# Terbium(III) Luminescence Study of Tyrosine Emission from *Escherichia coli* Glutamine Synthetase†

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ABSTRACT: Radiationless energy transfer from tyrosine to Tb(III) in *Escherichia coli* glutamine synthetase and its two mutants (W57L and W158S) has been utilized to assess the tyrosine residue(s) responsible for the observed tyrosine emission and to investigate its spatial relationships to the two metal binding sites of GS. The interference from tryptophan fluorescence was removed by chemical modification of the tryptophan residues by *N*-bromosuccinimide (NBS). The Tyr–Tb(III) distances measured by using Förster energy-transfer theory were in good agreement among the three enzymes with average distances of 10.7 and 11.2 Å from Tyr to the two metal binding sites. The $pK_a$ value for the ionization of tyrosine was determined from fluorescence titration experiments to be ~10 for both mutant enzymes. The similarities in $pK_a$ values and Tyr–Tb(III) distances observed for all three enzymes lead to the conclusion that the same tyrosine residue(s) is (are) most likely responsible for the Tyr emission. According to the crystal structure distances from tyrosine residues to the two metal binding sites of GS, it is believed that Tyr-179 is the main contributor to the observed Tyr emission. The fact that an intense Tyr emission was observed for W57L GS but not for W158S GS indicates that Trp-57 is much more effective than Trp-158 in quenching the Tyr-179 emission probably through a Förster-type energy transfer. Furthermore, modification of Trp-57 by NBS causes no significant increase in Tyr-179 emission while replacement of Trp-57 by leucine does. This may indicate that oxidized Trp-57 is also an effective quencher for Tyr-179 emission.

*Escherichia coli* glutamine synthetase (GS) is a dodecameric enzyme which catalyzes the formation of glutamine from glutamate, $NH_4^+$, and ATP. The enzyme has been shown to contain two metal binding sites of unequal affinity per subunit designated as the $n_1$ and $n_2$ sites (Ginsburg, 1972; Hunt et al., 1975). Occupation of both sites by metal ions is required for the expression of catalytic activity (Hunt et al., 1975). Introduction of different chromophoric metal ions [e.g., Mn(II), Co(III), Cr(III), and the lanthanide ions] to the two binding sites provides a variety of methodologies to investigate the spatial relationships among active-site components and nearby protein residues (Villafranca & Ash, 1976; Villafranca et al., 1977, 1978; Balakrishnan & Villafranca, 1979; Gibbs et al., 1984).

Many of the trivalent lanthanide ions [Ln(III)] possess magnetic and spectroscopic properties which make them useful as probes of metalloproteins. Tb(III) ion has the ability to luminesce in solution at room temperature, and its fluorescence is dramatically enhanced when bound to proteins due to a Förster-type energy transfer. These properties have been successfully applied to the determination of the metal–metal or metal–tryptophan distances in enzymes (Horrocks & Sudnick, 1981; Rhee et al., 1981; Horrocks et al., 1975; Horrocks & Collier, 1981).

GS was found to bind Tb(III) sequentially as illustrated by a biphasic Tb(III) luminescence titration curve (Eads, 1985). This sensitized Tb(III) luminescence is believed to result mainly from an energy transfer from Trp residues to Tb(III) since the emission of phenylalanine and tyrosine in proteins is usually very small. GS contains two Trp residues, Trp-57 and Trp-158, per subunit. Mutants with Trp-57 replaced by leucine (W57L GS) or Trp-158 replaced by serine (W158S GS) have been constructed to study the individual effect of each Trp residue on the energy transfer to Tb(III) and their spatial relationships to the two metal ion binding sites (Atkins et al., 1991; Lin et al., 1991). The main concern of this paper is characterization of the Tyr fluorescence of wild-type and mutant GS. Comparison of the experimental Tyr–Tb(III) distances for GS and its mutants with the crystal structure coordinates provides information to assess the Tyr residue(s) responsible for this Tyr emission.

