

Direct Kinetic Evidence for Triplet State Energy Transfer from *Escherichia coli* Alkaline Phosphatase Tryptophan 109 to Bound Terbium*

(Received for publication, March 29, 1995, and in revised form, June 20, 1995)

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The addition of excess Tb³⁺ to metal-depleted *Escherichia coli* alkaline phosphatase results in enhanced luminescence from enzyme-bound terbium, which increases with sample deoxygenation and exhibits a tryptophan-like excitation spectrum. Following pulsed excitation at 280 nm, the time-resolved terbium emission shows a negative prefactor associated with a submillisecond rise time, which is independent of the concentration of dissolved oxygen. The absence of a build-up phase and similarity in lifetime in the decay kinetics of directly excited (488 nm) terbium allows for the assignment of the submillisecond component in the 280 nm excited sample to bound terbium. The results of the steady state and time-resolved experiments suggest that the time evolution of alkaline phosphatase-bound terbium emission is determined by energy transfer ($k_{ET} \sim 360$ and 120 s^{-1}) from the triplet state of tryptophan to terbium followed by terbium decay. This model is based on the observations that 1) the tryptophan phosphorescence lifetime (previously assigned to Trp¹⁰⁹) corresponds to the longer component of the terbium emission and 2) the long-lived emission is enhanced, as is the Trp¹⁰⁹ phosphorescence, by deoxygenation. An energy transfer mechanism involving the Trp¹⁰⁹ triplet state is shown to be inconsistent with a dipole-dipole process and is best understood as a through-space electron exchange over a donor-acceptor distance of 9–10 Å.

Metalloenzyme research has been greatly aided by the isomorphic replacement of intrinsic and spectroscopically silent metals (*i.e.* Ca²⁺, Mg²⁺, and Zn²⁺) with optical and magnetic probes such as Mn²⁺, Co²⁺, Cd²⁺, Eu³⁺, and Tb³⁺. Because of its similar size and preference for strong oxygen donor groups as ligands, the use of the extrinsic luminescent probe Tb³⁺ as a replacement for Ca²⁺ is particularly well established (1–4). For the typical concentrations used in terbium-protein systems, $<10^{-3} \text{ M}$, the emission of free terbium in solution is generally not observed following direct UV excitation with conventional sources because of its low extinction coefficient ($\epsilon_{285} \approx 0.4 \text{ M}^{-1} \text{ cm}^{-1}$ for Tb³⁺ complexes of diethylenetriaminopentaacetic acid

(5)), whereas, when bound to a protein and in proximity to photoexcited aromatic residues, energy transfer is very efficient (3). The enhanced terbium luminescence thus observed has been used analytically to determine binding constants (6, 7) and, most importantly, assuming that a Förster-type dipole-dipole energy transfer mechanism is established, to extract intraprotein donor-acceptor pair distances (for example see Refs. 8 and 9).

Although enhanced terbium luminescence has found extensive use in metalloenzyme studies, the nature of the energy transfer mechanism and the identification of the molecular donor states involved is far from clear. For some terbium-substituted metalloenzymes, energy transfer has been convincingly established to proceed by way of a long-range nonradiative transfer from protein aromatic singlet states (5, 10). At shorter donor-acceptor distances, however, a Dexter exchange mechanism is suggested (2). The observation of increased terbium luminescence following deoxygenation of terbium containing samples of synthetic peptides (4) and several calcium-binding proteins (11) provides strong evidence that protein triplet states may be involved. Indeed, for nonbiological systems the involvement of aromatic triplet states as donors has long been known (12–15). A recent detailed investigation (16) has considered the mechanisms of dipole-dipole, electron exchange, superexchange, external heavy atom effect, and electron transfer in the interpretation of fluorescence quenching of one to one lanthanide complexes of an indolyl-EDTA¹ derivative where the metal ion is held in a cofacial geometry about 6 Å from the center of the indole ring. It was found for terbium complexes that the majority of the observed energy transfer derived from the indole triplet state and was not dipole-dipole in nature. Of particular relevance to the work reported here is the kinetic study of terbium luminescence complexed to elastase (17), where the observation of an oxygen-sensitive growth of emission following UV excitation was interpreted as evidence of energy transfer from a triplet state donor via electron exchange. Whether or not these results are general or specific for the terbium-elastase system is open to question.

