Mutations in Transhydrogenase Change the Fluorescence Emission State of TRP^{72} from ^1L_a to ^1L_b

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ABSTRACT The dI component of *Rhodospirillum rubrum* transhydrogenase has a single Trp residue (Trp^{72}), which has distinctive optical properties, including short-wavelength fluorescence emission with clear vibrational fine structure, and long-lived, well-resolved phosphorescence emission. We have made a set of mutant dI proteins in which residues contacting Trp^{72} are conservatively substituted. The room-temperature fluorescence-emission spectra of our three Met^{97} mutants are blue shifted by ~4 nm, giving them a shorter-wavelength emission than any other protein described in the literature, including azurin from *Pseudomonas aeruginosa*. Fluorescence spectra in low-temperature glasses show equivalent well-resolved vibrational bands in wild-type and the mutant dI proteins, and in azurin. Substitution of Met^{97} in dI changes the relative intensities of some of these vibrational bands. The analysis supports the view that fluorescence from the Met^{97} mutants arises predominantly from the ^1L_b excited singlet state of Trp^{72}, whereas ^1L_a is the predominant emitting state in wild-type dI. It is suggested that the sulfur atom of Met^{97} promotes greater stabilization of ^1L_a than either ^1L_b or the ground state. The phosphorescence spectra of Met^{97} mutants are also blue-shifted, indicating that the sulfur atom decreases the transition energy between the ^3L_a state of the Trp and the ground state.

INTRODUCTION

Measurements of fluorescence and phosphorescence from tryptophan residues in proteins have been extremely helpful in understanding many biological processes. Changes in emission intensity, wavelength maximum, energy transfer efficiency, lifetimes, and anisotropy have provided valuable information on the catalytic, folding and ligand-bound intermediates associated with biological reactions. Importantly, the two types of emission can report on protein motions taking place on different time scales, fluorescence in the subnanosecond to nanosecond range, and phosphorescence in the submillisecond to second range.

Fundamental aspects of Trp fluorescence and phosphorescence are now becoming better understood, opening up new areas for the exploration of protein structure and dynamics (1). Fluorescence arises in the electronic transition to the ground state from one of two close-lying excited singlet states (designated ^1L_a and ^1L_b), and phosphorescence in the transition from the triplet state, ^3L_a, to the ground state. The excited and ground states of the Trp have different permanent dipole moments, and therefore interact to different extents with polar and polarizable elements in the protein environment; these interactions determine spectral energies and cause inhomogeneous broadening of vibrational bands. The dipole moment of indole is 5.0–7.0 D in ^1L_a, ~2.5 D in ^1L_b, 2.13 D in the ground state, and 1.4–1.5 D in ^3L_a, respectively (1). The orientations of the dipole moments relative to the long axis of the indole ring are similar but not identical. Understanding spectral energies and vibrational fine structure in the context of tryptophan model compounds, and in relation to emerging quantum mechanics/molecular mechanics calculations (2,3), is key to improved interpretation of Trp emission in proteins in terms of the local molecular structure. Fluorescence emission from Trp in proteins is inhomogeneously broadened due to the range of environments experienced by the fluorophore in the typically large ensemble of conformational substates. The phenomenon obscures vibrational fine structure and generally limits investigation. In one approach to overcome this problem vibrational fine structure in an otherwise broad emission was shown by red-edge excitation at low temperatures (4). However, Trp residues in some proteins, particularly those with very blue fluorescence (i.e., short λ_{max}), display a fine structure in their emission spectra even from broad-band excitation at ambient temperatures. This is usually attributed to a highly nonpolar and/or rigid environment of the indole side chain in these proteins. The single Trp residue (Trp^{48}) of the electron transport protein, azurin from *Pseudomonas aeruginosa*, is a well-known example of a blue-emitter, and it has been the subject of considerable investigation (5–7). The dI component of the proton-translocating, nicotinamide-nucleotide transhydrogenase from *Rhodospirillum rubrum* (see Jackson (8) and Jackson et al. (9) for reviews on this enzyme) emits from its single Trp residue (Trp^{72}) at only a slightly longer wavelength than azurin, and also shows a clear vibrational fine structure (10).

Single amino acid substitutions of bulky nonpolar, with smaller polar, residues in the vicinity of Trp^{48} in azurin shifted λ_{max} to the red, and blurred the vibrational fine structure, consistent with increased local electrostatic interactions, and a looser organization around the Trp (11,12). It

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had been thought that azurin might be one of the few proteins to emit fluorescence from its ^1L_b state (1,6,13,14). However, recent excitation anisotropy spectra suggest that emission is essentially from ^1L_a in both azurin and transhydrogenase dI (15). Remarkably, substitution of Met^{97}, which neighbors Trp^{72} in dI, with a Val residue led to a 4–5 nm blue shift of λ_{max}, and to a change in the fluorescence-anisotropy spectrum indicating a sizable contribution to the emission from ^1L_b (15). In the current work it is shown that this blue shift in λ_{max} is a general consequence of substituting Met^{97} with an amino acid residue having a nonpolar side chain, i.e., of removing the S atom from the Trp environment. In contrast, other conservative mutations in the vicinity of Trp^{72} have a minimal effect on the fluorescence spectra. Low-temperature emission spectra of the dI mutants, recorded at a higher resolution than has previously been achieved with protein solutions, show that, in addition to the blue shift of the emission bands, substitution of Met^{97} also leads to distinctive changes in vibrational-band intensities that provide further evidence on the change in character of the emitting state.