## Experimental Procedures

*Materials.* The site-directed mutagenesis, expression, and purification of *E. coli* glutamine synthetase in a low state of adenylylation were as described in the preceding papers (Atkins et al., 1991; McNemar et al., 1991; Lin et al., 1991). Metal-free enzyme was prepared by dialysis against 3 mM EDTA, 10 mM Hepes, and 100 mM KCl, pH 7.0, followed by extensive dialysis against the same buffer without EDTA. A Tb(III) solution was prepared from the chloride salt obtained from Aldrich. *N*-Bromosuccinimide was purchased from Sigma.

*Methods.* The fluorescence spectra of GS and Tb(III) were measured on a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with a differential corrected spectra unit. The pH of the solution was adjusted by successively adding a small amount of 1 M KOH and was measured by an Orion Model 601A digital pH meter. Fluorescence spectra were recorded after conformational changes induced by metal binding were complete, at least 20 min after metal addition.

*Förster Theory.* Equations describing Förster theory are discussed in the preceding papers (Atkins et al., 1991; McNemar et al., 1991; Lin et al., 1991).

*Modification of Trp Residues of Glutamine Synthetase by NBS.* Glutamine synthetase was modified by NBS as described by Spande and Witkop (1967) with the modifications listed here and in the legends to the figures. A solution of GS (1–10 μM, subunit concentration) in 10 mM Hepes buffer, pH 7.0, containing 100 mM KCl was incubated with a 2–

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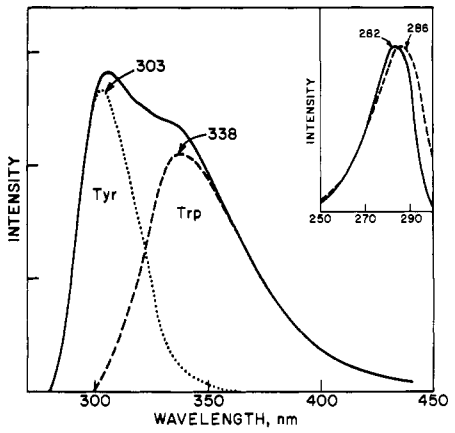


FIGURE 1: Fluorescence spectrum ($\lambda_{ex}$ = 275 nm) for 1 $\mu$M W57L GS in 10 mM Hepes/0.1 M KCl, pH 7.0, at 23 °C. Inset: excitation spectrum of W57L GS with $\lambda_{em}$ = 303 nm (—) or $\lambda_{em}$ = 338 nm (---).

10-fold excess of NBS. The optical spectrum was taken as well as the fluorescence spectrum. Under all conditions, the oxidation was completed within 1 min of addition. The activity of the enzyme was 85–95% of that of the unmodified enzyme.

## Results

*Excitation and Emission Spectra of Glutamine Synthetase and Mutant Enzymes.* Proteins contain three amino acid residues which may contribute to their fluorescence: tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe). The emission of Tyr and Phe is small and frequently undetectable, and the protein fluorescence emission is usually dominated by the tryptophan fluorescence. However, an intense Tyr emission was observed for one of the mutants of glutamine synthetase, W57L GS. Figure 1 shows the fluorescence spectrum ($\lambda_{ex}$ = 275 nm) of W57L GS in 10 mM Hepes/0.1 M KCl, pH 7.0, at 23 °C. The spectrum consists of two overlapping bands, centered at 303 and 338 nm, which correspond to the emission of Tyr and Trp residues, respectively. The excitation maxima for these two bands (Figure 1, inset) are centered at 282 ($\lambda_{em}$ = 303 nm) and 286 nm ($\lambda_{em}$ = 338 nm).

The emission spectra of W158S GS and wild-type GS show no significant tyrosine emission (not shown). Each spectrum has only a single broad band with emission maxima at 324 and 329 nm for W158S GS and wild-type GS, respectively. In contrast to W57L GS, the spectra show no noticeable Tyr emission. However, the excitation spectra of these two enzymes monitored at two different wavelengths (300 and 340 nm) also show differences in excitation maxima as observed for W57L GS. Excitation maxima of 283 ($\lambda_{em}$ = 300 nm) and 287 nm ($\lambda_{em}$ = 340 nm) were observed for W158S GS and 282 ($\lambda_{em}$ = 300 nm) and 286 nm ($\lambda_{em}$ = 340 nm) for wild-type GS (data not shown). These results indicate the presence of Tyr emission for these two enzymes but with much lower intensity.

