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# The Emitting State of Tryptophan in Proteins with Highly Blue-Shifted Fluorescence\*\*

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The absorption band of the indole side chain of tryptophan (Trp), at approximately 280 nm, comprises two overlapping transitions to the  $^1L_a$  and  $^1L_b$  excited states. Compared with the ground state, the dipole moment of  $^1L_a$  is large, giving Trp fluorescence its high sensitivity to changes in the micro-environment, as reflected in a range of Stokes shifts.<sup>[1]</sup> In contrast, the dipole moment of  $^1L_b$  is small and comparable with that in the ground state. Given the differences in the properties of these states, fluorescence data for proteins can only be properly interpreted if the emitting state is known.<sup>[1]</sup>

For 3-methylindole (3MI) in solid argon, the  $^1L_b$  0–0 transition is 250 cm<sup>−1</sup> lower in energy than the  $^1L_a$  transition,<sup>[2]</sup> and  $^1L_b$  is the emitting state similar to when it is in the gas phase or when dissolved in a fluorinated hydrocarbon.<sup>[1b]</sup> In cyclohexane, near degeneracy of the  $^1L_a$  and  $^1L_b$  0–0 transitions was observed.<sup>[1b]</sup> In more-polar organic solvents or in water, emission is from  $^1L_a$  because this state is much more sensitive to solvent relaxation than  $^1L_b$ , and excited-state conversion between  $^1L_a$  and  $^1L_b$  is extremely fast.<sup>[3]</sup> For a Trp residue buried in a protein with a local environment dominated by hydrocarbon side chains, the net effect of long-range electrostatic interactions could, in principle, result in

either  $^1L_a$  or  $^1L_b$  being the emitting state. There is a consensus view that Trp residues with long-wavelength fluorescence (320–350 nm) emit from  $^1L_a$ , but short-wavelength Trp emission ( $\approx$  310 nm) is more contentious.<sup>[1b,c]</sup> Herein, we determine the emitting state of the short-wavelength Trp in azurin, in a dI protein from transhydrogenase and, notably, in a newly isolated mutant of dI, which has the shortest-wavelength Trp emission of any protein described to date. Of the three proteins, only the last yields evidence of  $^1L_b$  emission.

Azurin from *Pseudomonas aeruginosa* harbors a single Trp residue and is the protein that emits light at the shortest blue-shifted wavelength that has been previously reported.<sup>[4]</sup> The emission maximum ( $\lambda_{\text{max}}$ ) is at 306–308 nm and the emission band shows vibrational fine structure.<sup>[4]</sup> Because of these features, its emission has often been assigned to the  $^1L_b$  state.<sup>[1b,5]</sup> As the transition dipole moments of the  $^1L_a$  and  $^1L_b$  states are almost perpendicular to each other, anisotropy excitation spectra can conclusively determine which state is emitting.<sup>[6]</sup> In Figure 1, the excitation spectra of holozurin and of the neutral Trp analogue, *N*-acetyl-tryptophan amide (NATA), in polypropylene glycol (PG) glass at  $-55^\circ\text{C}$  are presented. The 0–0  $^1L_b$  transition is relatively strong and sharp<sup>[1b]</sup> and is evident as a shoulder at 291 nm. If  $^1L_b$  is the emitting state, excitation at this wavelength should result in a high intrinsic anisotropy ( $r_0$ ). However, for both apo (not shown) and holozurin, a sharp dip in  $r_0$  is observed at the  $^1L_b$  absorption.<sup>[7]</sup> This pattern is essentially the same as that recorded with the  $^1L_a$ -emitting NATA<sup>[1b]</sup> when the indole ring is completely exposed to a polar environment (Figure 1a). No evidence for mixed emission was obtained as  $r_0$  is nearly independent of emission wavelength (Figure 1b,d). The data are, therefore, inconsistent with previous suggestions that azurin fluorescence emission is from  $^1L_b$ . Experiments performed at  $15^\circ\text{C}$ , where some depolarization owing to rotational mobility of the Trp side chain and protein rotation is expected, also led to a dependence of  $r_0$  on  $\lambda_{\text{ex}}$ , which clearly suggests that emission is from  $^1L_a$  (see Figure S1 in the Supporting Information). Anisotropy excitation spectra of the F110S azurin mutant,<sup>[4c]</sup> whose Trp residue experiences a uniquely rigid local environment,<sup>[8]</sup> again were similar (Figure 1e,f).

