of the β-subunit induced by coenzyme binding. The nonexponential nature of the decay in fluid solutions provides direct evidence for structural heterogeneity in the bienzyme complex, revealing at least two main conformers slowly interconverting in the millisecond time scale.

Bacterial tryptophan synthase is an $\alpha_2\beta_2$ complex that catalyzes the last two steps in the biosynthesis of L-tryptophan (Yanofsky & Crawford, 1972; Miles, 1979, 1991). The α-subunit catalyzes the reaction:

indole-3-glycerol phosphate ⇌ indole + D-glyceraldehyde 3-phosphate

The β-subunit, that contains a pyridoxal 5′-phosphate (PLP)[1] molecule bound via a Schiff's base to Lys-87, catalyzes the reaction:

indole + L-serine → L-tryptophan + H_2O

The $\alpha_2\beta_2$ complex catalyzes the overall reaction:

indole-3-glycerol phosphate + L-serine → L-tryptophan + H_2O

Subunit association increases the rates of the α and β reactions by about 2 orders of magnitude. Moreover, substrate and substrate analogs of one subunit affect the thermodynamic and kinetic properties of the other (Kirschner et al., 1975, 1991; Lane & Kirschner, 1981, 1983a,b, 1991; Kawasaki et al., 1987; Dunn et al., 1987, 1991; Houben & Dunn, 1990; Mozzarelli et al., 1989, 1991). The molecular basis of these regulatory properties is still essentially unknown. In fact, comparison of the three-dimensional structures of the $\alpha_2\beta_2$ complex from *Salmonella typhimurium*, obtained either in the absence or in the presence of the α-subunit ligand indole-3-propanol phosphate, indicates only small conformational changes localized near the α-active site (Hyde et al., 1988; Hyde & Miles, 1990). The $\alpha_2\beta_2$ complex in the crystal has been demonstrated to be catalytically competent and allosterically regulated by α-subunit ligands (Ahmed et al., 1987; Mozzarelli et al., 1989). Circular dichroism measurements have provided some evidence of conformational changes induced by ligand binding to isolated α-subunits and the $\alpha_2\beta_2$ complex (Heyn & Weischet, 1975; Lane & Kirschner, 1983a).

The molecular description of intersubunit communications requires the application of physicochemical techniques sensitive enough to detect very small conformational or microenvironmental changes. The luminescence of aromatic amino acids and prosthetic groups in proteins can often provide such unique structural and dynamic information on the macromolecule. Fluorescence and phosphorescence spectra can be related to the nature of the chromophore environment, whereas spectral relaxations and emission quantum yields are parameters affected by the protein flexibility and quenching interactions with distal centers in the molecule (Lakowicz, 1983; Galley, 1976; Strambini & Gabellieri, 1991). In tryptophan synthase, the separation between tryptophan at position 177 of the β-subunit and the pyridoxal ring of the coenzyme is about 20 Å. This distance is well within the range of single–singlet and triplet–singlet energy transfer. Measurements of transfer efficiencies could thus provide unique information on the separation and mutual orientation between indole and pyridoxal rings (Lane, 1983). Furthermore, in the last decade, Trp phosphorescence of proteins in solution at ambient tem-

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; PG, propylene glycol.

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perature has shown considerable potential for the study of protein conformation/flexibility (Strambini, 1989). In particular, since the lifetimes of the excited triplet state are dramatically reduced in fluid media through the enhancement of radiationless transition to the ground state, a strong correlation exists between lifetime and microvsicosity of the embedding matrix (Strambini & Gonnelli, 1985). This correlation has been exploited as a monitor of the protein flexibility near Trp residue, and for revealing subtle conformational changes in enzymes as a result of their interaction with substrates, effector molecules, and metal ions (Cioni & Strambini, 1989; Cioni et al., 1989; Strambini & Gonnelli, 1990).

In order to gain new insight into the regulatory mechanism that implies a fine-tuning of the interactions between α- and β-subunits of tryptophan synthase, we have characterized the fluorescence and phosphorescence properties of Trp-177 and of bound coenzyme in its Schiff's base and reduced forms either in the absence (present paper) or in the presence of substrates or substrate analogs of α- and β-subunits (Strambini et al., 1992).

MATERIALS AND METHODS

N,*N*-Bis(2-hydroxyethyl)glycine (Bicine) from Sigma, L-valine from Fluka, pyridoxal 5′-phosphate (PLP) from Boehringer, and spectroscopic-grade propylene glycol (PG) from Merck were of the best available quality and were used without further purification.

The holo-$\alpha_2\beta_2$ complex of tryptophan synthase from *Salmonella typhimurium* was a generous gift of Dr. Edith W. Miles; the purity of each preparation of the $\alpha_2\beta_2$ complex was established to be greater than 95% by SDS gel electrophoresis on 10–15% gradient gels (E. Miles, personal communication; Miles et al., 1989; Kayastha et al., 1991). Enzyme activity was assayed according to Higgins et al. (1979). Apoenzyme was prepared by extensive dialysis against 1 M KSCN, 100 mM Tris, 100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.8 (Miles & Moriguchi, 1977). The residual enzymatic activity was 5% of the holoenzyme. Full recovery of the activity was obtained upon addition of 0.2 mM PLP to the apoenzyme solution. Reduced holo-$\alpha_2\beta_2$ was obtained by adding a small amount of $NaBH_4$ to an holoenzyme solution, followed by dialysis against 50 mM Bicine/1 mM EDTA, pH 7.8.