In the current work we extend the above observations implicating aromatic triplet states in the enhanced luminescence of terbium-protein complexes by using a protein that is well characterized with respect to tryptophan room temperature phosphorescence (RTP). For this purpose the metalloenzyme *Escherichia coli* alkaline phosphatase (AP) was chosen. Of its three tryptophan residues per subunit, Trp¹⁰⁹, Trp²²⁰, and Trp²⁶⁸,

* This research was supported by Grant AG09761 from the National Institute on Aging and by Contract N00014-91-J-1938 from the Office of Naval Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: indolyl-EDTA, *S*-N-[2-[bis(carboxymethyl)amino]-3-[3-indolyl]propyl]-N-(2-carboxymethyl)glycine; AP, *E. coli* alkaline phosphatase; holoAP, AP with a full complement of metals as found in the native state; apoAP, metal-depleted AP; TbAP, apoAP in the presence of excess terbium; RTP, room temperature phosphorescence.

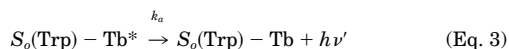
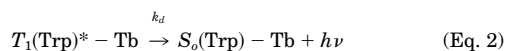
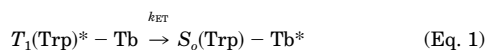
only Trp¹⁰⁹ shows RTP (18, 19) with a remarkably long (2 s) lifetime in extensively deoxygenated samples. Given the proximity of Trp¹⁰⁹ to the AP metal-binding sites, we anticipated its phosphorescence to be useful in elucidating the nature of energy transfer to AP-bound terbium (TbAP). We note that a recent report (20) suggests that only the singlet state of Trp¹⁰⁹ plays a role in enhancing the luminescence from TbAP. In the present work we report direct kinetic evidence for the enhanced luminescence in TbAP originating at least in part via energy transfer from the triplet state of Trp¹⁰⁹. Our investigation broadens the important conclusions of the terbium-elastase study and highlights the need for caution in the application of the Förster equation for distance determinations in terbium-substituted metalloenzymes.

MATERIALS AND METHODS

AP was purchased from Sigma (Type III-S) as a suspension in 2.5 M ammonium sulfate and exchanged into 10 mM Tris, pH 8.0, the buffer in which all spectroscopic measurements were performed. Protein concentrations were determined spectrophotometrically with a Shimadzu UV-260 or Cary 2400 UV-VIS based on $A_{278}^{0.1\%} = 0.72$ (21) and a molecular weight of 94,058 (22). Metal-depleted AP (apoAP) was prepared using the method of Bosron *et al.* (23) using the chelating agent 8-hydroxyquinoline-5-sulfonic acid. Atomic absorption analysis (Perkin-Elmer 7000) of apoAP found less than 0.01 g atom of zinc and 0.01–0.02 g atom of magnesium/mol of protein. ApoAP activity (24) was found to be less than 1% of native AP (holoAP), consistent with complete metal removal. Terbium-substituted AP was made by slowly adding, with stirring, an appropriate amount of a concentrated stock solution of TbCl₃ (Aldrich). Prior to spectroscopic measurements in the absence of oxygen, the capped sample cuvette was allowed to attain equilibrium with a continuous stream of purified argon (25). The oxygen content of an air saturated solution is taken to be 260 μ M (26).

Steady state emission spectra were recorded using a SPEX Fluorolog II spectrofluorimeter. Time-resolved phosphorescence measurements and decay analysis were made using instrumentation and methods described previously (27). Spectra were not corrected for photomultiplier wavelength response. The UV excitation source used for the time-resolved experiments was the frequency-doubled output of a neodymium ion (Nd³⁺) doped yttrium aluminum garnet (Y₃Al₅O₁₂) laser pumped dye laser. Data were collected using photon counting techniques in which the count rate was kept well below the instrumental bandwidth to prevent counting errors. For experiments where direct excitation of terbium was required, an argon ion laser operating at 488 nm was used. An optical chopper with a small aperture running at 48 Hz was used to produce a short pulse (60–80 μ s) from this continuous wave source.