The dI component of transhydrogenase from *Rhodospirillum rubrum* is the only other known protein showing  $\lambda_{\text{max}} = 310$  nm and emission featuring vibrational fine structure<sup>[9]</sup> (Figure 2). The single Trp72 is embedded in the  $\beta$  sheet of a Rossmann fold.<sup>[10]</sup> The phosphorescence lifetime of Trp72 is very long, 2.3 s at  $20^\circ\text{C}$ , suggesting a highly rigid environment.<sup>[11]</sup> Similar to the data recorded for azurin, the excitation

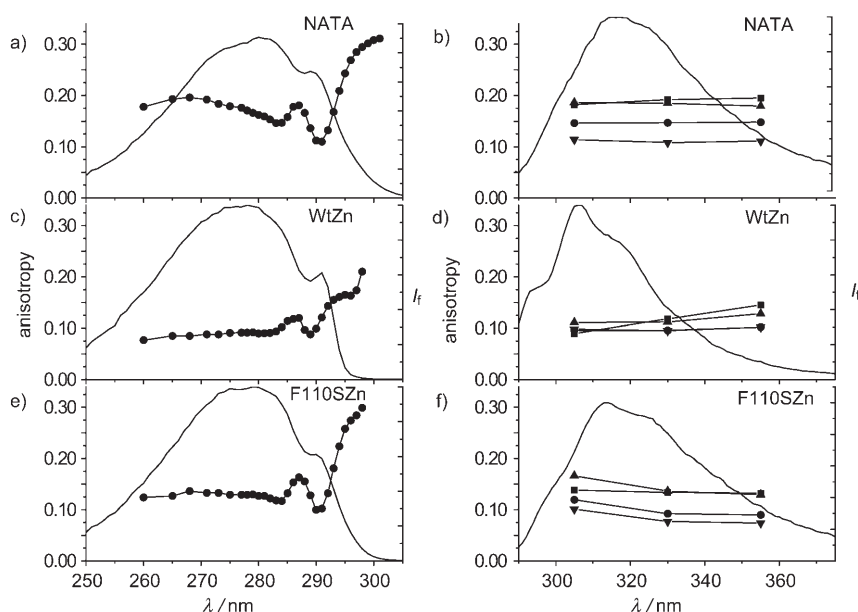
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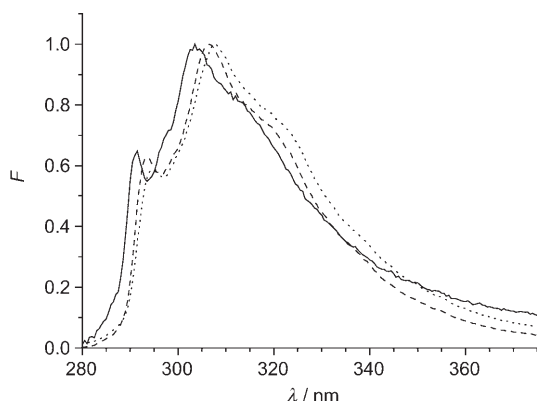
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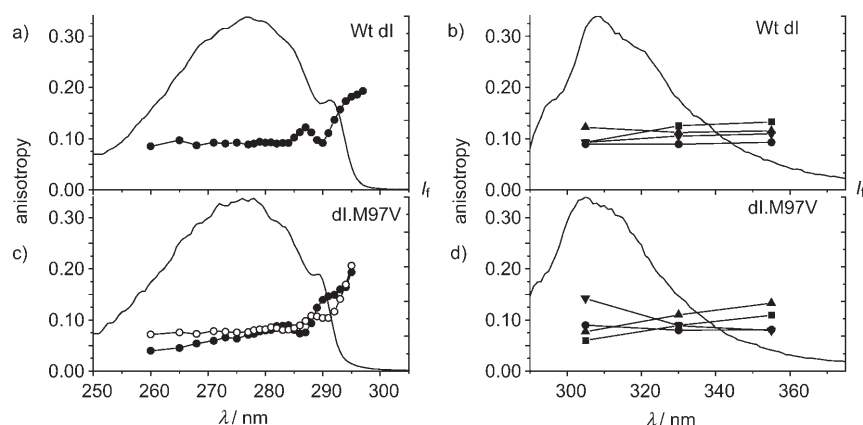
**Figure 1.** Fluorescent properties of NATA, WtZn azurin, and F110SZn azurin in PG glasses at  $-55^{\circ}\text{C}$ . a, c, e) Excitation spectra (emission at 320 nm) and excitation anisotropy values (●). For the latter, the emission was collected at 350 nm (NATA) or at 305 nm (azurin). b, d, f) Emission spectra ( $\lambda_{\text{ex}}=285$  nm) and the emission-wavelength dependence of the anisotropy upon excitation at 270 nm (■), 283 nm (●), 287 nm (▲), and 290 nm (▼).  $I_f$ =fluorescence intensity.



