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Steady-State Fluorescence and Phosphorescence Spectroscopic Studies of Bacterial Luciferase Tryptophan Mutants

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Bacterial luciferase, which catalyzes the bioluminescence reaction in luminous bacteria, consists of two nonidentical polypeptides, α and β. Eight mutants of luciferase with each of the tryptophans replaced by tyrosine were generated by site-directed mutagenesis and purified to homogeneity. The steady-state tryptophan fluorescence and low-temperature phosphorescence spectroscopic properties of these mutants were characterized. In some instances, mutation of only a single tryptophan residue resulted in large spectral changes. The tryptophan residues conserved in both the α and the β subunits exhibited distinct fluorescence emission properties, suggesting that these tryptophans have different local environments. The low-temperature phosphorescence data suggest that the tryptophans conserved in both the α and the β subunits are not located at the subunit interface and/or involved in subunit interactions. The differences in the spectral properties of the mutants have provided useful information on the local environment of the individual tryptophan residues as well as on the quaternary structure of the protein.

KEY WORDS: Bacterial luciferase; tryptophan; steady-state fluorescence and phosphorescence; site-directed mutagenesis.

INTRODUCTION

Bacterial luciferase catalyzes the light-emitting reaction in luminous bacteria utilizing reduced flavin mononucleotide ($FMNH_2$), aliphatic aldehyde, and molecular oxygen as substrates to produce a green–blue light at 490 nm [1]. The enzyme consists of two nonidentical polypeptides, α and β, related in sequence (~30% identity) with a combined molecular weight of 76,000. Although the structure and function of bacterial luciferase have been investigated extensively over the last three decades [2, 3], very little detailed structural information is known.

The spectral properties of the aromatic amino acid residues of proteins provide extremely useful information, as they serve as both structural elements and intrinsic probes. In the absence of X-ray crystallographic data, steady-state fluorescence and low-temperature phosphorescence spectroscopy in conjunction with the application of molecular biology techniques such as site-directed mutagenesis furnishes attractive tools for the study of protein structure and function. In addition, the involvement of aromatic residues at the flavin binding site of luciferase has been implicated in an early study [4].

In the current study, mutants of *Xenorhabdus luminescens* luciferase with each of the tryptophans replaced by tyrosine were generated by site-directed

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mutagenesis and purified to homogeneity. Luciferase from *X. luminescens* contains eight tryptophans, six in the α and two in the β subunit [5]. Tryptophans at positions 182, 194, 250, and 277 in the α subunit and at positions 182 and 194 in the β subunit are conserved residues found in all luciferases isolated from different bacterial species [2]. Only tryptophans at positions 40 and 131 in the α subunit are not conserved. The steady-state fluorescence and phosphorescence properties were found to vary significantly with the different mutations. These tryptophan mutants have provided a unique opportunity to study the contribution of individual tryptophans to the spectral properties of luciferase.

MATERIALS AND METHODS

Chemicals. Oligonucleotides used for site-directed mutagenesis were supplied by the Sheldon Biotechnology Center, McGill University (Montreal, Quebec). Oxidized flavin mononucleotide of >90% purity was purchased from Fluka Chemical Co (Ronkonkoma, NY) and used without further purification. Flavin mononucleotide was reduced by H_2 with Pt as catalyst. Tetradecanal and decanal were obtained from Aldrich Chemical Co (Milwaukee, WI). The glycerol solvent used for the cryogenic phosphorescence experiments was the highest-grade product from Aldrich (Milwaukee, WI). The emission samples contained 70/30 (v/v) glycerol/buffer (50 m*M* phosphate, pH 7.0).

Preparations of Luciferase Mutants. Tryptophan mutants, in which a single tryptophan at different positions was mutated to a tyrosine residue, were prepared by site-directed mutagenesis according to Kunkel [6] and Kunkel *et al.* [7]. A *SacI*/*Xba*I restriction fragment containing the *X. luminescens lux*A and *lux*B genes coding for the α and β subunits was inserted into the single-stranded DNA from the M13 (mp19) sequencing vector and used as the template. The mutant DNA was analyzed by nucleotide sequencing to ensure that a single point mutation has been introduced to convert a tryptophan codon to a tyrosine codon. The modified genes were transferred into the pT7 plasmid and expressed using the bacteriophage T7 promoter-RNA polymerase system [8] in *Escherichia coli* K38.