MATERIALS AND METHODS

Site-directed mutagenesis of the *R. rubrum* transhydrogenase dI protein was carried out, essentially as described (16), using oligonucleotide primers purchased from AltaBioscience (UK) (I3A, M97V, L343V, L339A, and L339V), or were constructed by York Biosciences (UK) (M97A and M97L). The parent plasmid, harboring the gene encoding wild-type *R. rubrum* dI, was pCD1 (10). The DNA of all constructs made by PCR was sequenced to confirm fidelity. The plasmids encoding the mutant proteins were designated pKTJ3 (I3A), pKTJ8 (M97A), pKTJ7 (M97L), pKTJ4 (M97V), pKTJ5 (L343V), pKTJ1 (L339A), and pKTJ2 (L339V). Each plasmid was transformed into competent cells of *Escherichia coli* strain C600. Cells were then grown, induced with isopropyl β-D-thiogalactoside, harvested, frozen, and disrupted, as described (10). Mutant proteins, M97L, M97V, and L343V, were found in the soluble fraction of the cell extract, and were purified, as described (17). Mutants, I3A and M97A, were found in inclusion bodies, and were refolded and purified in a procedure developed by S.J. Whitehead, as described (18). Mutants, L339A and L339V, were also found in inclusion bodies but could not be refolded. After chromatography, I3A, M97A, M97L, M97V, and L343V were >95% pure as judged by SDS-PAGE stained with PAGE Blue-83 (Fluka, UK). Protein solutions were either supplemented with 25% glycerol and stored at −20°C for up to ~6 months or precipitated with ammonium sulfate (to 90% saturation) and stored at 4°C for up to ~8 weeks. Both storage procedures were shown to yield similarly active proteins (see below). Recombinant dIII of *R. rubrum* transhydrogenase was prepared and stored as described (19). Protein concentrations were determined using the microtannin assay (20), and are expressed as monomers.

Wild-type copper-free azurin from *Pseudomonas aeruginosa* was purified using published protocols (21). Zn-azurin was prepared by incubating the copper-free protein with $ZnCl_2$ at a molar ratio of 1:2. The C112S mutant of azurin was isolated following the procedure of Sandberg et al. (22). A plasmid harboring the wild-type gene, a generous gift from Prof A. Desideri (Universita di Roma, ''Tor Vergata''), was subjected to site-directed mutagenesis using the Stratagene (UK) QuikChange kit. The DNA of the resulting plasmid was sequenced to confirm that no errors were introduced by the PCR.

Purified and mutant dI proteins (from 5 to 500 nM) were reconstituted with suspensions of dI-depleted *R. rubrum* membranes (containing 10.5 μM bacteriochlorophyll) in 50 mM MOPS, 2 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, pH 7.2, at 25°C, as described (10). Reverse transhydrogenation activity in the reconstituted system was measured by following the reduction of acetyl pyridine adenine dinucleotide (200 μM) by NADPH (200 μM) at 375 nm. Complexes of purified dIII (40 nM) and either wild-type or mutant dI (from 5 to 300 nM) were formed in 50 mM MOPS, 2 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, pH 7.2, at 25°C, as described (19). Cyclic transhydrogenation in the complexes was assayed by following the reduction of acetyl pyridine adenine dinucleotide (200 μM) by NADH (200 μM) using $NADP^+$ tightly bound to the dIII.

The binding affinities for NADH of the wild-type and some mutant dI proteins were measured by isothermal titration calorimetry using a MicroCal (UK) MCS system. Protein solutions were dialyzed against 20 mM MOPS, 10 mM $(NH_4)_2SO_4$, 0.5 mM dithiothreitol, pH 7.2, concentrated to 50–100 μM using a VivaSpin filter (Sartorius, UK) (10 kDa cut-off), and introduced into the calorimeter cell at 25°C. NADH, dissolved in the dialysate, was injected in microliter volumes as described (23). Alternatively, NADH-binding affinities were estimated from the degree of Trp fluorescence quenching using NADPH to compensate for the inner-filtering effect (10). Experiments were carried out in 20 mM Tris-HCl, pH 8.0, 10 mM $(NH_4)_2SO_4$, 0.5 mM dithiothreitol using 1.0 μM dI (wild-type or mutant) at 25°C.

For thermostability determinations, the dI proteins were dialyzed against 30 mM HEPES, 10 mM $(NH_4)_2SO_4$, 0.5 mM dithiothreitol, pH 8.0 and their concentrations adjusted to 12.5 μM. The dependence of heat capacity on temperature was measured in a MicroCal VP-DSC, and the transition temperatures determined as described (18).

Routine fluorescence emission and excitation spectra (Table 1) were recorded with a Photon Technology International model C-61/2000 fluorimeter set to 2 nm bandwidth at 25°C. Stored protein solutions were diluted to 1.0 μM using 20 mM MOPS, pH 7.2, 10 mM $(NH_4)_2SO_4$, 1.0 mM dithiothreitol.

Fluorescence excitation anisotropy was measured at 15°C as described (15). Solutions contained 11–27 μM protein in 30 mM HEPES, pH 8.0, 10 mM $(NH_4)_2SO_4$, 1.0 mM dithiothreitol, and 70% (v/v) glycerol.

The fluorescence spectra shown in Fig. 3 and Figs. S1–S6 in Supplementary Material, Data S1 were acquired with a laboratory-built fluorimeter (24) using laser-pulsed excitation at 288 nm to minimize contributions from Tyr fluorescence, and CCD-camera recording. The bandwidth was 0.2 nm, and all spectra were corrected for instrument response. Stored solutions of the dI and azurin proteins were dialyzed against 100 mM (Na^+) phosphate, pH 7.2, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, concentrated using VivaSpin filters (10 kDa cut-off), and then supplemented with glycerol. The solution used to record the fluorescence spectra contained 25 μM protein monomers, 40 mM (Na^+) phosphate, pH 7.2, 4 mM $(NH_4)_2SO_4$, 0.4 mM dithiothreitol, and 60% (v/v) glycerol.

Phosphorescence spectra and decays were recorded in similar solution conditions but at a dI concentration of between 1.2 and 1.5 μM, using the apparatus described previously (24). Protein samples for phosphorescence experiments were placed in 5 × 5 mm^2 quartz cuvettes, and deoxygenated by repeated cycles of mild evacuation followed by inlet of pure nitrogen, as described previously (25).