Decay kinetics of enhanced terbium luminescence were analyzed using a simple model that assumes irreversible energy transfer from the excited triplet state of Trp¹⁰⁹ $T_1(\text{Trp})^*$ to terbium-bound AP:



where k_d and k_a are rates for the excited state deactivation of the donor $T_1(\text{Trp})^*$ and acceptor Tb^* , respectively, in the absence of energy transfer, k_{ET} . The rate equations for the de-excitation of $[T_1(\text{Trp})^*]$ and $[\text{Tb}^*]$ are:

$$d[T_1(\text{Trp})^*]/dt = -(k_d + k_{\text{ET}})[T_1(\text{Trp})^*] = -\Gamma_d[T_1(\text{Trp})^*] \quad (\text{Eq. 4})$$

$$d[\text{Tb}^*]/dt = k_{\text{ET}}[T_1(\text{Trp})^*] - k_a[\text{Tb}^*] \quad (\text{Eq. 5})$$

which are solved to give

$$[T_1(\text{Trp})^*](t) = D_0 \exp\{-\Gamma_d t\} \quad (\text{Eq. 6})$$

$$[\text{Tb}^*](t) = C \exp\{-\Gamma_d t\} + (A_0 - C) \exp\{-k_a t\} \quad (\text{Eq. 7})$$

where the concentration C is a collection of time-independent terms,

$$C = (D_0 k_{\text{ET}})/(k_a - \Gamma_d) \quad (\text{Eq. 8})$$

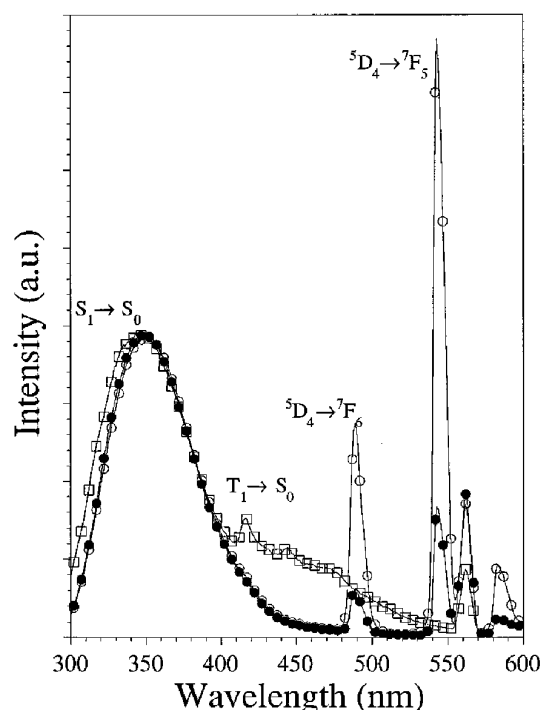


FIG. 1. Total luminescence spectra of 4 μ M apoAP + 80 μ M Tb³⁺ in 10 mM Tris, pH 8.0, observed at 1.8 nm resolution using 280 nm excitation with a 3.6 nm band pass. Filled circles (●) refer to an air-equilibrated sample; open circles (○) refer to a deoxygenated sample. The spectrum of the air-saturated and deoxygenated samples have been normalized with respect to the integrated fluorescence from 300 to 450 nm. Also shown under identical conditions is the total luminescence spectra of deoxygenated holoAP (□). The emission of free Tb³⁺ under the same experimental conditions is indistinguishable from baseline at these instrumental settings.

D_0 and A_0 can be respectively identified with the initial Trp¹⁰⁹ triplet state population, $[T_1(\text{Trp})^*]_0$ (following intersystem crossing from $S_1(\text{Trp})$), and the initial excited terbium population, $[\text{Tb}^*]_0$, which arises either through direct excitation at 280 nm or via energy transfer from one or more fast decaying protein excited state(s). Note that for $A_0 = 0$ the prefactors associated with decay rates k_a and Γ_d have equal and opposite signs, with the larger decay rate always having the negative amplitude. In practice, Equation 7 must be modified (as discussed below) to account for heterogeneity of the system.

RESULTS

Luminescence—The replacement of the native AP metals with Tb³⁺ results in terbium emission when the sample is excited at 280 nm as shown in Fig. 1. Terbium absorbs only very weakly at 280 nm (5) relative to protein aromatic groups and is only marginally directly excited under the conditions used here. Furthermore, the addition of 80 μ M Tb³⁺ to a 4 μ M solution of holoAP did not generate any appreciable terbium emission. It is clear from these experiments that terbium specifically binds to apoAP and that the enhanced luminescence arises from sensitized excitation, likely by energy transfer from phosphatase aromatic residues. Consistent with this statement is the observation of a tryptophan-like excitation spectrum of bound terbium (data not shown).