*pH Dependence of the Emission Spectra of W57L GS.* Ionization of L-tyrosine produces a weakly fluorescent tyrosinate which has an excitation maximum at 295 nm and an emission maximum at 345 nm (Shimizu & Imakuvo, 1977). The p$K_a$ for the ionization of Tyr residues in most proteins is usually in the range of 9–12 (Tanford, 1962). Monitoring the Tyr fluorescence provides a sensitive means to determine the p$K_a$ of Tyr residues in proteins. Figure 2 shows the emission spectra ($\lambda_{ex}$ = 275 nm) of W57L GS at various pH values. As expected, the intensity of the Tyr emission of W57L GS decreases as the pH of the solution increases. At pH 11.9, Tyr emission essentially disappears (Figure 2G), indicating that the fluorescent Tyr residues have been converted to

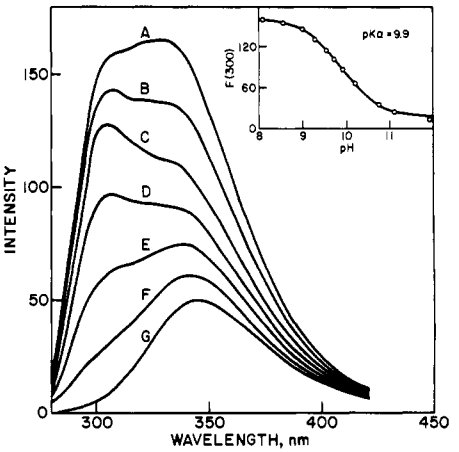


FIGURE 2: Fluorescence spectra ($\lambda_{ex}$ = 275 nm) of 1 $\mu$M W57L GS in 10 mM Hepes/0.1 M KCl, 23 °C, at pH (A) 8.10, (B) 9.00, (C) 9.27, (D) 9.72, (E) 10.21, (F) 10.76, and (G) 11.90. Inset: pH titration curve for the fluorescence intensity at 300 nm.

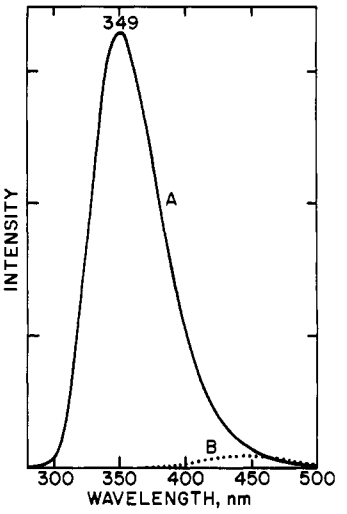


FIGURE 3: Fluorescence spectra ($\lambda_{ex}$ = 280 nm) of (A) 10 $\mu$M L-tryptophan and (B) 10 $\mu$M L-Trp and 0.1 mM NBS in aqueous solution at 23 °C.

tyrosinate, and the remaining fluorescence, which has an excitation maximum of 286 nm, is primarily from the emission of Trp-158. The p$K_a$ value of 9.9 was determined from the pH profile (Figure 2, inset) which is a resonable ionization constant for a Tyr residue in proteins. This process is reversible. Lowering the pH of the solution causes increases in Tyr emission with the same p$K_a$ value.

*Chemical Modification of Tryptophan by NBS.* The indole ring of Trp can be oxidized by NBS to form an oxindole derivative (Witkop, 1961). The oxidation of Trp results in a decrease in absorbance at 280 nm and also a decrease in Trp fluorescence. Upon oxidation, Trp fluorescence centered at 349 nm is red-shifted to about 450 nm (Figure 3) with a dramatic decrease in intensity. The excitation and emission spectra of the oxidized Trp show an emission maximum at 458 nm and two excitation maxima at 268 and 342 nm (not shown). These spectral characteristics of NBS-oxidized Trp provide models for the expected changes in GS fluorescence when the protein is oxidized with NBS.