Pyridoxal 5′-phosphate Schiff's base with L-valine was prepared in 50 mM Bicine/1 mM EDTA according to Benecky et al. (1985).

All samples for luminescence studies were prepared in 50 mM Bicine buffer containing 1 mM EDTA, pH 7.8. For low-temperature studies, propylene glycol was added to the buffer solution to a defined proportion of 50/50 (v/v). Propylene glycol has been shown (Strambini & Gabellieri, 1990), and confirmed in the present study, to lead to about a 10% increase of phosphorescence lifetimes and small effects on the relative amplitude of multicomponent decays.

In all cases, the protein concentration was about 15 μM, as determined by a molar absorptivity at 280 nm of 90 800 M^{-1} cm^{-1} for holo-$\alpha_2\beta_2$, 87 800 M^{-1} cm^{-1} for reduced holo-$\alpha_2\beta_2$, and 78 900 M^{-1} cm^{-1} for apo-$\alpha_2\beta_2$. At this concentration and employing sample cells with 2-mm path length, inner filter artifacts were found to be negligible. Furthermore, to avoid photochemistry of the pyridoxal moiety, all preparative steps were carried out in the dark, and sample irradiation was kept to a minimum using new stock for every measurement. Controls were carried out at the end of each experiment to verify that the spectroscopic properties of the sample were not altered.

To obtain reproducible phosphorescence data in fluid solution, it is paramount that, besides using very pure reagents, all dissolved oxygen has been thoroughly removed. The procedure followed to obtain satisfactory deoxygenation was described previously (Strambini & Gonnelli, 1990; Strambini et al., 1990).

Steady-state fluorescence intensity and spectra at room temperature were obtained with a JASCO FP-770 spectrofluorometer. For phosphorescence and low-temperature fluorescence studies, the apparatus employed was a home-made fluorometer/phosphorimeter described elsewhere (Strambini, 1983; Strambini & Gonnelli, 1990). Pulsed excitation for phosphorescence decays was provided by a frequency-doubled flash-pumped dye laser (UV-500 M, Candela) tuned at 295 nm with a pulse duration of 1 μs and an energy per pulse typically of 1–10 mJ. Analysis of decay curves in terms of a sum of exponential components was carried out by a nonlinear least-squares fitting algorithm implemented on the program Global Analysis (Global Unlimited, LFD University of Illinois, Urbana).

RESULTS

A low-temperature glass is a suitable reference state for characterizing the luminescence of chromophores free from thermally activated and diffusional quenching processes. In a propylene glycol/buffer glass at 160 K, the distinct contribution of Tyr, Trp, and coenzyme to the fluorescence and phosphorescence of tryptophan synthase can be selectively studied by an appropriate choice of the excitation wavelength. Moreover, the temperature dependence of the luminescence is very informative on quenching interactions and on protein conformational states. These measurements allow correct interpretation of the luminescence properties of tryptophan synthase at ambient temperature where the structure and the function of the enzyme are usually investigated.

Coenzyme Luminescence. Selective excitation of pyridoxal 5′-phosphate luminescence can be achieved at wavelengths greater than 325 nm. Fluorescence and phosphorescence spectra of the Schiff's base of pyridoxal phosphate with either L-valine or the enzyme and of reduced holotryptophan synthase are shown in Figure 1. Relative luminescence intensities are presented in Table I.

The fluorescence spectrum of PLP–protein species exhibits two bands at 365 and 490 nm, and a broad phosphorescence peak at 466 nm. These bands are, respectively, 1–2, 7–8, and 10–11 nm red-shifted with respect to the PLP–valine complex. The two fluorescence bands originate from different tautomers of the Schiff's base (Kallen et al., 1985), the red band being associated with the ketoenamine form. A drastic reduction in intensity of the blue fluorescence band is observed in the PLP–protein complex. The intensity ratio F_{365}/F_{490} changes from 11 ± 0.5 in the PLP–valine complex to 2.3 ± 0.2 in the PLP–protein species. The difference in intensity ratio may be due to either an altered tautomeric equilibrium or a change in fluorescence quantum yields or both. At excitation wavelengths greater than 360 nm, the luminescence is only comprised of the red fluorescence band with no detectable phosphorescence associated with it (data not shown).

Reduced coenzyme tryptophan synthase excited at 330 nm possesses a single, intense fluorescence band at 365 nm, and a phosphorescence band at 477 nm with an intensity 6.5 times greater than that of the Schiff's base–coenzyme $\alpha_2\beta_2$ complex.