Deoxygenation of the TbAP sample leads to a 4–5-fold enhancement of terbium emission (Fig. 1). Previously it was suggested (4) that increases in the emission of terbium complexed to model calcium-binding peptides following deoxygenation were caused by sample precipitation (*i.e.* an artifact of the deoxygenation procedure). We note that the enhanced terbium emission observed here following deoxygenation cannot be ascribed to protein aggregation. This is clearly demonstrated by the similar magnitude of elastic scattering seen at 560 nm

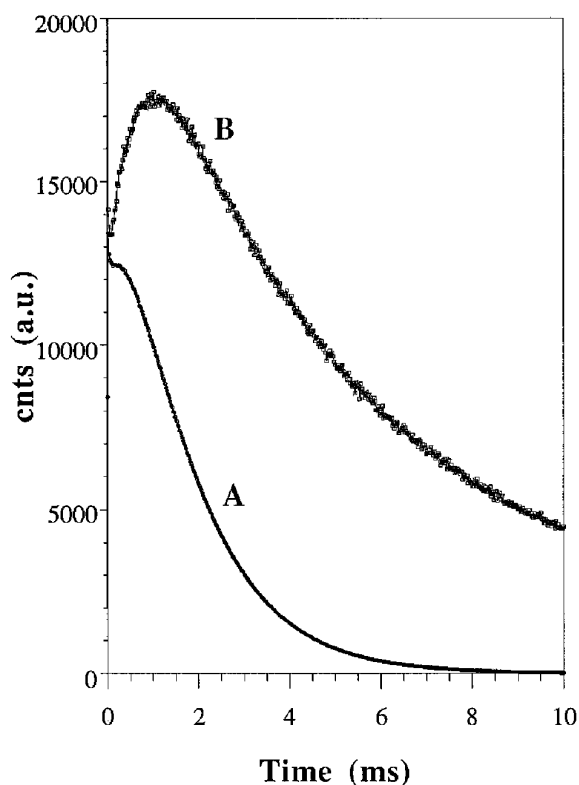


FIG. 2. Terbium emission transient observed at 544 nm following pulsed excitation at 280 nm. A, air-equilibrated solution. B, deoxygenated sample.

(second order of the 280 nm excitation) under the two conditions (Fig. 1).

Also shown in Fig. 1 is the total luminescence spectrum of holoAP under deoxygenated conditions. The phosphorescence ($T_1 \rightarrow S_0$) of Trp¹⁰⁹, the only AP residue that shows RTP (18, 19), is clearly shown to arise around 415 nm. When terbium is substituted for the native AP metals the phosphorescence is quenched.

Decay Kinetics—Because the emission of terbium in solution is insensitive to the presence of oxygen (11), the induced sensitivity upon binding to AP coupled with the excitation spectrum strongly suggest that the emission is partially sensitized by triplet state energy transfer from an AP tryptophan residue. When excited at 280 nm, the terbium emission shows an oxygen insensitive submillisecond lifetime associated with a negative prefactor (Equations 7 and 8) and oxygen-dependent long decay components associated with positive prefactors (Fig. 2). The decay transients require three lifetime components to account for heterogeneity in k_{ET} . For both deoxygenated and aerated samples, we find a single luminescent rise time and two oxygen-dependent decay times. The functional form used, justified below, to fit the data is given by: $[Tb^*](t) = C_1 \exp(-\Gamma_{d1} t) + C_2 \exp(-\Gamma_{d2} t) + (2A_0 - C_1 - C_2) \exp(-k_a t)$ where $\Gamma_{di} = k_d + k_{ETi}$ for $i = 1, 2$ and the values for the various parameters are given in Table I. Judged by their oxygen sensitivity, by the lifetime that is orders of magnitude longer than the nanoseconds expected from singlet state decay, and by their steady state excitation spectrum, we assign the longer lifetime components, Γ_{d1} and Γ_{d2} in the above equation, to an alkaline phosphatase tryptophan triplet state. To verify this statement and identify the specific tryptophan involved, we have monitored the quenched Trp¹⁰⁹ phosphorescence in TbAP at 440 nm following 280 nm excitation (Fig. 3, A and C). The longer lived components were found to exhibit tryptophan-like phosphores-

TABLE I

Results of analysis of observed kinetic transients

For the TbAP sample the lifetime given is associated with either a positive amplitude, (+), or a negative amplitude, (−), in accordance with the equation given in the text. O₂ present refers to a sample oxygen concentration of 260 μM, whereas O₂ absent is a deoxygenated sample. λ_{Ex} , excitation wavelength; λ_{Em} , emission wavelength.