The transformed *E. coli* cells were grown in the presence of ampicillin and kanamycin (50 μg/ml each) at 32°C to an OD (660 nm) of about 2.6 and lysed by ultrasonic disruption. All the tryptophan mutants prepared in this work were catalytically active. Luminescence activity was determined by the flavin injection assay described elsewhere [9]. The enzymes were purified according to the procedure described by Gunsalus-Miguel *et al.* [10]. The purified wild-type and mutant enzymes had purities of greater than 95% based on analyses by SDS–polyacrylamide gel electrophoresis. Protein concentration was determined by measuring the absorbance at 280 nm using extinction coefficients of $7.72\times10^4\ M^{-1}\ cm^{-1}$ for the wild-type and $7.28\times10^4\ M^{-1}\ cm^{-1}$ for the mutant enzymes. The enzymes were stored at −20°C in the presence of 30% glycerol (v/v) and 10 m*M* β-mercaptoethanol until use.

Steady-State Fluorescence and Phosphorescence. The apparatus for the steady-state phosphorescence consisted of several modules including a phosphorescence scope, a low-temperature sample compartment, and grating monochromators for excitation and emission. The light source was a 100-W high-pressure OSRAM HBO mercury arc lamp. The excitation and emission wavelengths were selected by a 0.25-m and a 0.5-m Bausch & Lomb monochromator, respectively. The phosphorescence scope consisted of a pair of rotating choppers synchronized 90° out of phase. The emission sample was a 4-mm-i.d. Suprasil quartz tube immersed in liquid nitrogen in a Dewar equipped with optical windows for both excitation and emission. The signals were detected by a photomultiplier tube and amplified by a custom-built DC amplifier. The output was recorded by an *XY* recorder.

The steady-state fluorescence apparatus was a Hitachi fluorometer (Model F-3010) equipped with a magnetic stirring unit. The excitation source was a 150-W xenon lamp. The temperature of the sample chamber was regulated by a circulating water bath and measured by a digital thermometer. All the fluorescence experiments were carried out at room temperature (25 ± 1°C). The wavelength dispersion was typically 3 nm for excitation and 5 nm for emission unless specified otherwise. The background emission and scattered light were subtracted from the fluorescence signals. In fluorimetric titrations dilution of the sample due to addition of quencher were corrected for changes in volume. The inner filter effect due to competitive absorption was corrected as described by Lakowicz [11]. The fluorescence and phosphorescence spectra were not corrected for the variations of instrumental sensitivity with emission wavelength.

Fluorescence quenching data were analyzed by linear regression. Parameters defining the linear functions were derived with a conventional spreadsheet program from Microsoft (Redmond, WA) performing multiple regressions in which the slope and intercept as well as the standard errors could be obtained simultaneously.

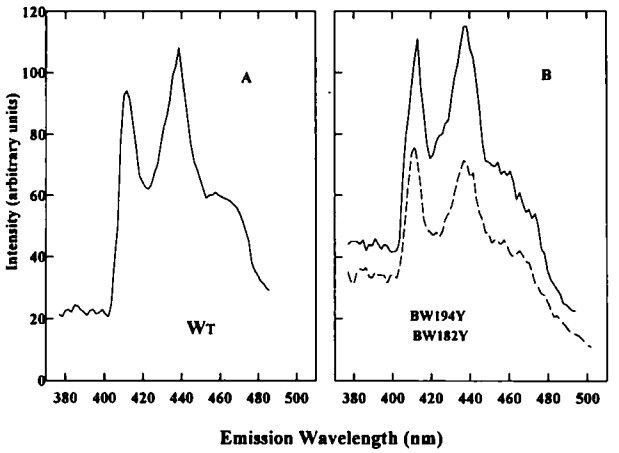

Fig. 1. Low-temperature phosphorescence spectra of the wild-type *X. luminescens* luciferase (A) and tryptophan mutants (B) excited at 280 nm at 77 K. The samples were dissolved in phosphate buffer (0.05 *M*, pH 7.0) containing 70% (v/v) glycerol. The concentration of luciferase was 1×10^{-5} *M* in all cases.