*Modification of Trp Residues of Wild-Type and Mutant Glutamine Synthetase by NBS.* As demonstrated above, W57L GS possesses an intense Tyr emission (Figure 1), while the presence of Tyr emission in W158S GS and wild-type GS is not as prominent. Selective modification of Trp residues by NBS was carried out to decrease the Trp emission and

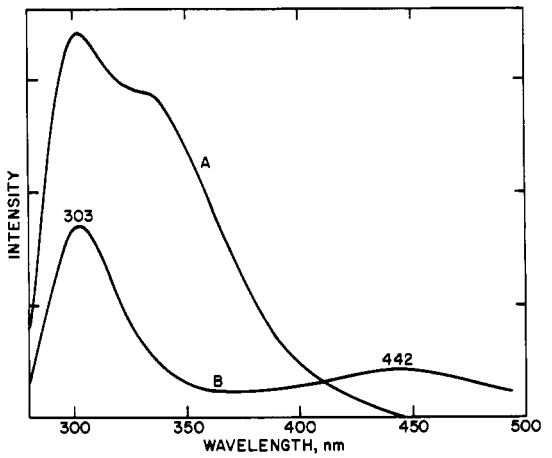


FIGURE 4: Fluorescence spectra ($\lambda_{ex}$ = 275 nm) of (A) 1 $\mu$M W57L GS and (B) 1 $\mu$M W57L GS and 50 $\mu$M NBS in 10 mM Hepes/0.1 M KCl, pH 7.0, at 23 °C.

make the Tyr emission more prominent. Figure 4 shows the effect of NBS on the emission spectrum of W57L GS. After complete oxidation of Trp-158, a distinct Tyr emission centered at 303 nm appears. The excitation maximum for this Tyr emission is 282 nm which is the same as that of W57L GS monitored at 303 nm (Figure 1, inset). The weak fluorescence centered at 442 nm, which has an excitation maximum of 370 nm, is due to the emission of oxidized Trp-158.

A similar effect of NBS on the emission spectrum has also been observed for W158S GS and wild-type GS. Upon complete modification of Trp residues by NBS, a distinct Tyr emission centered at 303 nm, which also has an excitation maximum of 282 nm, has been observed for both enzymes (data not shown). The excitation and emission maxima of the oxidized Trp residues for both enzymes are the same as those of NBS-treated W57L GS (370 nm for excitation and 442 nm for emission).

*pH Profile of NBS-Treated W57L GS.* The emission spectra ($\lambda_{ex}$ = 275 nm) of NBS-treated W57L GS at various pH values were determined. The Tyr emission at 300 nm decreases as the pH of the solution increases. A tyrosinate emission centered at 345 nm is observed at pH 11.9 which has an excitation maximum of 295 nm. The pH profile for the variation of the Tyr fluorescence intensity at 300 nm is very similar to the pH dependence of the native W57L (Figure 2), except that a p$K_a$ value of 10.2 was determined. This compares to the p$K_a$ value of 9.9 for the native W57L emission. As before, this ionization process is reversible with the same p$K_a$ value.

*Determination of Tb(III)–Tyr Distances for NBS-Treated Glutamine Synthetase.* Tb(III) is significantly sensitized to luminescence when bound to proteins upon irradiation of the absorption bands of the aromatic amino acid residues (Trp, Tyr, Phe) (Brittaine et al., 1976). This phenomenon is generally attributed to a Förster-type energy transfer, and the sensitized Tb(III) luminescence has been utilized to determine the distances between the Tb(III) ion and Trp residues in proteins (Horrocks & Sudnick, 1981; Horrocks & Collier, 1981). In this report, a similar approach has been adopted to determine the Tyr–Tb(III) distances in NBS-treated glutamine synthetase and its mutants.