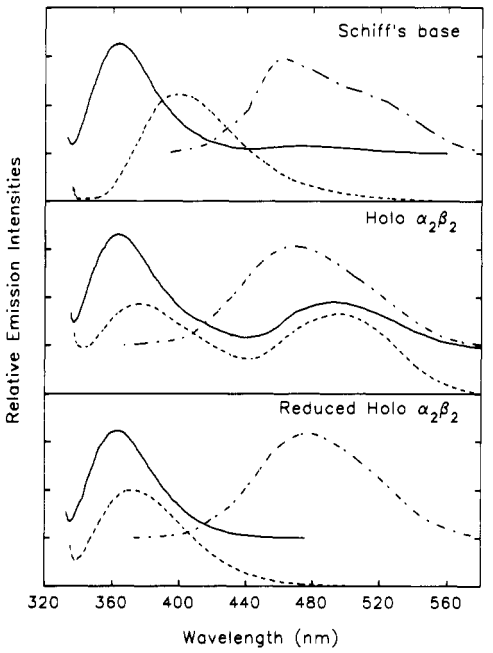

FIGURE 1: PLP fluorescence and phosphorescence spectra for the Schiff's base with L-valine, the Schiff's base, and the reduced form with the $\alpha_2\beta_2$ complex at different temperatures. Fluorescence (—, - - -) and phosphorescence (– · –) spectra of solutions containing the species indicated in the figure in equimolar coenzyme concentration were recorded in PG/bicine buffer (50/50, v/v) at 160 (—) and 273 K (- - -). The excitation wavelength was 330 nm. Relative intensities are given in Table I. These spectra are not corrected for instrumental response.

Table I: Luminescence of PLP–Valine and Protein-Bound Coenzyme in Propylene Glycol/Buffer at 160 and 273 K under Steady-State Excitation (λ_{ex} = 330 nm)

	Fluorescence (160 K)				
	λ_{max} (nm)	F_{max} (% intensity reduced holo-$\alpha_2\beta_2$)			
PLP–valine	364, 483	91.4, 8.2			
holo–$\alpha_2\beta_2$	365, 490	13.5, 5.8			
reduced holo-$\alpha_2\beta_2$	364	100			
apo–$\alpha_2\beta_2$	365	6.0			
	Phosphorescence (160 K)				
	λ_{max} (nm)	P_{max} (% intensity reduced holo-$\alpha_2\beta_2$)	τ_1 (s)	τ_2 (s)	f_2
PLP–valine	455, 515 (SH)	10.9	0.23	1.26	0.21
holo-$\alpha_2\beta_2$	466	15.6	0.20	1.01	0.48
reduced holo-$\alpha_2\beta_2$	477	100	0.14	1.67	0.13
apo-$\alpha_2\beta_2$	457	17.4	0.31	1.50	0.30
	Fluorescence (273 K)				
	λ_{max} (nm)	F_{max} (% value at 160 K)			
PLP–valine	400, 508	6.7, 1.8			
holo-$\alpha_2\beta_2$	376, 502	7.0, 17			
reduced holo-$\alpha_2\beta_2$	370	19.9			
apo-$\alpha_2\beta_2$	368	69.1			

Control experiments with the apoprotein gave, upon excitation at 330 nm, an unexpected reduced coenzyme-like luminescence with a fluorescence band (λ_{max} = 365 nm) and a phosphorescence band (λ_{max} = 457 nm). The intensities of these bands are, respectively, around 6% and 17% of those of the reduced form (Table I). Since this emission could not be eliminated by extensive dialysis against 1 M KSCN, it probably represents a small fraction of coenzyme that has undergone an irreversible reaction with some active-site residue.

The decay of coenzyme phosphorescence, measured at λ_{max}, was found in all the species to depart from a single exponential. The weak signal was fitted to a sum of two exponential functions. The lifetimes and the corresponding amplitudes are reported in Table I. Short-lived components (0.15–0.30 s) generally dominate the emission, except for the Schiff's base PLP–protein species where the amplitude are comparable. The long-lived component has a typical lifetime of 1.0–1.15 s, similar to that of pyridoxal analogs at 77 K.

An increase in the temperature of the glass leads to spectral red shifts and quenching of both fluorescence and phosphorescence intensities, the effects being most dramatic with the triplet state. At 195 K, on the cold side of the glass transition temperature, the residual phosphorescence intensity is only a few percent of the cold temperature value with minor differences between L-valine- and protein-bound forms. A clear distinction between L-valine- and protein-bound coenzyme is, however, evident in the temperature dependence of the fluorescence emission. Fluorescence maxima and residual intensities at 273 K are given in Table I. The PLP–valine complex undergoes larger red shifts of the blue and the red fluorescence bands. Furthermore, although the residual intensity of the blue band is around 7% in both PLP derivatives, the ketoenamine band is quenched about 10 times less in the PLP bound to the protein. Both shifts and intensities demonstrate a protection of bound ketoenamine tautomer against solvent-mediated quenching reactions (presumably excited-state proton transfer). The different yield may also in part reflect a concomitant change in tautomeric equilibrium.

A smaller red shift and a relatively large residual intensity were found for the fluorescence of the reduced coenzyme–protein species. Finally, the weak anomalous fluorescence associated with the apoprotein is least quenched and red-shifted.

Low-Temperature Trp Luminescence. Fluorescence and phosphorescence spectra (λ_{ex} = 298 nm) of apo-, holo-, and reduced holotryptophan synthase in PG/buffer at 195 K are shown in Figure 2. The partly structured fluorescence spectrum of apoenzyme presents band maxima peaked at 314 and 327 nm. The corresponding phosphorescence spectrum has a single, relatively narrow, 0–0 vibronic band centered at 418.5 nm. At lower temperature, 160 K, in addition to Trp phosphorescence, there is a small contribution in the region between 365 and 410 nm with spectral and decay properties typical of ionized Tyr (data not shown).