Table I. Tryptophan-to-Tyrosine Phosphorescence Ratios and Positions of the 0–0 Tryptophan Vibronic Band of the Mutants and Wild-Type Luciferase Measured at 77 K in 70/30 (v/v) Glycerol/Buffer Glass

Protein[a]	Trp/Tyr phosp. ratio (λ_{ex} = 280 nm)	0–0 band (nm) (λ_{ex} = 296 nm)
TME[b]	—	406
Wild type	3.4	412
AW40Y	3.1	411
AW131Y	2.6	412
AW182Y	3.3	411
AW194Y	3.2	412
AW250Y	3.0	409
AW277Y	3.6	412
BW182Y	1.2	412
BW194Y	1.4	412

[a]Mutants in the α and β subunits are designated A and B, respectively, with the locations of the tryptophan-to-tyrosine mutation (W→Y) on the polypeptide chain given by the numerical values.
[b]L-Tryptophan methyl ester.

RESULTS

Low-Temperature Phosphorescence. Phosphorescence spectra obtained at 77 K in a rigid glass (70% glycerol:30% buffer, w/w) with an excitation wavelength of 280 nm for both the wild-type *X. luminescens* luciferase and the tryptophan mutants showed both tyrosine and tryptophan emission (Fig. 1). The tryptophan 0–0 vibronic band, which appeared at 410 nm, was broad as a consequence of emission heterogeneity. The tryptophan 0–0 bands observed with the mutants varied in both position and bandwidth and, in particular, with respect to intensity relative to that of tyrosine. With mutations of tryptophan in the α subunit, tyrosine phosphorescence observed at wavelengths below 400 nm was weak and invariant relative to the tryptophan emission (Table I). In contrast, tyrosine phosphorescence of mutants BW182Y and BW194Y with tryptophans (W) in the β subunit replaced by tyrosine was increased considerably relative to the tryptophan phosphorescence (Fig. 1, Table I).

With the excitation wavelength above 295 nm, tryptophan phosphorescence can be selectively excited. The phosphorescence spectra of the wild-type luciferase and mutants excited at 296 nm were essentially free of tyrosine emission at wavelengths below 400 nm. This allows observation of the spectral features due to excited state interactions that occur specifically between tryptophans. With wild-type and mutant *X. luminescens* luciferases, the tryptophan 0–0 vibronic band appeared typically between 410 and 412 nm (Table I). For comparison, L-tryptophan methyl ester (TME) showed a 0–0 vibronic band at 406 nm. This revealed that the local environment surrounding most of the emitting tryptophans was hydrophobic in nature. The only exception was mutant AW250Y, which exhibited a 0–0 band skewed to the blue at 409 nm (Fig. 2).

The tryptophan mutants exhibited rather large variations in phosphorescence spectral properties, especially emission heterogeneity, although only a single tryptophan was mutated. Mutant AW182Y showed a narrow 0–0 vibronic band compared to wild-type and other mutant luciferases (Fig. 2), suggesting that the environments of the emitting tryptophans in the mutant are more homogeneous. In contrast, AW131Y and AW194Y had very broad 0–0 bands with flattened tops (Fig. 2), reflecting increasing phosphorescence heterogeneity. In some instances, the heterogeneity appeared as a blue shoulder of the 0–0 band which could not be further resolved.