In order to calculate distances using the Förster equation, the quantum yields of Tyr and Tb(III), the overlap integral, and the efficiencies of energy transfer have to be determined. The quantum yields of Tyr residues of NBS-treated wild-type and mutant glutamine synthetase were determined from the relative areas of the emission spectra of the enzyme and an aqueous L-tryptophan solution as described by Chen (1967). The results are summarized in Table I. The quantum yields of Tb(III) bound to GS were estimated to be 0.21 and 0.36 for 1 and 2 equiv of Tb(III) per subunit (McNemar, 1989) from the change in the luminescent exponential decay constant as the $H_2O$:$D_2O$ ratio was varied. Because Tb(III) binds at the same sites in wild-type and mutant enzymes, these quantum yields were also used for the mutant enzymes.

Table I: Summary of the Quantum Yields of Tyr, $\phi_{Tyr}$, Efficiencies of Energy Transfer, $E$, Critical Distances for 50% Energy Transfer, $R_0$, and Tb(III)–Tyr Distances, $r$, for NBS-Treated Glutamine Synthetase and Its Mutants

| enzyme | equiv of Tb(III) | $\phi_{Tyr}$[a] | $E$[a] | $R_0$ (Å) | $r$ (Å)[b] |
|---|---|---|---|---|---|
| W57L | 1 | 0.017 | $1.16 \times 10^{-4}$ | 2.58 | 11.7 |
| | 2 | 0.017 | $1.25 \times 10^{-4}$ | 2.58 | 11.6 |
| W158S | 1 | 0.0037 | $8.10 \times 10^{-5}$ | 2.40 | 9.6 |
| | 2 | 0.0040 | $4.80 \times 10^{-5}$ | 2.45 | 10.7 |
| wild type | 1 | 0.0011 | $1.17 \times 10^{-5}$ | 1.64 | 10.9 |
| | 2 | 0.0011 | $9.76 \times 10^{-6}$ | 1.64 | 11.2 |

[a] With an error of ±10%. [b] With an error of ±1 Å.

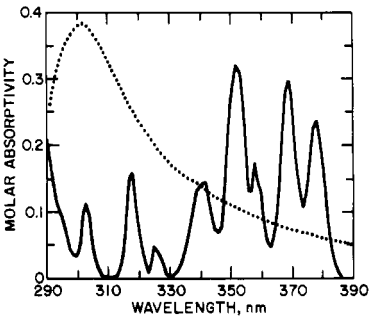


FIGURE 5: Spectral overlap of the emission spectrum [(···) $\lambda_{ex}$ = 275 nm] of NBS-treated W57L GS and absorption spectrum (—) of a 1:1 Tb(III)–EDTA complex. $\epsilon_{353nm}$ = 0.3 $M^{-1}$ $cm^{-1}$.

The overlap in the fluorescence spectrum of NBS-treated W57L GS and the absorption spectrum of the Tb(III)–EDTA complex are illustrated in Figure 5. The Tb(III)–EDTA absorption spectrum was used due to the weak absorption properties of Tb(III) which precludes use of the spectrum of Tb(III) bound to GS. The overlap integrals estimated from eq 3 of McNemar et al. (1991) were the same for all three enzymes and equal to $1.0 \times 10^{-19}$ $cm^6$ $mol^{-1}$.

Tb(III) fills the two metal binding sites of GS sequentially, and the resulting Tb(III) luminescence is easily measurable. The Tb(III) luminescence spectrum of NBS-treated W57L GS shows four peaks at 488, 544, 583, and 618 nm. The emission spectrum is nearly identical with that shown for Tb(III)–GS in Figure 1 of the accompanying manuscript, except that the band at 488 nm has a greater intensity for the NBS-treated sample (McNemar et al., 1991). These emission bands are characteristic of Tb(III) luminescence (Horrocks & Sudnick, 1981). In order to calculate the efficiency of energy transfer, peak areas of Tyr fluorescence and Tb(III) luminescence were determined as GS was titrated with Tb(III). The $A_{Tb(III)}$ was corrected for the fractional occupancy of each metal ion binding site. The values of $E$ were then calculated by eq 4 of McNemar et al. (1991), and the results are summarized in Table I.