Whereas the fluorescence spectrum of Trp-177 is within the norm for proteins at low temperature, the phosphorescence spectrum is unusually red-shifted relative to both solvent-exposed residues (10–11 nm) and residues buried in hydrophobic pockets (6–7 nm) (Hersberger et al., 1980). The lower spectral energy implies a stabilization of the excited triplet state by strong anisotropic polar interactions that might arise from the proximity of charged groups. Moreover, the constancy in wavelength and the narrow width of the 418.5-nm phosphorescence band in all three protein forms emphasizes that the indole ring of Trp-177 is in a well-defined polar site of the protein interior and that this microenvironment is not altered either by reduction of the coenzyme or by its removal.

The coenzyme contribution alters the phosphorescence spectrum by adding a broad band in the wavelength range 440–550 nm. Relative to the coenzyme phosphorescence (λ_{ex} = 330 nm), this contribution is red-shifted and its intensity exceeds that anticipated from direct excitation of the coenzyme, suggesting that a different process is involved in the

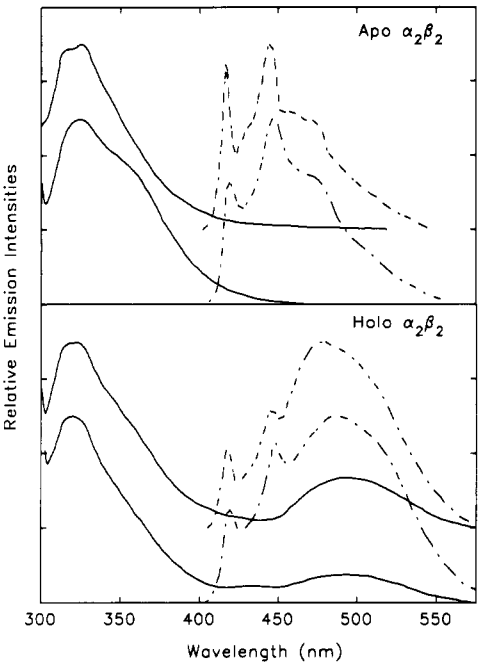

FIGURE 2: Tryptophan fluorescence and phosphorescence spectra of apo- and holotryptophan synthase at different temperatures. Fluorescence (—) and phosphorescence (– · –) spectra of the species indicated in the figure were recorded in PG/buffer at 195 (upper traces) and 273 K (lower traces). The excitation wavelength was 298 nm.

Table II: Triplet Lifetimes and Relative Fluorescence (F) and Phosphorescence (P) Intensities of Trp-177 as a Function of Temperature

	apo-$\alpha_2\beta_2$	holo-$\alpha_2\beta_2$	reduced holo-$\alpha_2\beta_2$
	T = 195 K, PG/Buffer		
F_{325}/F_{325}(apo)	1.0	0.28 ± 0.04	0.25 ± 0.03
P_{419}/F_{325}	1.0 ± 0.03	0.22 ± 0.01	0.96 ± 0.04
$\sum P_i/F\tau_i$	1.0	0.97	1.05
τ_1 (s), f_1 (419 nm)	1.85, 0.17	0.40, 0.50	1.38, 0.27
τ_2 (s), f_2 (419 nm)	4.28, 0.83	3.31, 0.50	4.26, 0.73
τ_1 (s), f_1 (520 nm)		0.40, 0.88	
τ_2 (s), f_2 (520 nm)		2.27, 0.12	
	T = 273 K, Buffer		
$[\sum P_i/F\tau_i(273\text{ K})]/[\sum P_i/F\tau_i(195\text{ K})]$	0.93 ± 0.08	1.02 ± 0.1	0.95 ± 0.12
τ_1 (ms), α_1 (420 nm)	17.3, 0.45	28.2, 0.46	29.5, 0.56
τ_2 (ms), α_2 (420 nm)	55.5, 0.55	48.5, 0.54	79.4, 0.44
τ_{av} (ms)	38.3	39.1	51.4
	T = 293 K, Buffer		
F_{325}/F_{355}(Trp)	1.51 ± 0.05	0.79 ± 0.04	0.70 ± 0.03
τ_1 (ms), α_1 (420 nm)	4.7, 0.71	14.6, 0.66	11.6, 0.58
τ_2 (ms), α_2 (420 nm)	16.8, 0.29	23.6, 0.34	25.2, 0.42
τ_{av} (ms)	8.2	17.6	17.3

generation of this band (see below).

In reduced PLP–tryptophan synthase complex, the coenzyme band (364 nm) is so intense that it makes the fluorescence contribution of Trp appear as a shoulder at 315–320 nm.

Both Trp-177 fluorescence and phosphorescence emissions are quenched by the coenzyme in holoprotein species. Relative to the apoenzyme, the fluorescence intensities of holoenzyme and reduced holoenzyme are 0.28 ± 0.04 and 0.25 ± 0.03, respectively (Table II). Quenching of the triplet-state emission is measured accurately from the ratio of phosphorescence and fluorescence intensity, P/F, that allows normalization of the phosphorescence intensity to the fluorescence emission. This ratio was found to be reduced to 0.22 ± 0.01 only in the Schiff's base coenzyme–protein species.