Sample	λ_{Ex}	λ_{Em}	τ O ₂ absent	τ O ₂ present
	nm	nm	ms	ms
TbAP	280	544	(−) 0.72	(−) 0.72
			(+) 2.8	(+) 1.3
			(+) 8.5	(+) 2.3
	280	440	(+) 9.0	(+) 2.0
			(+) 12.0	(+) 3.0
AP	280	440	(+) 0.43	
			(+) 0.93	
TbCl ₃	488	544	(+) 1950	(+) 0.2
				(+) 3.3

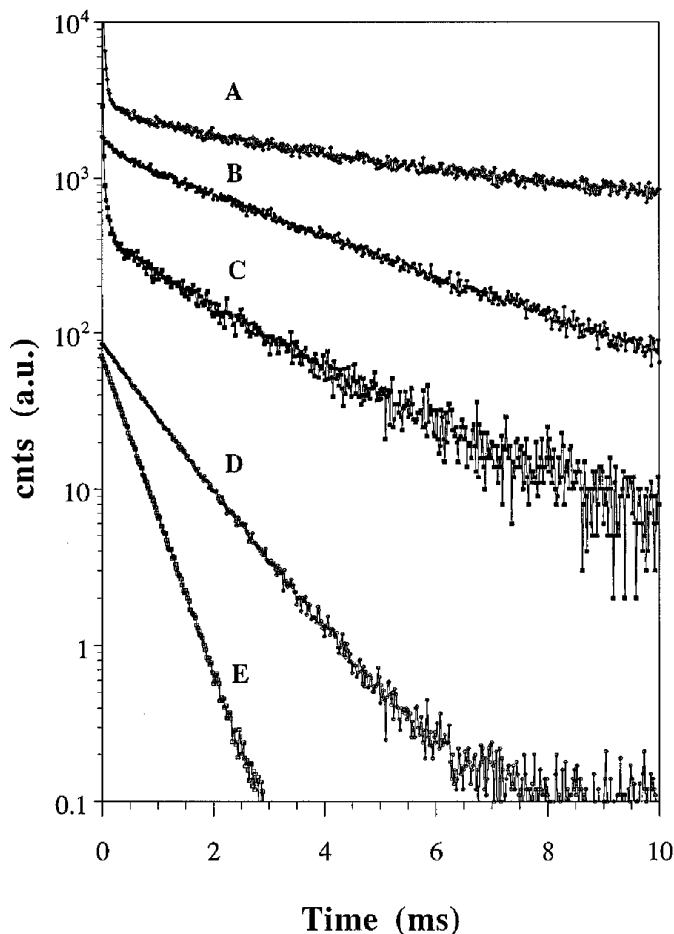


FIG. 3. Phosphorescence decay kinetics of Trp¹⁰⁹ in TbAP and AP as well as of terbium. A, B, and C show the phosphorescence of Trp¹⁰⁹ observed at 440 nm following 280 nm excitation of TbAP in deoxygenated (A) and air-equilibrated samples (C) and of native AP in an air saturated solution (B). Shown in D and E are terbium transients observed at 544 nm following pulsed excitation at 488 nm with TbAP (D) or a TbCl₃-containing solution (E). For D and E the decay rates are not affected by dissolved oxygen.

cence spectra (assigned to Trp¹⁰⁹) and have lifetimes of 9 and 12 ms in deoxygenated and 2 and 3 ms in oxygenated samples. The reasonable agreement between the long-lived components observed at 440 nm (Trp¹⁰⁹) and at 544 nm (bound terbium) for the deoxygenated and air-equilibrated samples indicate that they both arise from the Trp¹⁰⁹ triplet state. Also included for comparison in Fig. 3 is the transient (B) of native AP in an

air-saturated sample observed at 440 nm following 280 nm excitation. The major component of this decay has a lifetime of 3.3 ms. We note that the 3.3 ms lifetime of native AP in the air-equilibrated sample (260 μM O_2) compared with 2.0 s in deoxygenated solution yield a value for the rate constant of Trp^{109} phosphorescence quenching by oxygen of $1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, in excellent agreement with the literature values (28, 29).

A further check of our assignment of the 280 nm excitation-induced transient is afforded by observing the terbium decay at 544 nm following direct excitation of this cation into the weakly allowed transition at 488 nm. The results of this experiment (Fig. 3D) can be fit to 2 lifetimes, 430 (minor component) and 930 μs (Table I). The 430- μs lifetime is assigned to free $\text{Tb}^{3+}(\text{aq})$ because it is in good agreement with the 420 μs found for a TbCl_3 solution (Fig. 3E) and with literature values (30). Because the lifetime of terbium is linearly related to the number of hydroxyl oscillators in its ligand field (30, 31), the longer lifetime of terbium in TbAP relative to terbium free in solution is evidence for complexation. The notable absence of a decay component associated with a negative prefactor for directly excited terbium in TbAP is further proof of the participation of a protein aromatic residue in the excitation process serving as energy donor.