The critical distance for 50% energy transfer, $R_0$, was calculated from eq 2 of McNemar et al. (1991). The orientation factor, $\kappa^2$, was taken as $^2/_3$, the value for an isotropic donor and an isotropic acceptor. According to Horrocks and Collier (1981), it is unlikely that the true value of $\kappa^2$ deviates from this value sufficiently to cause a significant error in the

Table II: Summary of the Crystal Structure Distances (in Å) between Various Tyr Residues and the Two Metal Binding Sites from *S. typhimurium Glutamine* Synthetase

| Tyr residue | $n_1$ | $n_2$ |
|---|---|---|
| 179 | 7.6 | 11.2 |
| 238 | 11.3 | 16.2 |
| 326 | 16.2 | 15.0 |
| 100 | 17.2 | 13.9 |
| 319 | 19.5 | 20.2 |
| 334 | 21.8 | 15.7 |
| 397 | 24.0 | 20.2 |

calculation of $R_0$. A value of 1.36 was used for the refractive index $n$. The Tyr–Tb(III) distance was then estimated by eq 1 of McNemar et al. (1991), and the resulting $R_0$ values and distances are also summarized in Table I. The average distances of Tyr residue to $n_1$ and $n_2$ sites of GS are 10.7 and 11.2 Å, respectively.

## Discussion

Tyrosine emission in proteins is usually small, and a number of reasons have been suggested for the lack of Tyr emission (Lakowicz, 1983; Edelhoch et al., 1968; Wever & Rosenheck, 1964; Cowgill, 1976). Tyr fluorescence can be quenched by nearby charged and uncharged amino groups and by charged carboxyl or neutral carboxylate groups. Furthermore, energy transfer is also expected to be efficient since the critical distance for Tyr–Trp transfer is about 14 Å (Lakowicz, 1983), a size comparable to the diameter of many proteins. Finally, the hydroxyl group could be hydrogen-bonded to nearby peptide bonds, and such hydrogen-bonded phenols are nonfluorescent (Cowgill, 1976). As a result, wild-type and W158S glutamine synthetases exhibit only a very weak Tyr emission even though there are 17 Tyr residues per subunit (Colombo & Villafranca, 1986). The observation of an intense Tyr emission in W57L GS is unexpected. This fact may indicate that Trp-57 plays an important role in quenching the nearby Tyr residues probably through Förster-type energy transfer, although quenching by indirect involvement of Trp-57 in changing the local environment experienced by the nearby Tyr residues is also possible. No significant increase in Tyr emission was observed upon replacement of Trp-158 by serine in W158S GS. Thus, Trp-158 does not seem to have a significant effect in quenching the nearby Tyr residues.

The Tyr–Tb(III) distances obtained from sensitized Tb(III) luminescence experiments for NBS-treated glutamine synthetase and its mutants are in good agreement. These results indicate that the Tyr emission in the three enzymes may originate from the same Tyr residue(s). The Tyr–Mn(II) crystal structure distances from *S. typhimurium* GS for several Tyr residues located near the active site are listed in Table II (Almassy et al., 1986). Because the amino acid sequences of *E. coli* and *S. typhimurium* GS differ by only 10 residues (Colombo & Villafranca, 1986; Janson et al., 1986), the two enzymes likely have very similar three-dimensional structures. The efficiency of Förster energy transfer is inversely proportional to the sixth power of the donor–acceptor distance; therefore, the closest Tyr residue(s) will predominate in the energy transfer. Using the crystal structure coordinates, it is evident from Table II that Tyr-179 will be the main source for energy transfer while Tyr-238 and Tyr-100 may have some minor contributions to the engergy transfer to metal ions bound to the $n_1$ and $n_2$ sites, respectively. The average distance from Tyr to the second Tb(III) binding site of GS for the three enzymes determined from the sensitized Tb(III) luminescence experiments is 11.2 Å which is in excellent agreement with the distance from Tyr-179 to the $n_2$ sites of GS shown in Table II. The average distance from Tyr to the first Tb(III) binding site of GS is 10.7 Å which is longer than the crystal structure distance of 7.6 Å from Tyr-179 to the $n_1$ site. The discrepancy may result from a slightly different conformation of GS in solution and in the crystal and/or slightly different Tb(III) binding sites and Mn(II) binding sites due to an additional protein ligand present when Tb(III) is present.