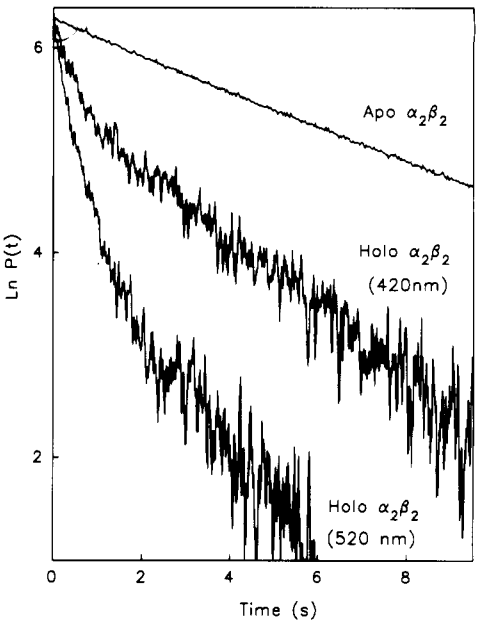

FIGURE 3: Decay of tryptophan phosphorescence intensity for apo- and holo-$\alpha_2\beta_2$ complex. The time course of Trp phosphorescence was recorded upon steady-state excitation (298 nm). With the holoprotein, the emission was collected over a narrow bandwidth (10 nm) centered either at 420 or at 520 nm, whereas the emission from the apoprotein was collected over the entire spectrum. The samples were in PG/Bicine buffer at 195 K.

The decay of Trp phosphorescence following steady-state excitation was measured at 160 and 195 K. Although at the colder temperature the Tyr⁻-like contribution precludes an unambiguous assignment of decay kinetics, the data are nevertheless useful for obtaining the cold temperature limiting lifetime. All decays were fitted to a biexponential function. The lifetimes and preexponential terms obtained at 160 K are as follows: τ_1 = 1.90 s, τ_2 = 5.02 s with f_2 = 0.87 for the apoprotein; τ_1 = 1.53 s, τ_2 = 4.95 s with f_2 = 0.62 for the reduced form; τ_1 = 0.42 s, τ_2 = 3.31 s with f_2 = 0.38 for the Schiff's base form. For apo and reduced forms, the large amplitude of the 5 s component, f_2 (a lifetime typical of unperturbed Trp in proteins), accounts quite well for the Trp contribution to the overall intensity at 419 nm. By contrast, the f_2 of the 3.31 s component of the Schiff's base form represents only a fraction of Trp phosphorescence so that the remaining part of Trp intensity must decay with a shorter lifetime. Thus, a clear reduction in the triplet lifetime of Trp-177 is evident only in the Schiff's base PLP–enzyme species. This conclusion is fully consistent with the observation that the phosphorescence is quenched only by oxidized coenzyme.

As mentioned above, by raising the temperature to 195 K, the phosphorescence intensity at 419 nm becomes exclusive of Trp. At this warmer temperature, the lower solvent viscosity reduces the triplet lifetime of both apo and reduced forms (Table II). The change reflects an increased flexibility of the polypeptide chain near the triplet probe (Strambini & Gonnelli, 1985).

An important feature of the decay (because unexpected in a protein with a single Trp) is its clear departure from a monoexponential law. The deviation is present in all three forms of the enzyme and becomes even greater in fluid solutions. A representative decay for the three enzyme forms is shown in Figure 3, and the corresponding kinetic parameters, derived from a biexponential fitting, are reported in Table II. In apo and reduced forms, most of the intensity decays with

a lifetime of 4.3 s. In the Schiff's base PLP–enzyme complex, the decay is much more rapid with lifetimes of 0.40 and 3.30 s, both rates being practically unchanged relatively to 160 K. Assuming an unperturbed lifetime of 4.3 s, the reduction corresponds to phosphorescence quenching rates, $K_q = 1/\tau_{\text{Schiff's base}} - 1/4.3$, of 2.26 and 0.07 s^{-1}, respectively. Since the intensity/lifetime ratio $[\sum P_i/F\tau_i(\text{Schiff's base})]/[\sum P_i/F\tau_i(\text{apo})]$ is roughly 1, the two rates of quenching do account fully for the reduction in steady-state phosphorescence intensity associated with the Schiff's base form.

Regarding the nature of the triplet quenching interaction, multiple evidence points to triplet–singlet dipole–dipole (Forster type) energy transfer (Galley & Stryer, 1969) between the phosphorescent state of tryptophan and the red-absorbing ketoenamine tautomer of the Schiff's base, which is the only tautomer that satisfies the requirement of emission–absorption spectral overlap. Energy transfer entails the observation of sensitized delayed fluorescence from the ketoenamine tautomer with decay kinetics identical to the triplet donor. The difference spectrum between the phosphorescence of apo and Schiff's base forms of tryptophan synthase, after equating the intensity at 419 nm, is a broad band practically coincident (λ_{max} = 480 nm) with the red fluorescence band of the coenzyme (data not shown). Such delayed emission does not represent coenzyme phosphorescence because the latter, apart from being blue-shifted by 25–30 nm, is almost totally quenched at 195 K (direct excitation at 330 nm). Furthermore, the delayed emission is long-lived with lifetime components indistinguishable from Trp phosphorescence. An interpretation of this phenomenon is offered under Discussion.