RESULTS

Biological properties of mutants of transhydrogenase dI with single amino acid substitutions around Trp^{72}

A set of mutants of the dI protein of *R. rubrum* transhydrogenase was isolated with single amino acid substitutions in the hydrophobic core of domain dI.1. The mutants are designated, M97V (whose fluorescence anisotropy spectrum was presented in Broos et al. (15)), I3A, M97A, M97L, L343V, L339A, and L339V. Fig. 1 shows that, in wild-type dI, Ile^3, Met^{97}, Leu^{339}, and Leu^{343} are all in van der Waals contact with Trp^{72}. The mutants I3A, M97A, M97L, M97V, and L343V were active when reconstituted into either intact

TABLE 1 Catalytic and physical properties of wild-type and mutant dI proteins

Protein	V_{max} (reverse)*	[dI] at half V_{max} (reverse)†	V_{max} (cyclic)‡	[dI] at half V_{max} (cyclic)§	T_m (°C)¶	K_d NADH (μM)‖	λ_{max} (nm)**
Wild-type	2.9	20	2920	72	59.2	19 [15]	308.5
I3A	2.0	18	2910	66	55.3	20 [nd]	309
M97A	1.8	nd	2860	nd	nd	nd [nd]	304
M97L	1.9	nd	2650	nd	nd	nd [nd]	305
M97V	2.1	20	3280	64	56.3	23 [15]	304
L343V	2.0	17	2780	72	58.5	18 [13]	308.5

Details of the experiments are given in the Methods section. nd, not determined.

*Reverse transhydrogenation was measured with saturating concentrations of either wild-type or mutant dI. Rates are given as mol acetyl pyridine adenine dinucleotide reduced mol^{-1} bacteriochlorophyll min^{-1}.

†The concentration of dI (nM) required to give half-maximal rates of reverse transhydrogenation.

‡Cyclic transhydrogenation was measured with complexes made from dIII and saturating concentrations of dI. Rates are expressed as mol acetyl pyridine adenine dinucleotide reduced mol^{-1} dIII min^{-1}.

§The concentration of dI (nM) required to give half-maximal rates of cyclic transhydrogenation.

¶T_m, the endothermic transition peak, was measured by differential scanning calorimetry.

‖The dI affinity for NADH was measured either from Trp fluorescence quenching or (values in brackets) by isothermal titration calorimetry.

**Determined using a Photon Technology International model C-61/2000 fluorimeter using 280 nm excitation light, and a 2-nm bandwidth.

transhydrogenase (see Diggle et al. (10) or dI_2dIII_1 complexes (19)), and they had similar thermostability, and bound NADH with a similar K_d, to the wild-type protein (Table 1). In contrast, the mutants L339A and L339V could not be refolded from the inclusion bodies in which they were expressed, and further work on these two proteins was abandoned.

Fluorescence spectra

λ_{max} values for wild-type dI, I3A, M97A, M97L, M97V, and L343V in buffered solution at 298 K are listed in Table 1. The values for L343V and I3A were not significantly different from wild-type dI but those for the three M97 mutants were all shifted ~4 nm to the blue. Thus, M97A and M97L share with M97V (15) the distinction of having shorter λ_{max} values than any other protein described in the literature. With the exception of M97V, the fluorescence intensities of the wild-type and mutant proteins were all similar within error. In replicate protein preparations the fluorescence intensity of M97V was typically ~40% lower than in the other proteins. When the wild-type and mutant proteins were unfolded with 6 M guanidinium hydrochloride, the λ_{max} values were all shifted to 352 nm, and the vibrational fine structure was lost. The fluorescence excitation spectra (with emission measured at 308 nm), and the absorption spectra, of L343V and I3A were similar to those of wild-type dI but those of the three Met^{97} mutants were blue-shifted by ~1 nm.

A set of fluorescence excitation anisotropy experiments, similar to those recently described for the M97V of dI (15), was carried out with M97A and M97L (Fig. 2); the results were essentially the same. The transition dipole moments for ^1L_a and ^1L_b are almost orthogonal (26). When, therefore, ^1L_b is preferentially excited at ~290 nm, and when molecular rotation is slow relative to the excited-state lifetime, fluorescence anisotropy will be high if emission is from ^1L_b, and low if emission is from ^1L_a (27). Thus, when the emission was measured at 305 nm, there was an increase in anisotropy as the excitation wavelength was scanned across the 0,0 band for ^1L_b absorption, from ~287 to 293 nm. This suggests emission from ^1L_b. However, when the emission was measured at 320 nm, where a larger contribution from ^1L_a is expected, the anisotropy decreased when the excitation was scanned across the same region. The results contrast with those obtained with wild-type dI and with azurin, where only ^1L_a emission was detected (15). Excitation anisotropy experiments with L343V produced similar spectra to those obtained with the wild-type protein (data not shown).

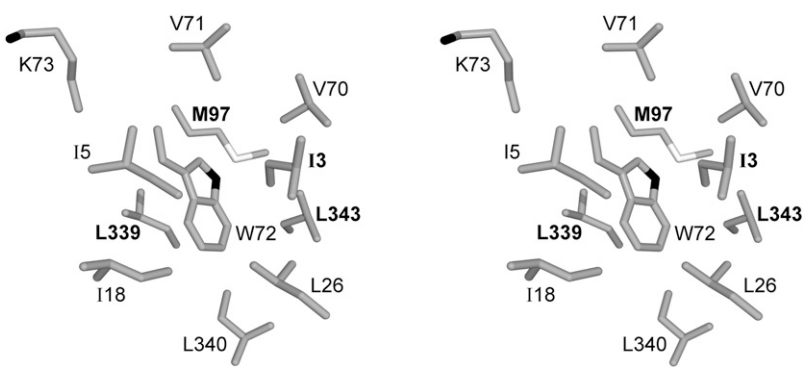

FIGURE 1 Stereo-view of amino acid residues around Trp^{72} in wild-type dI (PDB 1F8G). Carbon atoms are gray, nitrogen, black, and sulfur, white. Amino acid residues mutated in this study (Ile^3, Met^{97}, L^{343}, and L^{339}) are labeled in bold letters.