Steady-State Fluorescence Properties of the Wild-Type and Mutant Luciferases. The fluorescence spectrum of the native *X. luminescens* luciferase excited at 296 nm displayed spectral features typical of tryptophan emission with a maximum at 340 nm (Fig. 3). Because of the asymmetrical band shape, positions of fluorescence bands were more properly represented by the centers of the full-width at half-height (FWHH). In the presence of 30% (v/v) glycerol, the fluorescence maximum of the wild-type luciferase was shifted to the blue by about 4 nm (Fig. 3). The addition of 1 *M* potassium

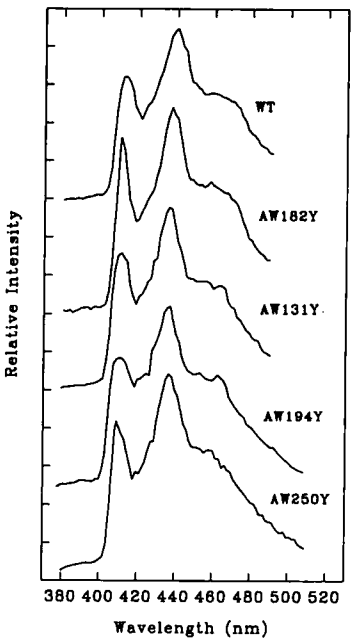

Fig. 2. Phosphorescence spectra of wild-type and mutant luciferases excited at 296 nm at 77 K. The experimental conditions were the same as described in the legend to Fig. 1.

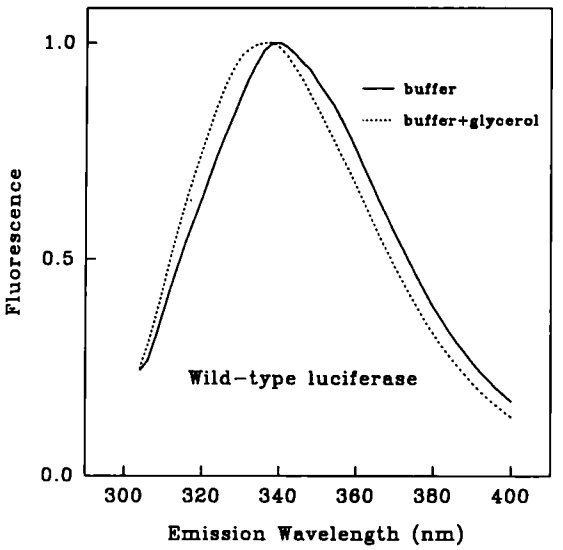

Fig. 3. Fluorescence spectra of the wild-type *X. luminescens* luciferase excited at 296 nm. Spectra were taken at 25°C in phosphate buffer (pH 7.0, 0.05 *M*) in the absence (——) and presence (------) of 30% (v/v) glycerol. Both spectra were normalized to 1 at the fluorescence maximum for comparison. The concentration of the protein was 5×10^{-7} *M* in both cases. The bandpass was 3 nm for excitation and 5 nm for emission.

iodide gave rise to a 30% quenching in the fluorescence intensity which was accompanied by a blue shift of 9–10 nm in the emission maximum (Fig. 4). The overall fluorescence spectrum of the *X. luminescens* luciferase appeared to be comprised of a set of individual intrinsic emitters with emission maxima ranging from 330 nm to 345 nm, with the more exposed tryptophans being preferentially quenched causing the blue shift.

Quantitative measurements of the fluorescence quenching by various quenchers sometimes provide more specific information on the structure of a protein. The Stern–Volmer constants derived from quenching of the fluorescence of luciferase and L-tryptophan methyl ester by a number of quenchers are given in Table II. The values of K_q obtained for the different quenchers from the Stern–Volmer plot for quenching luciferase fluorescence were smaller than the values for quenching the TME fluorescence. The efficiency of iodide ion in quenching the intrinsic fluorescence of luciferase was about 10% of that for quenching of TME emission. With cesium ion, the quenching of luciferase was reduced to 3% of the quenching of free tryptophan. As the iodide and cesium ions are similar in size, the difference in the ability of the two ions to quench the fluorescence of *X. luminescens* luciferase relative to that of TME suggests that the local environment in luciferase surrounding the emitting tryptophans may interact unfavorably with the