Trp Luminescence in Fluid Solutions. A temperature increase above the glass transition affects the luminescence of Trp-177, leading to spectral shifts, reduction in fluorescence and phosphorescence intensities, and shortening of triplet lifetimes. Spectra, intensities, and decay kinetics of apo, reduced, and Schiff's base coenzyme forms of tryptophan synthase were obtained over a wide temperature/viscosity range both in PG/buffer ($T \leq 273$ K) and in buffer ($T \geq 273$ K).

At ambient temperature in buffer, the fluorescence spectra of apo and Schiff's base coenzyme protein are only 2–3 nm red-shifted relative to the glass state (Figure 2). Differential temperature-induced quenching of the Trp and coenzyme fluorescence alters the shape of the spectra with respect to those obtained at low temperature. In Table II, the relative fluorescence intensity of Trp-177 in buffer at 293 K is compared to that of an equimolar solution of Trp in the same buffer. The results show that the apoprotein Trp-177 is about 1.5 times more fluorescent than the free amino acid. Binding of the coenzyme reduces the quantum yield to about 50% with a minor difference between reduced and Schiff's base coenzyme complexes. It should be noted that, relative to low temperature, the smaller quenching in buffer as well as the greater similarity in the extent of quenching between Schiff's base and reduced coenzyme probably reflects the temperature-induced decrease (≈55%) in the Trp fluorescence quantum yield.

Trp-177 was found to be phosphorescent even in buffer at ambient temperature with a spectrum modestly (1.5 nm) red-shifted relative to the glassy state (Figure 2). This result suggests that the Trp-177 microenvironment is conserved over a very large temperature range in each enzyme form. The 480-nm shoulder in the spectrum of the Schiff's base form, due to coenzyme-delayed fluorescence, although attenuated, is still observable at 273 K but becomes negligible at ambient temperature.

As generally found in proteins, the decreased solvent viscosity at higher temperature has dramatic effects on the phosphorescence quantum yield and lifetime (Strambini & Gabellieri, 1990). In all three forms of tryptophan synthase, the reduction in phosphorescence intensity was found to be proportional to the shortening of the triplet lifetime. As a consequence, the intensity/lifetime ratio, $\sum P_i/F\tau_i$, remains fairly constant across the temperature range explored (Table II).

As stated before, the decay of phosphorescence in fluid solutions is highly nonexponential. A biexponential fitting of decays was adopted throughout in spite of the fact that, in the temperature range 230–260 K, a χ^2 of 3.7 and the asymmetry in the plot of residuals do show the presence of additional components (data not shown). Since at higher temperature a biexponential fitting is adequate ($\chi^2 = 1.2$), we must conclude that rapid interconversion between conformers narrows down the distribution to two main conformational states. The amplitudes and the lifetimes of the two components in buffer are reported in Table II. The two components are almost equally represented ($\alpha_1 \approx \alpha_2$) at 273 K. At 293 K, the equilibrium shifts in favor of the short-lived component particularly for apo and Schiff's base proteins. In the absence of quenching by the coenzyme, the average lifetime, $\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$, is a measure of the average flexibility of the polypeptide chain around the triplet probe (Strambini & Gonnelli, 1985). The values of τ_{av} for apo and reduced forms of tryptophan synthase are very similar at low temperature, whereas at ambient temperature τ_{av} reveals a greater flexibility for the apoprotein. A more rigid structure is also inferred for the holo-$\alpha_2\beta_2$ complex. At 293 K, where the lifetime is only marginally influenced by energy transfer to the coenzyme, τ_{av} is almost identical to that of the reduced holo-$\alpha_2\beta_2$ complex, and double that of the apoprotein (Table II).

DISCUSSION

The crystallographic structure of the holo-$\alpha_2\beta_2$ bienzyme complex (Hyde et al., 1988) shows that Trp-177 is localized at the end of an helix of the N-domain of the β-subunit close to the α-subunit interface. The indole ring is buried between the helix and four-strand β-sheet which, together with an adjacent tier of α-helical rods, form a compact agglomerate of secondary structure [see Figure 4 of Strambini et al. (1992)]. Trp-177 is about 20 Å away from the α-active site and about 23 Å from the pyridoxal moiety, a distance that, being within the range of energy transfer, allows for extensive interaction between chromophores.

The luminescence characteristics of Trp-177 do reflect its particular location in the macromolecule. Its interaction with the coenzyme is amply demonstrated by the large extent of quenching of both fluorescence and phosphorescence intensities. The compactness and structural rigidity of the site are inferred both from the modest shifts in fluorescence and phosphorescence spectra over the transition from a glass to a fluid solution and from the long-lived phosphorescence lifetime at ambient temperature. The correlation between decay kinetics and local viscosity (Strambini & Gonnelli, 1985) provides even a direct measure of the polypeptide flexibility near the chromophore. From an average triplet lifetime of 17 ms at ambient temperature, we derive an effective viscosity of about 400 P, a value not unusual within α/β-structural knots.

In the following discussion, we will first focus on the luminescent properties of tryptophan synthase at cold temperature and then at ambient temperature.