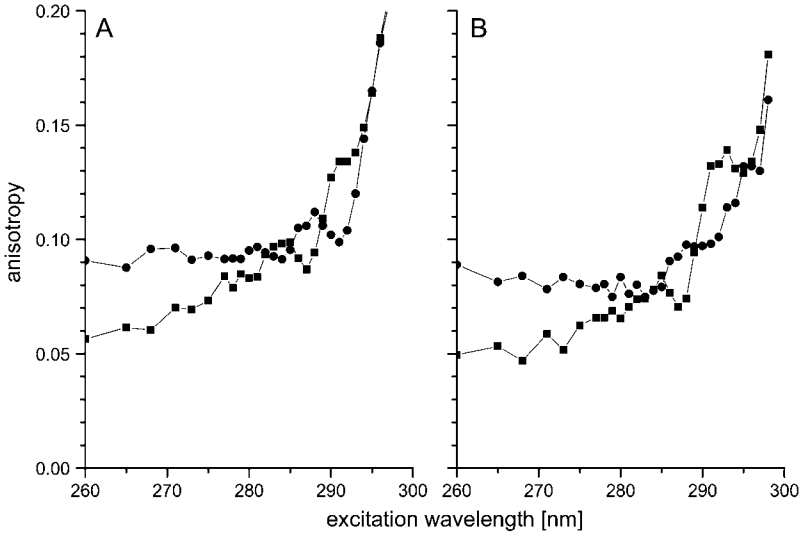

FIGURE 2 Fluorescence excitation anisotropy spectra of the M97A (*A*) and M97L (*B*) mutants of dI in 70% (v/v) glycerol at 15°C. ■, emission measured at 305 nm; ●, emission measured at 320 nm.

Fluorescence spectra of wild-type dI and M97L recorded in a glycerol/phosphate glass (Tg ≈ 190 K) at 160 K and in fluid solutions at 295 K, using a narrow band-pass of 0.2 nm, are shown in Fig. 3. The spectra of azurin under similar conditions are also shown in the figure; these experiments were conducted with Zn-azurin, which has a much higher fluorescence yield than the native Cu form (6,28). Equivalent spectra of M97V and L343V and also of the C112S mutant of azurin, which does not bind the metal ion (22), together with series of fluorescence spectra at intermediate temperatures, are shown in the Figs. S1–S6 in Data S1. The technical improvements in this work led to a much better spectral resolution than that obtained in earlier experiments, for example those on Cu-azurin carried out at 77 K (5). Four vibrational bands (Fig. 3, *A–D*) are clearly evident between 290 and 310 nm in the low-temperature emission spectra of all the chosen proteins, and a group of overlapping peaks is seen at longer wavelengths (310–350 nm). Although the temperature reduction and the narrow instrument bandwidth led to a considerable enhancement of spectral resolution, comparisons with the emission spectra of indole and 3-methyl indole in a cold helium jet and in solid argon (2,29), show that the protein spectra still suffer a significant degree of inhomogeneous broadening. Thus, the FWHM (full width at half maximum) values of bands A–D are several hundred cm^{-1}, as compared to ~5 cm^{-1} for the vibrational bands in the model spectra (2,29), and many of the bands in Fig. 3 are expected to have contributions from more than one vibrational mode.

There is a strong similarity between the spectra of L343V and those of wild-type dI (Figs. S5 and S1 in Data S1, respectively), and between the spectra of M97V and those of M97L (Figs. S4 and S2 in Data S1, respectively). The spectra of I3A resembled those of wild-type dI but the mutant protein was rather unstable in the buffer used in these experiments (data not shown). The wavelength maxima of peaks A, B, C, and D at 160 K in dI and its mutants, and in Zn-azurin and C112S-azurin, are listed in Table 2.

Thanks to the enhanced resolution of the 160 K spectra (Fig. 3), it can be seen that there are two factors contributing to the 4–5 nm blue shift of λ_{max} in the room-temperature spectra of M97L and M97V, summarized in Table 1. First, compared to wild-type dI there is a blue shift in the origin (band A) of the spectrum of 2.5 nm for M97L and of 3.1 nm for M97V. At least in the case of the moderately well-resolved peaks, the shift is seen to be maintained at higher temperatures. Second, the 160 K spectra show that in M97L and M97V (Fig. 3, *middle* and Fig. S4 in Data S1, respectively) there is an increase in the intensity of peak C relative to that of peak D, when compared with wild-type dI (Fig. 3, *top*). Due to thermal broadening, C and D coalesce to give the dominant single peak (largely responsible for λ_{max}) in the spectra recorded at 295 K. The envelopes of the spectra at 295 K indicate that the relative increase in intensity of peak C persists at the higher temperature, where it contributes to the overall shift in the λ_{max} of the M97 mutants.

A similar correlation between the spectral blue-shift and the increase in intensity of band C over that of band D is also observed with azurin. Relative to wild-type dI, the spectra of Zn-azurin (Fig. 3, *bottom*) and of C112S-azurin (Fig. S6 in Data S1) are also shifted to the blue by 0.9–1.2 nm, and the intensity of peak C is increased compared with that of peak D. However, these differences are not as marked as they are in the M97 mutants of dI. The spectral energy and the shape of the vibrational envelope of azurin are therefore intermediate between those of wild-type dI and those of M97L and M97V.

In all the chosen proteins the vibrational bands of fluorescence broadened with increasing temperature, and the well-resolved peaks shifted slightly to the red. The patterns of thermal broadening of peak A, and of its red shift, are shown for wild-type dI, M97L and M97V in Fig. 4. In all three proteins both parameters change over a wide temperature