**Figure 2.** Normalized emission spectra of Wt apo-azurin (----;  $\lambda_{\text{max}}=306.5$  nm), Wt dI (.....;  $\lambda_{\text{max}}=308.0$  nm), and dI.M97V (—;  $\lambda_{\text{max}}=303.5$  nm) in 10 mM HEPES, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , and 1 mM dithiothreitol, pH 7.4, and room temperature. Excitation: 275 nm; excitation and emission band passes were at 2 and 1 nm, respectively; step size: 0.5 nm.

anisotropy spectra of dI in a glass (Figure 3) and at  $15^{\circ}\text{C}$  (see Figure S1 in the Supporting Information) suggest that  $^1\text{L}_a$  is the emitting state, and no indications for mixed emission were found.

In wild-type (wt) dI, the S atom of Met97 contacts the pyrrole N atom of Trp72.<sup>[10]</sup> We have substituted Met97

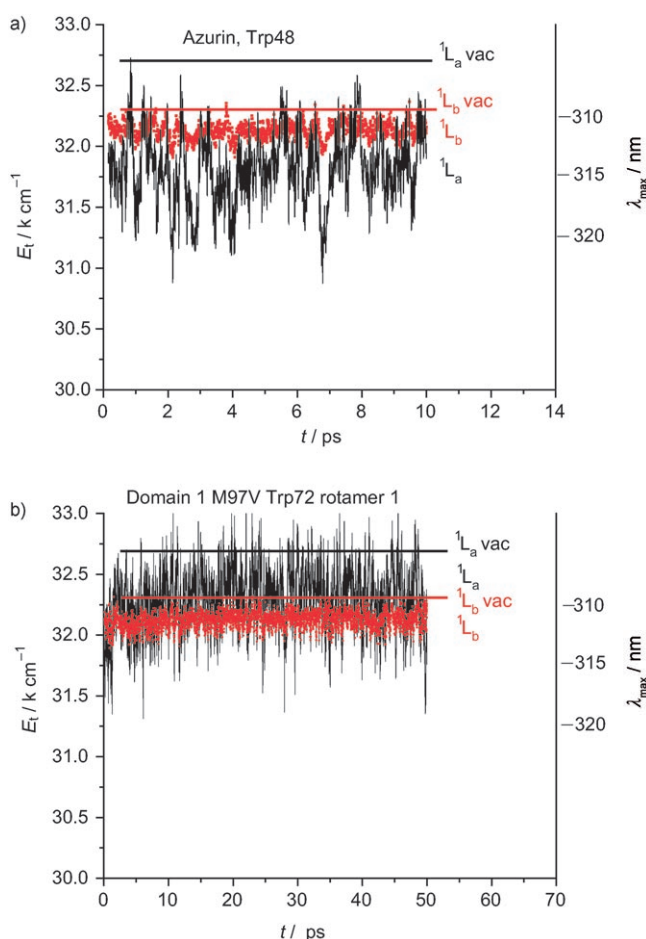


**Figure 3.** Fluorescent properties of transhydrogenase dI and mutant M97V in PG glasses at  $-55^{\circ}\text{C}$ . a, c) Excitation spectra (emission at 320 nm) and excitation anisotropy values (●). The emission was collected at either 305 nm (●) or 320 nm (○). b, d) Emission spectra ( $\lambda_{\text{ex}}=285$  nm) and the emission-wavelength dependence of the anisotropy upon excitation at 270 nm (■), 283 nm (●), 287 nm (▲), and 290 nm (▼).