Table III: Theoretical Computation of Fluorescence and Phosphorescence Quenching by Förster-Type Energy Transfer to the Coenzyme[a]

	J[b] (M^{-1} cm^3)	R_0[c] (Å)	E[d] (%)	k_T[e] (s^{-1})
	Singlet–Singlet Energy Transfer			
holo-$\alpha_2\beta_2$	3.8×10^{-15}	24.5	59 (52)	
reduced holo-$\alpha_2\beta_2$	3.5×10^{-15}	24.1	57 (46)	
	Triplet–Singlet Energy Transfer			
holo-$\alpha_2\beta_2$	7.8×10^{-15}	35.6	92 (78)	2.56 (2.26, 0.07)

[a] Experimental values are shown in parentheses. [b] $J = \int_0^\infty F_d(\lambda)\epsilon_a(\lambda)\lambda^4\, d\lambda / \int_0^\infty F_d(\lambda)\, d\lambda$, where ϵ_a is the molar absorption coefficient of the acceptor and F_d is the fluorescence intensity of the donor corrected for the instrumental response at each wavelength. [c] $R_0^6 = (8.8 \times 10^{-25})k^2 n^{-4}\phi J)$ in centimeters. $\phi = 0.31$ is the quantum yield of the apoenzyme on the basis of $\phi = 0.20$ for free Trp (Eftink & Ghiron, 1976). n is the refractive index of the medium and is assumed to be 1.4. k^2 (0.82) is the orientation factor (Lackowicz, 1983) for the transition dipole moment ^{1}La (Eftink et al., 1990) of tryptophan and that of the PLP moiety. Small changes in the orientation of the latter (Metzler et al., 1988) corresponding to different tautomers were neglected. [d] $E = R_0^6/(R_0^6 + r^6)$, where r (23 Å) is the distance between the two chromophores. [e] $k_T = (1/\tau_d)(R_0/r)^6$, where $\tau_d = 5$ s is the unperturbed phosphorescence lifetime of the donor. For computing R_0, $\phi = k_p\tau_d = 0.45$ is the phosphorescence quantum yield of Trp on the basis of a radiative transition probability $k_p = 0.09$ s^{-1} (Galley & Stryer, 1969). The orientation factor k^2 (2.57) and the overlap integral J were evaluated considering the transition dipole moment (perpendicular to the plane of the indole ring) and the emission spectrum of the phosphorescent state, respectively.

Trp-177 is highly fluorescent in apotryptophan synthase from *Salmonella typhimurium*, in contrast with the 7–8 times weaker yield reported for the *Escherichia coli* protein (Lane, 1983). The sequence homology between the two enzyme forms is about 85% and 96.5% for the α- and β-chains, respectively, and the three-dimensional structure is expected to be very similar. The low yield in *E. coli* enzyme indicates that the aromatic residue must be in proximity of an internal quenching group (Tyr^- or His^+). Such juxtaposition is absent in the enzyme from *S. typhimurium*, and the reason could be either a different local structure or a specific amino acid replacement. The latter possibility seems, however, unlikely since all amino acids present in a sphere of 10 Å surrounding Trp-177 are conserved.

Binding of pyridoxal 5′-phosphate to the apoenzyme leads to a 3-fold decrease in Trp-177 fluorescence. Trp fluorescence quenching by pyridoxal phosphate may take place, if one excludes the formation of dark ground-state complexes, by one of two mechanisms: singlet–singlet energy transfer or electron exchange. The extent of singlet–singlet energy transfer can be predicted theoretically from the distance and orientation between chromophores deduced from the crystallographic coordinates (Hyde et al., 1988; Brookhaven protein data bank, file 1WSY.PDB). The requirement of emission/absorption spectral overlap is satisfied by both reduced and Schiff's base coenzyme complexes, and the separation between chromophores is close to the critical interaction radius ($R_0 \approx 20$ Å) estimated for the reduced coenzyme (Churchich, 1965).

The efficiency of energy transfer for the Schiff's base and reduced form of the coenzyme–protein complex, calculated according to Föster's theory (Stryer, 1978; Dale & Eisinger, 1974), is reported in Table III. In the same table, J, the spectral overlap between donor emission and acceptor absorption, is also shown. The agreement with experimental efficiencies is good and supports a mechanism of fluorescence quenching based on singlet–singlet energy transfer.

Förster-type, triplet–singlet, energy transfer, observed previously in a protein–dye conjugate (Galley & Stryer, 1969) but, to our knowledge, never reported in a native system, is clearly implicated in quenching of Trp phosphorescence by the Schiff's base coenzyme–protein complex. The rate of triplet–singlet energy transfer, k_T, and the energy-transfer efficiency, E, reported in Table III, are referred to the ketoenamine tautomer, the only species that satisfies the requirement of spectral overlap. The agreement with experiment is quite satisfactory in the case of the rapid and dominant transfer rate (2.26 s^{-1}). By identifying the rate of 2.26 s^{-1} with the velocity of transfer to the ketoenamine tautomer, we can compute a maximum efficiency (at 195 K) $E = 1 - \tau/\tau_0 = 1 - 0.4/4.3 = 0.90$, which is close to the theoretical value. The presence of other tautomers of PLP will decrease the actual value of E. From $E_{obs} = 0.78$, it is possible to anticipate that only 85% of the Schiff's base is present as ketoenamine, a prediction that is in substantial agreement with the preexponential terms of the phosphorescence decay.