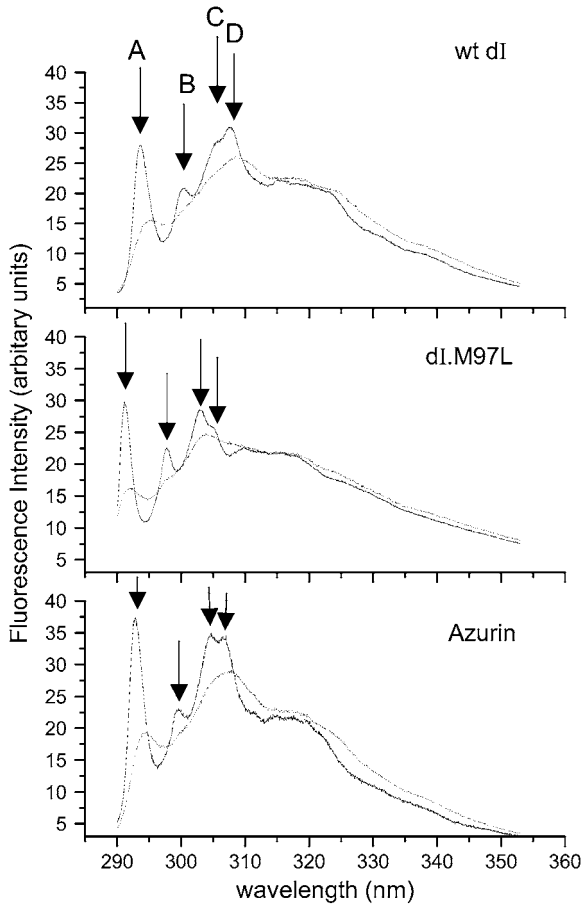

FIGURE 3 High-resolution fluorescence emission spectra of (*top*) wild-type dI, (*middle*) the M97L mutant of dI, and (*bottom*) Zn-azurin. Solid lines, 160 K; dashed lines, 295 K. Experimental conditions are described in Materials and Methods. The peaks identified by the arrows (A, B, C, and D) are referred to in the text.

range, reflecting the distribution of conformations around the Trp that are sampled by the respective proteins while in the excited and ground states. The red shift is more pronounced in M97V than in wild-type dI and M97L, and becomes evident at lower temperatures, perhaps indicating that the Trp environment is loosened more by the Val substitution.

Phosphorescence spectra and lifetimes

The phosphorescence spectrum of wild-type dI in a 77 K glass is exceptionally well resolved, and this is taken to indicate a very homogeneous structural environment around Trp^{72} (30). Fig. 5 shows a phosphorescence spectrum recorded at lower temperature and at a better resolution than in previous work, and an equivalent spectrum of M97V. The λ_{00} (wavelength maximum of the 0,0 band) of wild-type dI (labeled peak A′ in Fig. 5) is centered at 408.2 nm, which is blue-shifted relative to that of Trp in a nonpolar solvent. It is very similar to that in the earlier data (408.0 nm), but the bandwidth is significantly decreased (2.2 nm compared with 3 nm).

Relative to wild-type dI, the phosphorescence spectrum of M97V is shifted further to the blue by 1.1 nm, whereas the bandwidth is slightly increased by 0.5 nm. The low-temperature phosphorescence spectrum of M97L (Fig. S7 in Data S1) is similar to that of M97V, whereas that of L343V (Fig. S7 in Data S1) is unchanged relative to wild-type dI—the widths and spectral positions of the 0,0 band are compared in Table 3. In a rigid glass (at 160 K) the phosphorescence lifetimes of wild-type dI and mutants are all in the region of 5 s, indicating that there are no nearby quenching groups such as Cys residues (31).

In buffer at ambient temperature the phosphorescence spectrum of wild-type dI remains quite well resolved, and the 0,0 band shifts only slightly to the red, consistent with a rigid well-packed local structure (30). The spectra of the dI mutant proteins, M97L, M97V, L343V, and I3A, all behave in the same way (Table 3). The phosphorescence decay of wild-type dI at ambient temperature is almost uniform and exceptionally slow. In close agreement with the earlier results (30), ~94% of the intensity had a lifetime (τ) of 2.95 ± 0.1 s, indicating a very rigid site around Trp^{72} (31,32). The substitution of Met^{97} for Leu, and particularly for Val, increased the phosphorescence decay rate (Table 4), the smaller τ suggesting a looser Trp^{72} environment. The faster phosphorescence decay in M97V compared with M97L is consistent with the more extensive thermal red shift in the fluorescence emission of the former mutant and its lower temperature onset (see above). In L343V, the ambient temperature phosphorescence

TABLE 2 Peak positions of, and intervals between, vibrational bands in fluorescence emission spectra of wild-type and mutant dI, and of azurin at 160 K

Protein	Peak A (nm)	Peak B (nm)	Peak C (nm)	Peak D (nm)	A–B (cm^{-1})	A–C (cm^{-1})	A–D (cm^{-1})
Wild-type dI	293.6	300.5	305.7	307.7	782	1348	1561
L343V	293.5	300.1	305.3	307.5	750	1317	1552
M97L	291.1	297.8	303.0	304.8	772	1349	1544
M97V	290.5	297.1	302.3	303.9	764	1343	1517
Zn-azurin	292.7	299.6	304.6	306.5	787	1335	1539
C112S azurin	292.7	299.4	304.5	306.6	765	1324	1549

The peak (or shoulder) positions are measured to the nearest 0.1 nm from the data shown in Fig. 3 for wild-type dI, M97L, and azurin, and from Figs. S4 and S5 in Data S1 for M97V and L343V. The peaks that lie >310 nm in wild-type dI, and their equivalents in the dI mutants and azurin, are not sufficiently resolved to estimate their wavelength maxima. Peak positions A and B are accurate to within ~0.2 nm, and therefore the intervals between the peaks to within ~40 cm^{-1}. Peak C is a shoulder in wild-type dI and L343V, and its wavelength is therefore more difficult to determine accurately; similarly peak D in M97L and M97V.