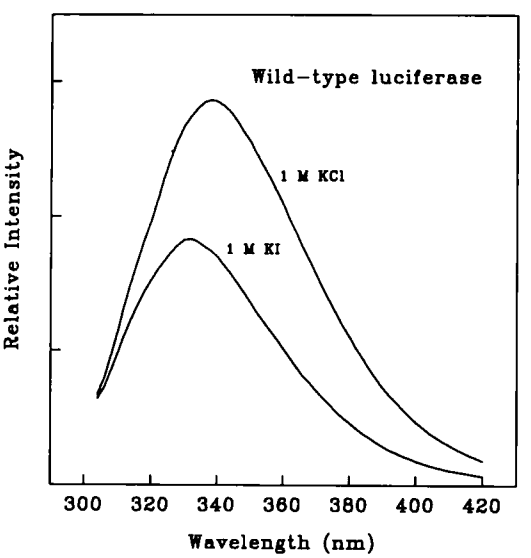

Fig. 4. Fluorescence spectra of wild-type luciferase in 0.05 M phosphate buffer (pH 7.0) in the absence and presence of 1 *M* potassium iodide. The spectra were taken with an excitation wavelength of 296 nm at 25°C. The concentration of the protein was 5×10^{-7} *M*.

positively charged ions, thus decreasing the effectiveness of cesium ion as a quencher. Although previous results indicate that at neutral pH the net charge of the exterior

Table II. Stern–Volmer Quenching Constants of the Wild-Type *X. luminescens* Luciferase Obtained with Different Quenchers[a]

	K_q (M^{-1})		
	I^-	Cs^+	Acrylamide
TME	6.9 ±0.2	1.7±0.1	16.3±0.4
Luciferase	0.61±0.02	(4.4±0.1)×10^{-2}	4.1±0.1

[a]The wavelengths of excitation and emission were 296 and 340 nm, respectively.

Table III. Positions of the Fluorescence Band of the Wild-Type and Mutant Luciferases[a]

Protein	Position (nm)
Wild type	340
AW40Y	339
AW131Y	338
AW182Y	335
AW194Y	339
AW250Y	338
AW277Y	338
BW182Y	339
BW194Y	338

[a]The excitation wavelength was 296 nm. The experimental conditions are the same as described in the legend to Fig. 5.

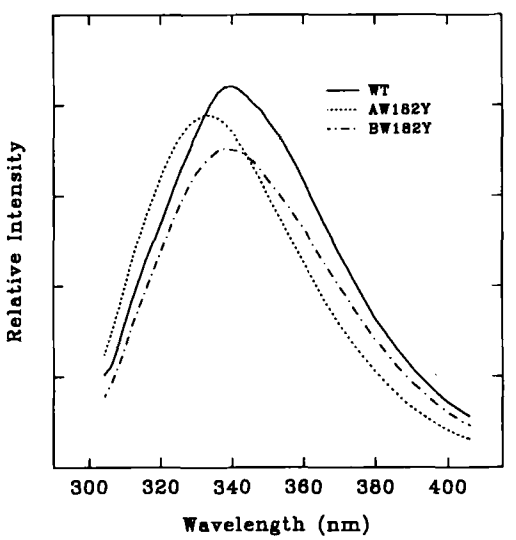

Fig. 5. Fluorescence spectra of the wild-type and mutant luciferases AW182Y and BW182Y excited at 296 nm (25°C). The proteins were in 0.05 *M* phosphate buffer (pH 7.0) at a concentration of 5×10^{-7} *M*.

of luciferase is negative [9], the presence of positively charged patches near the emitting tryptophan residues cannot be ruled out. Recently, fluorescence quenching of