with Val by site-directed mutagenesis. The  $\lambda_{\text{max}}$  of the mutant protein (dI.M97V) is shifted 4–5 nm to shorter wavelengths (Figure 2), making it the shortest-wavelength-emitting protein known. The  $\lambda_{\text{max}}$  is essentially similar when excited at 275 nm and 292 nm (see Figure S2 in the Supporting Information), confirming that fluorescence originates from Trp72 and not from Tyr residues. Remarkably, the anisotropy spectra of dI.M97V show that fluorescence is emitted, not only from the  $^1\text{L}_a$  state of the Trp, but also from  $^1\text{L}_b$ . Thus, in marked contrast to wt dI and azurin, the spectrum has a maximum in  $r_0$  when the protein is excited in the 0–0 band of  $^1\text{L}_b$  (290 nm) and emission is collected at 305 nm (Figure 3c). The maximum disappears when emission is collected at 320 nm. For azurin and wt dI, the excitation anisotropy spectra are essentially the same if emission is collected at either 305 or 320 nm. The dI.M97V data indicate that the emission is mixed: it is predominantly from  $^1\text{L}_b$  at approximately 305 nm and from  $^1\text{L}_a$  at around 320 nm. The observation is also reflected in the dependence of

$r_0$  on emission wavelength when excitation is at 290 nm (Figure 3d), a feature not seen for either wt dI or for azurin. Because of greater inhomogeneous broadening, absorption at the red edge is probably due to the  $^1\text{L}_a$  state. As with wt dI and azurin, excitation of dI.M97V at the red edge leads to increasing  $r_0$ , indicating  $^1\text{L}_a$  emission.

A quantum mechanical/molecular mechanical (QM/MM) treatment, based on the assumption that only electrostatic interactions of the Trp ring electron density with the surrounding protein and solvent affect the transition energy, predicts  $\lambda_{\text{max}}$  with encouraging accuracy.<sup>[12]</sup> Figure 4 shows typical trajectories of transition energies from  $^1\text{L}_a$  and  $^1\text{L}_b$  to



**Figure 4.** a) Simulated  $^1L_b$  (■) and  $^1L_a$  (—) transition energies for Trp48 of azurin during a dynamics trajectory. The horizontal lines give the equivalent calculated  $^1L_b$  and  $^1L_a$  transition energies of 3MI in vacuum. b) Simulated  $^1L_b$  (■) and  $^1L_a$  (—) transition energies for Trp72 of Val97 rotamer 1 of dI.M97V during a dynamics trajectory. The horizontal lines give the equivalent calculated  $^1L_b$  and  $^1L_a$  transition energies of 3MI in vacuum.  $E_t$  = transition energy.

the ground state, calculated by using this protocol (see the Supporting Information) for a) Trp48 in azurin, b) Trp72 in dI.M97 V (for wt dI, see Figure S3 in the Supporting Information). In all three cases, the protein stabilizes  $^1L_a$  more than  $^1L_b$  relative to values in vacuum, which is nicely consistent with the greater dipole moment and polarizability of  $^1L_a$ . For azurin, the  $^1L_a$  origin is on average approximately  $500\text{ cm}^{-1}$  below the  $^1L_b$  origin (nearly  $2.5\text{ kT}$  at room temperature) and this successfully predicts  $^1L_a$  emission. For dI proteins, the  $^1L_a$  and  $^1L_b$  states have rather similar transition

energies but, consistent with experimental findings,  $^1L_a$  is stabilized less in dI.M97V than in wt dI, indicating that  $^1L_b$  emission is favored and that  $\lambda_{\text{max}}$  is shifted to the blue portion of the spectrum.

In conclusion, our data show that Trp protein fluorescence featuring a blue-shifted and structured emission spectrum can result from  $^1L_a$  as was predicted theoretically.<sup>[1b,c]</sup> The proteins investigated are extreme in that they yield the highest degree of blue-shifting emission known and their triplet-lifetime values imply that the Trp residues are embedded in a very rigid microenvironment.<sup>[8,11]</sup> The large dipole moment of  $^1L_a$  and its high polarizability,<sup>[1b]</sup> compared with that of  $^1L_b$ , makes  $^1L_b$  protein emission very unlikely. However, a mutant protein was isolated showing  $^1L_b$  emission and featured the highest degree of blue shifting  $\lambda_{\text{max}}$  recorded to date. Thus, the protein matrix can present a platform for  $^1L_b$  emission, provided that minimal stabilization of the  $^1L_a$  state is offered.

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