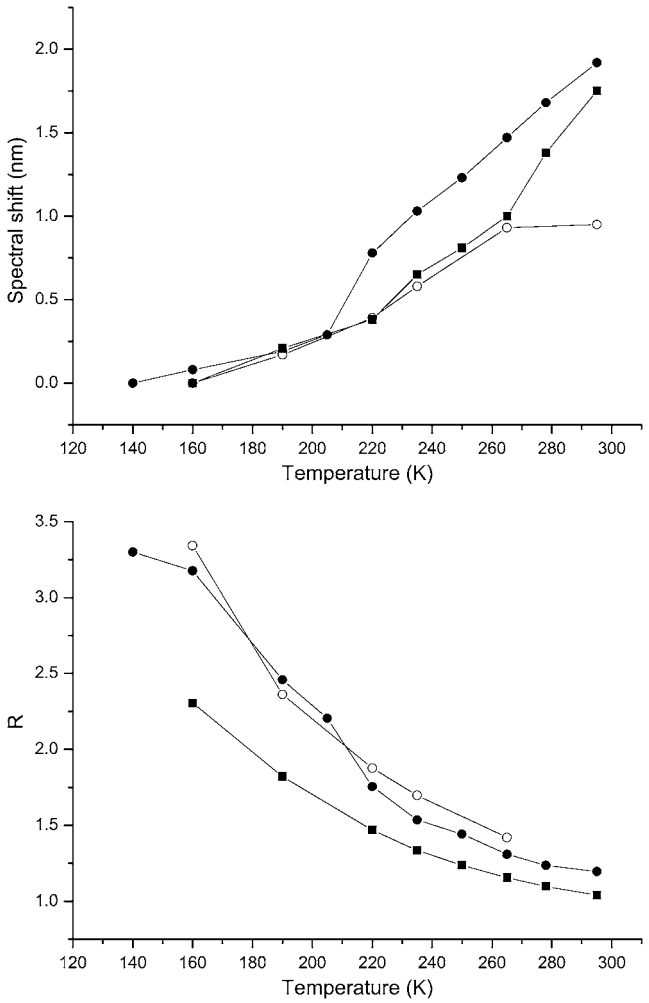

FIGURE 4 The dependence of fluorescence wavelength and band broadening on temperature. The spectral shift (*top*) is λ_{00} at the temperature shown relative to the value measured at the lowest temperature. The spectral broadening (*bottom*) is indicated by the intensity ratio, $R = I_{\lambda 00}/I_{\text{trough}}$. ■, wild-type dI; ●, M97V; ○, M97L.

decay was similar to that of wild-type dI but, in I3A, a relatively large fraction of the emission (54%) exhibited a much shorter lifetime presumably reflecting structural instability in this mutant as noted above.

DISCUSSION

The core of transhydrogenase domain dI.1, which hosts Trp^{72} (Fig. 1), comprises amino acid residues whose nonpolar character is highly conserved. The dI mutants, I3A, M97A, M97L, M97V, and L343V, with conservative single amino acid substitutions in domain dI.1, have similar properties and catalytic activities to those of the wild-type protein (Table 1). This shows that the mutants must have a similar fold to wild-type dI, and that the side-chain substitutions have little effect on biological function. All three of the Met^{97} substitutions resulted in a ~4 nm blue shift in the λ_{max} of Trp^{72} but neither I3A nor L343V had any effect on the protein fluorescence. The blue shift represents an increase in the energy of the transition between the lowest excited singlet state of the Trp and its ground state.

Shift of fluorescence from ^1L_a to ^1L_b on removal of Met^{97}

In a vacuum, the energy of the ^1L_b state of indole lies ~500 cm^{-1} below that of the ^1L_a state (1). Hence, fluorescence from indole in cold jets, and in nonpolar solvents is emitted from ^1L_b. However, the permanent dipole moment of ^1L_a is larger than that of ^1L_b, and polar interactions can therefore invert the energies of the two states. Accordingly, the fluorescence from Trp residues in proteins is generally found to be from ^1L_a. Fluorescence anisotropy experiments indicate that even very blue-emitters like azurin and wild-type dI are mainly ^1L_a, and to our knowledge, of all proteins, only the Met^{97} mutants of dI have been found to have ^1L_b character (Fig. 2 and Broos et al. (15)). It was concluded that, in wild-type dI, the two excited states are on the cusp of degeneracy, and that the change in the Trp^{72} environment caused by substitution of Met^{97} destabilizes ^1L_a to the point that ^1L_b becomes the predominant emitting state. This view is now supported by a comparison of the low-temperature high-resolution fluorescence spectrum of wild-type dI with those of the M97L and M97V mutants. Thus, in addition to the blue shift in the Met^{97} mutants (reflecting destabilization of ^1L_a), there is a distinctive change in the vibrational envelop which suggests an increase in ^1L_b character (Fig. 3 and Figs. S1, S2, and S4 in Data S1). Emission lines from indole in the $^1L_b \rightarrow S_0$ transition (in a cold expansion jet), and in the $^3L_a \rightarrow S_0$ transition (in solid argon), were assigned to Franck-Condon active, normal modes of ground-state vibrations (2). Note that the Franck-Condon factors and intensities of the ^1L_a transition are similar to those for the ^3L_a transition because the change in geometry is the same (2,29). Thus, taking band A (Fig. 3) as the fluorescence origin, a comparison with wavelength intervals given in (2) suggests that band C arises mainly from a clustering and broadening of modes 14, 15, and 16, and band D from modes 8, 9, and 10, together with an overtone of mode 26. Crucially, in the indole model systems modes 14, 15, and 26 are more intense, and modes 8, 9, and 10 are less intense, in the L_b transition than in the L_a transition. Consequently, the increase in the intensity of band C relative to band D observed with the M97 mutants confirms that their fluorescence emission has gained ^1L_b character. In contrast, the vibrational envelope of the low-temperature fluorescence spectrum of L343V is not significantly different to that of the wild-type protein (Figs. S1 and S5 in Data S1). Together with the unchanged fluorescence λ_{max} (Table 1), and the character of the excitation anisotropy spectrum (see Results), this indicates that ^1L_a is still the emitting state in L343V. By similar reasoning, the enhanced resolution of band B (mainly mode 26) in M97L and M97V relative to that