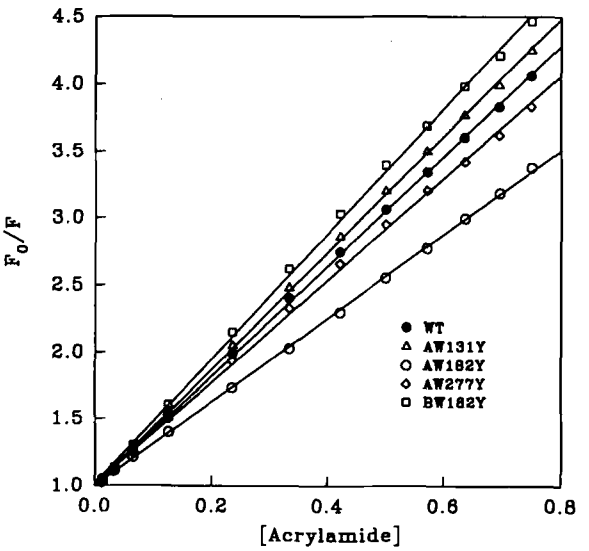

Fig. 6. The Stern–Volmer plots of the wild-type and mutant luciferases obtained by quenching with acrylamide. The excitation and emission wavelengths were 296 and 340 nm, respectively. The protein was dissolved in 0.05 *M* phosphate at pH 7.0 and the protein concentration was 5×10^{-7} *M* in all cases.

the tryptophan residue in luciferase from the firefly, *Luciola*, showed similar properties [12].

The positions of the fluorescence bands of all the tryptophan mutants obtained with the excitation wavelength at 296 nm are given in Table III. Mutation of a single tryptophan in most cases did not result in any appreciable change in the position of the fluorescence band. The only exception was found with mutant AW182Y. Mutation of Trp 182 in the α subunit but not Trp 182 in the β subunit caused a major blue shift of the fluorescence band to 335 nm, suggesting that this tryptophan is located in an unique environment of the protein (Fig. 5).

Mutations of tryptophan residues located at different positions in the α and β luciferase subunits not only caused changes in the fluorescence spectral features but also altered the intrinsic fluorescence quenching properties in response to externally added quenchers. Because of the heterogeneous environments of the emitters and interactions that cause static quenching, nonlinear Stern–Volmer plots with an upward or downward curvature are frequently observed [13]. One of the advantages with using acrylamide as a quencher is that it does not significantly interact with the proteins [13]. For all the mutant and native luciferases the Stern–Volmer plots obtained with acrylamide were strictly linear. A number of these plots are shown in Fig. 6. The vari-

Table IV. Fluorescence Quenching Constants of Wild-Type Luciferase and Tryptophan Mutants Derived from the Stern–Volmer Plot with Acrylamide[a]

Mutant	K_q (M^{-1})
AW40Y	4.7 ± 0.1
AW131Y	4.4 ± 0.1
AW182Y	3.1 ± 0.1
AW194Y	4.1 ± 0.1
AW250Y	4.3 ± 0.2
AW277Y	3.9 ± 0.1
BW182Y	4.6 ± 0.2
BW194Y	4.7 ± 0.2

[a]The excitation and emission wavelengths were 296 and 340 nm, respectively.

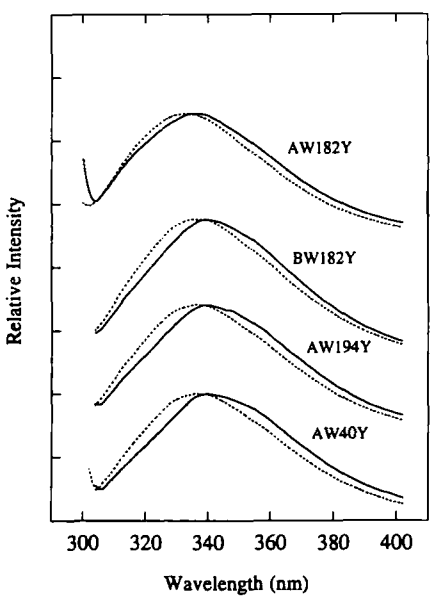

Fig. 7. Solvent-perturbed fluorescence spectra of the luciferase tryptophan mutants. The solid and dashed curves denote the spectra in the absence and presence of 30% glycerol (v/v), respectively. The experimental conditions were the same as described in the legend to Fig. 4.

ations of the quenching constants K_q derived from the Stern–Volmer plot (Table IV) reflect the different overall accessibility of these intrinsic groups to solvent. The mutant AW182Y had a value of 3.1 M^{-1} for K_q, significantly lower than the K_q of the other tryptophan mutants or wild-type luciferase.