In conclusion, theoretical predictions of energy transfer from both singlet and triplet states of Trp-177, on the basis of crystallographic coordinates, agree fairly well with experimental data. Since this is a rather rigorous double test of the actual distance/orientation between the two chromophores, the geometrical arrangement prevailing in solution is not substantially different from that present in the crystalline state.

β-Subunits in which other tautomeric forms of the coenzyme are present will not be involved in energy-transfer processes and should phosphoresce with an unperturbed lifetime of 5 s. However, from the decay of both phosphorescence (419 nm) and delayed fluorescence (520 nm), it is evident that energy transfer occurs at two distinct rates, 2.26 and 0.07 s^{-1}. A plausible explanation for the lack of an unperturbed phosphorescence decay component might be that the tautomeric interconversion occurs during the triplet lifetime, namely, that noninteracting tautomers change into the ketoenamines form (at a rate of 0.07 s^{-1}) during the long phosphorescence lifetime. At higher temperature, where the dynamic equilibrium between tautomers of the Schiff's base is certainly rapid with respect to the phosphorescence time scale, this hypothesis predicts a single average transfer rate. In fact, above the glass transition temperature ($T > 240$ K), the emission at 419 and 520 nm has undistinguishable lifetimes and amplitudes.

Trp Phosphorescence at Ambient Temperature. The transition from a glass to a fluid solution invariably causes an increase in radiationless deactivation of the excited triplet state of Trp. The lifetime then becomes a direct measurement of the acquired flexibility of the protein structure at the chromophore site. Side chains that are solvent-exposed or in peripheral mobile regions of a macromolecule are so dramatically affected in low-viscosity media that no phosphorescence can be measured from them (Strambini & Gabellieri, 1990). In tryptophan synthase, we observe a great reduction in triplet lifetime, but the protein exhibits detectable phosphorescence even at ambient temperature.

The most salient feature of the phosphorescence decay in fluid solution is its ubiquitous nonexponential nature. For a single Trp protein, a heterogeneous decay is unambiguous evidence for the existence of distinct molecular conformations at the site of the triplet probe either due to intrinsic protein chain flexibility or due to different rates of quenching by the coenzyme. In apo and reduced forms of tryptophan synthase, where the triplet state is not affected by the coenzyme, the multicomponent decay emphasizes that in solution the bien-

zyme complex exists in two or more conformations that differ in the flexibility of the N-domain of the β-subunit. These conformers do not interconvert in the millisecond phosphorescence time scale. Regarding the possible number of distinct conformers at ambient temperature, the phosphorescence is unable to resolve more than two components. Evidences that the actual distribution is more complex was obtained at lower temperatures (T = 260–220 K) where a biexponential fitting of the decay is clearly unsatisfactory.

Kinetic studies using the enzyme from *E. coli* (Lane & Kirschner, 1981, 1983a,b, 1991; Kirschner et al., 1991; Drewe & Dunn, 1985, 1986) have provided evidence that substrate–enzyme complexes exist in at least two conformational states. Moreover, the presence in the holoenzyme of two distinct absorption bands at 350 and 412 nm has been interpreted in terms of two protonated forms of the coenzyme, preferring a hydrophobic and a polar environment, respectively (Faeder & Hammes, 1970, 1971). Although it might be reasonable that distinct tautomers of the coenzyme stabilize distinct conformations of the β-active site, we find no simple correlation between the conformer distribution derived from absorption spectra (tautomer equilibria) and from phosphorescence decays (amplitudes).

The fraction of protein molecules in a given conformation (as inferred from the preexponential terms) as well as their respective flexibility is influenced by temperature and by the presence of coenzyme. Increases in temperature result in a greater average flexibility and in a shift of the equilibrium in favor of the short-lived conformer, effects that are also mimicked by the removal of the coenzyme. These findings establish a structure-tightening role of the coenzyme and are fully consistent with the greater thermal stability of the holoenzyme. Even if the energy transfer in the Schiff's base form of the enzyme precludes the determination of the intrinsic triplet lifetime, the structuring effect of the coenzyme appears equally important. Indeed, at 20 °C, when energy transfer no longer competes with intrinsic triplet deactivation processes, the average lifetime of the holoenzyme is practically identical to that obtained with the reduced coenzyme. An increased flexibility of the protein structure following the removal of the coenzyme is consistent with previous calorimetric studies of the $\alpha_2\beta_2$ complex (Wiesinger & Hinz, 1984). A similar conclusion was also reached by studying the thermal denaturation of isolated apo- and holo-β_2 dimers (Chaffotte & Goldberg, 1983).

In summary, characterization of the luminescence of Trp-177 and of pyridoxal phosphate has allowed us to determine a set of spectroscopic parameters which are directly correlated to the structure of the β-subunit. The efficiency of energy transfer from the singlet and triplet levels is related to the distance/orientation between indole and pyridoxal rings, whereas emission spectra and triplet lifetimes reflect respectively the nature and the dynamic makeup of the chromophore's environment. Consequently, the luminescence of Trp-177 represents an intrinsic probe suitably placed for reporting on structural changes at the active site of the β-subunit that might result from its association with the α-subunt and from the binding of substrates and allosteric effectors.

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