The p$K_a$ values for the ionization of Tyr obtained from fluorescence titrations are the same for both W57L GS and NBS-treated W57L GS ($pK_a = \sim 10$). This suggests that chemical modification of Trp residues by NBS does not affect the ionization of Tyr residues. The p$K_a$ value for the ionization of Tyr for NBS-treated W158S GS was also determined from fluorescence titration to be 10.2 (data not shown) which is similar to the results from the other mutant. The coincidence in the p$K_a$ and the Tyr–Tb(III) distances for both mutants indicates that the same Tyr residue (Tyr-179) may be responsible for the Tyr emission in both enzymes.

According to the crystal structure of *S. typhimurium* GS (Almassy et al., 1986), Tyr-179 is about equidistant to Trp-57 and Trp-158 (~18 Å). However, Tyr emission in NBS-treated W158S GS is only about 20% as intense as that of NBS-treated W57L GS [$\phi_{Tyr}$(W158S)/$\phi_{Tyr}$(W57L) ~ 20%]. In other words, Trp-57 is much more effective in quenching the emission of Tyr-179 than Trp-158. It is possible that Trp-158 has an unfavorable orientation for energy transfer ($\kappa = 0$ if the indole ring of Trp is perpendicular to the phenol ring of Tyr and no Tyr–Trp energy transfer will be expected) and is thereby a poor quencher. In addition, an intense Tyr emission was observed when Trp-57 was replaced by leucine (as in W57L) while modification of Trp-57 by NBS (as in NBS-treated W158S) exhibited a much weaker Tyr emission. This may indicate that oxidized Trp is also an effective quencher for Tyr emission. In fact, modification of Trp produced a new absorption band at 310 nm (Ohnishi et al., 1980) which overlaps significantly with the Tyr fluorescence spectrum centered at 303 nm. Therefore, effective quenching of Tyr emission by oxidized Trp is not unexpected.

Tyr residues have been reported to also react with NBS, but at a much lower rate. Ohnishi et al. (1980) have studied the relative reaction rates of Trp and Tyr with NBS in model compounds by stopped-flow methods and found that the reaction rate of Tyr is about $10^3$ times slower than that of Trp. It is possible that Tyr residues in GS might be partially modified especially when excess NBS is needed to completely oxidize the Trp residues (as in wild-type GS). Upon complete modification of free L-Tyr by NBS, Tyr emission at 303 nm disappears, and a very weak fluorescence band with an excitation maximum of 367 nm and an emission maximum of 460 nm is produced (data not shown). The fluorescence of oxidized Tyr is much weaker than that of oxidized Trp. Partial modification of Tyr residues will result in a smaller Tyr emission and hence a smaller quantum yield. However, the observation of a smaller value for $\phi_{Tyr}$ is not likely to cause a significant error in the determination of Tyr–Tb(III) distances. According to eq 1, 2, and 4 of McNemar et al. (1991) (with Tyr substituted for Trp), $r^6$ is proportional to $\phi_{Tyr}[(1/E) - 1]$, the efficiency of energy for Tyr–Tb(III) is $10^{-4}$ to $\sim 10^{-5}$ (Table I), and $1/E \gg 1$. Therefore, $r^6$ is proportional to $\phi_{Tyr}/E$ or $A_{Tyr}(\phi_{Tb(III)}/A_{Tb(III)})$. Because a smaller Tyr emission will also cause a corresponding decrease in Tb(III) luminescence, the ratio $A_{Tyr}/\phi_{Tb(III)}$ will be unchanged. As a result, the determined Tyr–Tb(III) distances will not be affected significantly by the variation in $\phi_{Tyr}$.

**Registry No.** GS, 9023-70-5; L-Tyr, 60-18-4; Tb, 7440-27-9.

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