With luciferase mutants that differ by only a single tryptophan, solvent-perturbed spectra may provide information on the solvent exposure of not only the mutated tryptophan but also the remaining ones. In the presence of perturbant solvent glycerol, a blue shift in the fluorescence maximum was found with all the tryptophan mutants. With the mutants containing Trp 182 on the α subunit, the shift was typically 4 to 6 nm, while the smallest shift (<2 nm) was observed with mutant AW182Y (Fig. 7). This suggests that Trp 182 showed a typical solvent-exposed feature, consistent with the above fluorescence quenching data. However, the results also indicate that the other tryptophans were at least partially buried, since the tryptophan fluorescence spectrum of AW182Y in the presence of glycerol showed a much smaller blue shift, revealing that the immediate environments of the remaining tryptophans may have restricted access to the solvent.

Table V. The Numbers of Tryptophan and Tyrosine Residues in the α and β Subunits of the Wild-Type *X. luminescens* Luciferase [5]

Subunit	Trp	Tyr
α	6	17
β	2	17

DISCUSSION

Mutation of tryptophan residues by protein engineering can provide specific information on the structure and function of luciferase as the tryptophan serves as an intrinsic probe. The spectroscopic properties of various tryptophan mutants observed at both singlet and triplet levels reflect contributions by a set of tryptophans in different local environments as well as the interactions at the excited states between them. As tryptophan residues are not expected to have identical luminescence quantum efficiencies, mutation of even a single tryptophan residue in proteins containing multiple tryptophans may significantly alter the spectroscopic properties of the protein. Consequently, phosphorescence and fluorescence studies can provide valuable information on the tertiary or quaternary structure of bacterial luciferase.

Excitation energy transfer between tyrosines, tryptophans, or tyrosines and tryptophans may occur at both the singlet and the triplet levels [14]. The presence of tryptophan residues in a protein provides an effective drainage for the tyrosine excitation energy at the singlet level [14], for which a critical transfer distance of about 15 Å has been estimated [15]. As a consequence of the electronic couplings between tryptophan residues, excitation energy within a protein can migrate between different sites separated by a distance usually greater than the critical radii of singlet–singlet energy transfer. With an increasing number of tryptophan residues in the pro-

tein or its subunit Tyr→Trp energy transfer becomes more efficient. The lower Tyr/Trp phosphorescence ratios found with mutants of the α subunit than those with the β subunit indicate that the energy transfers in the α subunit are more efficient. This difference can be explained by the fact that the relative number of tryptophan to tyrosine residues in the α subunit is greater than that in the β subunit (Table V). Substitutions of tryptophan residues in the β but not the α subunit give rise to a large increase in the Tyr/Trp phosphorescence ratio, showing that the two subunits have quite independent Tyr→Trp energy transfer processes. These results also show that the tryptophan residues in both subunits are not brought into close contact by the formation of the dimeric αβ structure.

Spectral heterogeneity decreases with increasing long-range electronic couplings between the emitters primarily at the singlet level [16]. Heterogeneous tryptophan emission as a well-resolved 0–0 vibronic band in phosphorescence spectrum is observed with proteins containing three or fewer tryptophans [17–20]. Tryptophan mutants that contain Trp 182 of the α subunit show either a broad 0–0 vibronic band or a discernible blue shoulder in the band in the phosphorescence spectra. This reflects the solvent-exposed nature of this residue, in agreement with the fluorescence data. From the steady-state phosphorescence spectra of the mutant luciferases, there is apparently a distribution of triplet energy levels among the various tryptophans. Mutant luciferases missing a tryptophan at position 131 or 194 in the α subunit showed a much broader 0–0 band than that of the native luciferase. Thus, it is possible that these groups (W131 and W194) are situated between tryptophans that differ in singlet or triplet energy, thus enhancing electronic couplings between the sites and reducing the spectral heterogeneity. A typical example for such a system is Trp 158 of bacteriophage T4 lysozyme reported by Maki and co-workers [20].