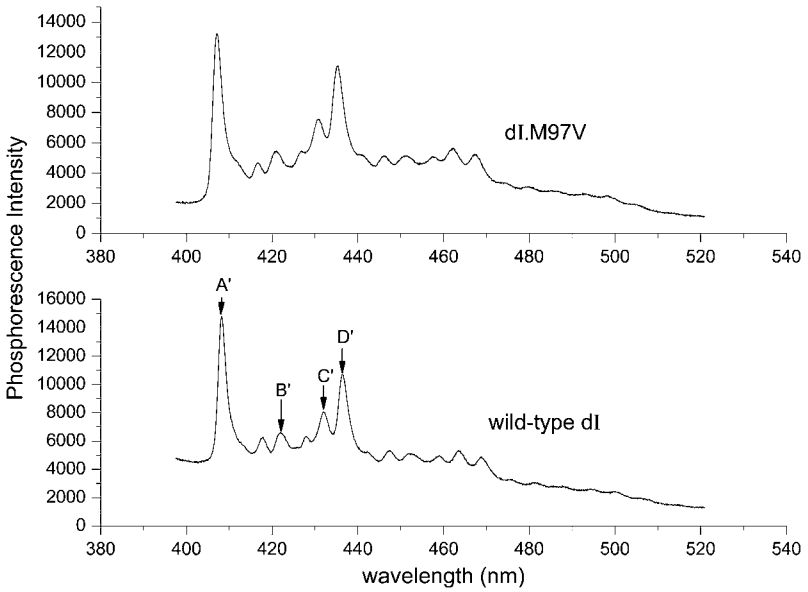

FIGURE 5 Phosphorescence spectra of wild-type dI and the M97V mutant in a glycerol/buffer glass at 77 K. (*Bottom*) wild-type dI; (*top*) M97V. Experimental conditions are described in Materials and Methods. The peaks identified by the arrows (A′, B′, C′, and D′) are referred to in the text.

in wild-type dI and L343V might also be a reflection of an increased contribution of ^1L_b to the emission.

On the possible origin of the blue shift in fluorescence and phosphorescence caused by removal of Met^{97}

The changes in the fluorescence properties of all three of the Met^{97} mutants seem to be similar, and independent of the replacement nonpolar side chain. It is therefore proposed that they result directly from loss of the S atom, and data obtained with model compounds may provide a helpful point of departure for understanding the effect. Thus, the energy of the ^1L_a state of jet-cooled 3-methyl indole is lowered more than that of ^1L_b in complexes with polar species linked through H-bonds to the indole N-H group (29,33,34). The dipole moments of ^1L_a and ^1L_b are both oriented approximately in the direction of the H-bond. The preferential stabilization of ^1L_a was therefore attributed to a greater interaction between the negative charge on the H-bond-accepting atom and the larger dipole moment of ^1L_a. The mean distance between the indole N atom of Trp^{72} and the S atom of Met^{97} in the four subunits of three x-ray structures of *R. rubrum* dI, all at 2.0 Å resolution or better (1F8G, 1L7D, and 1L7E), is 3.5 Å. Even allowing for coordinate error, this is significantly less than van der Waals distance (~4.4 Å), and suggests the existence of a hydrogen bond with the N-H group serving as donor and the S as acceptor; an earlier survey indicated that H-bonds involving the S atom of Met residues in proteins are not uncommon (35). The putative H-bond between Met^{97} and Trp^{72} in wild-type dI will therefore draw the S atom toward the indole N. Although sulfur is only weakly electronegative it is rather polarizable and, consequently, the dipole moment induced by Trp in the S atom will be greater and more stabilizing in ^1L_a than in ^1L_b and the ground state. The loss of this interaction in each of the Met^{97} mutants would preferentially raise the energy of ^1L_a, leading to a blue shift of λ_{max} and favoring emission from ^1L_b.

TABLE 3 Phosphorescence characteristics of wild-type and mutant dI, and Zn-azurin

	77 K		160 K	293 K	
Protein	λ_{00}* (nm)	Bandwidth$_{00}$† (nm)	Lifetime (s)	λ_{00}* (nm)	R‡
Wild-type dI	408.2	2.2	5.1	409.8	1.5
I3A	408.8	2.9	5.3	410.0	1.4
M97L	407.2	3.0	5.3	409.7	1.4
M97V	407.1	2.8	5.0	409.5	1.4
L343V	407.7	2.3	5.1	409.3	1.6
Zn-azurin	410.4	4.2	5.7	nd	nd

Details of the experiments are given in the Methods section.
*λ_{00} is the wavelength at which the intensity of the phosphorescence 0,0 transition (band A′ in Fig. 5) is at a maximum.
†The bandwidth of the phosphorescence 0,0 transition.
‡R, a measure of the spectral resolution, is the ratio of the phosphorescence intensity at the peak of the 0,0 transition to that at the trough at 417.5 nm in wild-type dI and the equivalent in the mutants.

TABLE 4 Lifetimes and amplitudes of components in the phosphorescence decay of wild-type and mutants of dI in buffer at 20°C

Protein	τ_1 (s)	α_1 (%)	τ_2 (s)	α_2 (%)	τ_3 (s)	α_3 (%)	τ_{av}† (s)
Wild-type	2.95 ± 0.1	94	0.48	6			2.80
I3A	2.30 ± 0.15	46*	0.01–0.02	54*			1.06
M97L	1.72 ± 0.11	93	0.145	7			1.61
M97V	2.04 ± 0.15	8.3	0.58	89	0.055	3	0.69
L343V	2.70 ± 0.08	96	0.49	4			2.61

*These amplitudes for I3A are estimated from the decrease in phosphorescence intensity relative to that expected for a fully native sample.
†Average lifetime = $\Sigma\alpha_i\tau_i$.

Other interpretations of the spectral changes caused by the Met^{97} mutations may be considered. The polarizability of the S atom in wild-type dI will also increase the local dielectric constant, and this could influence electrostatic interactions between Trp^{72} and the local electric field generated by protein matrix. Depending on the size of the Trp dipole, the strength of local electric field and the angle between them, this could either stabilize or destabilize ground and excited states (Fig. S8 in Data S1). In addition, substitution of Met^{97} with a residue of different bulk (Ala, Val, Leu) will loosen the packing of the local protein structure. In fact, this is evidenced by the more rapid decay of ambient-temperature phosphorescence in M97V and M97L (Table 4). The packing alteration may create small displacements of charged and polar groups in the protein that might preferentially influence the stability of ^1L_a. However, the observation that all three Met^{97} mutants give rise to a similar blue shift in the fluorescence λ_{max} suggest that this mechanism is not operative. Quantum mechanical/molecular mechanical calculations carried out on an M97V model of the crystal structure of wild-type dI predicted a small blue shift (~2 nm) (15). The calculations used a purely point-charge electrostatic framework, and did not take into account the polarizability properties of the S atom in the wild-type protein (3).