The significant blue shift of the 0–0 tryptophan phosphorescence vibronic band seen with mutant AW250Y suggests that Trp 250α may have the lowest energy due to its hydrophobic surroundings. Localization of the long-range singlet excitation energy at Trp 250α would reduce the ability of other tryptophans to serve as both singlet and triplet emitters. Removal of this tryptophan would reveal emitters of a higher energy level within the critical transfer distance, thus shifting the 0–0 band to the shorter wavelength.

Analysis of the amino acid sequences of the α and β polypeptides [5] shows that the two subunits have approximately 30% sequence identity, suggesting that the corresponding genes, *lux*A and *lux*B, respectively, occurred as the result of gene duplication. This led to a hypothesis that the two polypeptides forms a heterodimer with a quasi twofold rotation axis [2]. As a consequence, the amino acid residues that are conserved between α and β subunits were considered to be involved at the subunit interface and/or the active sites. Tryptophan residues at positions 182 and 194 are conserved in both the α and the β subunits [2, and the references therein] and the emission properties of the tryptophan mutants would be expected to be similar. However, it is clear that the tryptophan residues at positions 182 of the α and β subunits reside in different environments in terms of accessibility to solvent. These results indicate that luciferase does not possess a symmetry element with respect to the two subunits.

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REFERENCES

1. J. W. Hastings and Q. H. Gibson (1963) *J. Biol. Chem.* **238**, 2537–2554.
2. T. O. Baldwin and M. Z. Ziegler (1990) in F. Müller (Ed.), *Chemistry and Biochemistry of Flavoenzymes*, CRC Press, London, pp. 468–530.
3. E. A. Meighen and P. V. Dunlap (1993) *Adv. Microbiol. Physiol.* **34**, 1–67.
4. T. O. Baldwin, M. Z. Nicoli, J. E. Becvar, and J. W. Hastings (1975) *J. Biol. Chem.* **250**, 2763–2768.
5. R. Szittner and E. A. Meighen (1990) *J. Biol. Chem.* **265**, 16581–16587.
6. T. A Kunkel (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488–492.
7. T. A. Kunkel, J. D. Roberts, and R. A. Zabour (1987) *Methods Enzymol.* **154**, 367–382.
8. S. Tabor and C. Richardson (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1074–1078.
9. E. A. Meighen and J. W. Hastings (1971) *J. Biol. Chem.* **246**, 7666–7674.
10. A. Gunsalus-Miguel, E. A. Meighen, M. Z. Ziegler, K. H. Nealson, and J. W. Hastings (1972) *J. Biol. Chem.* **247**, 398–404.
11. J. R. Lakowicz (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
12. G. D. Kutuzova and T. O. Baldwin (1993) *J. Biolumines.* **8**, 95.
13. M. R. Eftink and C. A. Ghiron (1981) *Anal. Biochem.* **114**, 199–227.
14. J. W. Longworth (1971) in R. F. Steiner and I. Weinryb (Eds.), *Excited States of Proteins and Nucleic Acids*, Plenum, New York.
15. J. Eisinger, B. Feuer, and A. A. Lamola (1969) *Biochemistry* **8**, 3908–3915.
16. W. C. Galley (1976) in R. Chen and H. Edelhoch (Eds.), *Concepts in Biochemical Fluorescence*, Marcel Dekker, New York.

17. R. M. Purkey and W. C. Galley (1970) *Biochemistry* **9**, 3569–3575.
18. W. C. Galley, R. E. Williams, and L. Goodfriend (1982) *Biochemistry* **21**, 378–383.
19. M. R. Eftink, G. D. Ramsay, L. Burns, A. H. Maki, C. J. Mann, C. R. Matthews, and C. A. Ghiron (1993) *Biochemistry* **32**, 9189–9198.
20. S. Ghosh, L.-H. Zang, and A. H. Maki (1988) *J. Chem. Phys.* **88**, 2769–2775.