The results of experiments carried out in organic solvents suggest that the phosphorescence 0,0 band of a Trp residue in a nonpolar site in a protein should be around 410–412 nm (36,37). Depending on orientation, the local electric field arising from polar groups in the protein can shift the emission to either the red or the blue. The blue 0,0 emission band of wild-type dI, at ~408 nm, indicates a contribution from polar interactions to the energy of the $^3L_a \rightarrow$ ground state transition (30). The phosphorescence emission of Met^{97} mutants is shifted ~1 nm further to the blue (Fig. 5 and Fig. S7 in Data S1) and, as with the shift in the fluorescence λ_{max}, this can presumably be attributed to the loss of the sulfur atom. The blue shift in the spectra of the mutants calls for an even greater contribution of electrostatic interactions than in the wild-type protein. This may result simply from the expected decrease in the local dielectric constant on replacement of the polarizable S with an alkyl chain, without any change of field orientation or strength. Simple electrostatic calculations, using dipole moments and orientations taken from (1), show that a blue shift in both fluorescence and phosphorescence will result if the loss of the S atom causes a reduction of the dielectric constant, and if the field direction is between ~100° and 170° clockwise from the long axis of the indole side chain (Fig. S8 in Data S1).

The frequencies of vibrational normal modes of Trp residues in transhydrogenase dI and azurin

Despite the improved resolution achieved in the current investigation, the wavelength intervals between vibrational bands A, B, C, and D were insensitive to the Trp environment; within the experimental precision they were similar in wild-type dI, in all dI mutants and in azurin (Fig. 3 and Table 2). Interactions with the local protein matrix can affect the frequencies of vibrational normal modes of an amino acid residue but the effects are small, in the same order as the current instrument resolution (≈20 cm^{-1}). For example H-bond formation can shift frequencies by up to 50 cm^{-1}, and nonpolar interaction by rather less (29,38). The main limitation in the detection of changes in vibrational frequencies, however, is that the fine structure in the spectra reflects a clustering of ground-state vibrational modes of Trp, each broadened (100–300 cm^{-1}) by interactions with the protein matrix (2).

The profound effect that inhomogeneous broadening can have on the shape of the spectral envelope is shown by the differences seen in the vibrational fine structure of fluorescence and phosphorescence spectra (compare Figs. 3 and 5; and see Callis (1) and Callis et al. (39)). Although fluorescence and phosphorescence terminate on the same ground-state level, phosphorescence spectra are generally better resolved than fluorescence spectra because the smaller change in the permanent dipole moment makes the transition less sensitive to local electrostatic interactions. Thus, peaks A, B, C, and D in the160 K fluorescence spectra (Fig. 3) correlate with peaks A′, B′, C′, and D′ in the 77 K phosphorescence spectra (Fig. 5) in wild-type dI, M97V, M97L, and L343V (Fig. 6) but extra bands at 413.4, 417.8, 425.6, 428.0 nm (wild-type values), are resolved in the phosphorescence spectra of the dI proteins.

CONCLUSIONS

The substitution of Met^{97} in dI of *R. rubrum* transhydrogenase with Ala, Leu, or Val results in a ~4 nm blue shift of the λ_{max}

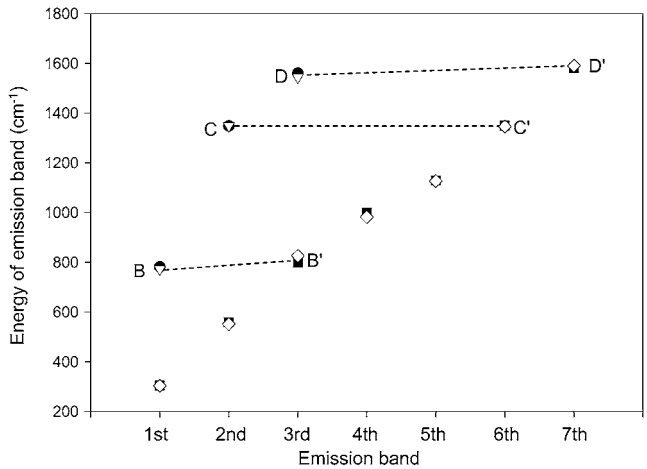

FIGURE 6 Correlation between fluorescence and phosphorescence emission bands. ●, Wild-type dI fluorescence; ∇, M97L fluorescence; ■, wild-type I phosphorescence; ◇, M97L phosphorescence. The emission bands are numbered as the 1st, 2nd, 3rd, etc. to the red of their respective origin. The energy of a band is calculated relative to that of its origin. The fluorescence bands (B, C, and D) (Fig. 3) and the phosphorescence bands (B′, C′, and D′) (Fig. 5) are also positioned relative to their origin (band A and band A′, respectively).

of Trp^{72} fluorescence but mutations of other residues had no effect. The excitation anisotropy spectra, and changes in the relative intensities of vibrational bands in the low-temperature emission spectra, indicate that the fluorescence-emission state of Trp^{72} is shifted from predominantly ^1L_a in wild-type dI to predominantly ^1L_b in the Met^{97} mutants. It is suggested that this results from the loss of the polarizable sulfur atom in the mutants raising the energy of ^1L_a above that of ^1L_b. To date, the Met^{97} mutants of dI are the only proteins known whose Trp emits from ^1L_b. The Met^{97} substitutions slightly increase the bandwidth of phosphorescence spectra of dI in low-temperature glass, and increase the phosphorescence decay rate at ambient temperature; both of these observations are consistent with a loosening of the local structure around Trp^{72}. As with the effect on fluorescence emission, a ~1 nm blue shift in the phosphorescence of the mutants is explained by altered electrostatic interactions between the electronic states of the chromophore and the local protein environment caused by loss of the polarizable sulfur atom of Met^{97}.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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