Inspection of Equation 7 according to the above decay component assignment shows that the decay associated with the negative prefactor arises from terbium (k_a in Equation 7), implying that C in our model is positive and therefore greater than A_0 . This is consistent with its increased contribution to the decay following deoxygenation (Fig. 2) because under these conditions k_d , and hence Γ_d , decreases.

DISCUSSION

The functional homodimer of AP contains two Zn^{2+} and one Mg^{2+} in each of two active sites (23). Catalytic activity in the native enzyme requires Zn^{2+} at site M1 (32). Although the role of the second Zn^{2+} (site M2) and Mg^{2+} (site M3) were once believed to be largely structural in nature (32), their close proximity to one another lends itself to the idea of a cocatalytic site (33).

The experiments presented here do not allow us to determine which metal-binding site contains the terbium(s) involved in triplet state energy transfer from Trp^{109} . Experiments where only one equivalent of terbium was added to apoAP (data not shown) still show the distinctive early rise of terbium luminescence upon pulsed excitation indicative of a triplet state donor. The analysis of the decay data again requires three lifetime components, one associated with the build-up and attributed to terbium decay and two with the decay of the enhanced emission fed by the donor tryptophan. We attribute the multiplicity of donor lifetimes in these experiments to a difference in terbium- Trp^{109} distances resulting in different energy transfer rates. This may occur when multiple terbium ions occupy distinct metal-binding sites or alternately when a single terbium residing at a single site adopts multiple distances with respect to Trp^{109} due to different protein conformations. The distances (obtained from the atomic coordinates deposited in the Brookhaven Protein Data Bank (34)) from the indole ring center (taken to be the midpoint of the $\text{C}_{\delta 2}$ - $\text{C}_{\epsilon 2}$ bond in the following discussion) to sites M1, M2, and M3 are 13.6, 9.7, and 9.4 Å. Below we show how the subtle differences in distance between Trp^{109} and terbium in M2 and M3 are consistent, when interpreted in terms of an exchange mechanism, with the observed multiple donor decay kinetics.

Long Terbium Lifetime—Strambini and co-workers (20) report that when terbium is added to nonphosphorescent apoAP (35) the lifetime of Trp^{109} is restored to a value close to that observed in holoAP (1.95 s) with an accompanying oxygen-

insensitive enhanced terbium emission. From these observations they concluded that terbium binds to site M1, thus restoring the phosphorescence of Trp^{109} , and that the enhanced luminescence is sensitized by a singlet state donor, there being no contribution from the Trp^{109} triplet state. These results are clearly in contradiction to our strong evidence for Trp^{109} triplet state involvement in energy transfer to bound terbium. Although the origin of this contradiction is not fully understood, it is not unreasonable to assume that metal contamination is responsible for the relatively strong Trp^{109} phosphorescence in the earlier TbAP study (20). A small portion of the AP sample, contaminated with zinc or another metal that does not act as a phosphorescence quencher, could explain the observation of a near 2-s lifetime, therefore obscuring contributions of Trp^{109} undergoing relatively rapid energy transfer to the bound terbium.

It should be noted that the 280 nm induced decays shown in Fig. 2 clearly have nonzero values at $t = 0$. Thus the contribution from either directly excited terbium or from energy transfer to terbium from fast decaying protein excited states, most likely singlet states, is significant.

Energy Transfer Mechanism—The rate of energy transfer is given by $k_{\text{ET}} = \Gamma_d - k_d$. Trp^{109} phosphorescence lifetime is little affected by exchanging AP metals with cations that show a low propensity for energy or electron transfer, such as Cd^{2+} (35), and therefore we assume k_d to be 0.5 s^{-1} upon terbium substitution, the value determined for the native enzyme in the absence of oxygen. The rates of energy transfer are found to be 360 and 120 s^{-1} . As an independent check we find k_{ET} to be 470 and 130 s^{-1} for the air-saturated TbAP sample, with $k_d = 300 \text{ s}^{-1}$ as determined above. The degree of correspondence between the two measurements is reassuring and demonstrates that k_{ET} is oxygen-independent.

Because the rate of energy transfer according to a dipole-dipole (Förster) mechanism is proportional to the overlap of the normalized donor emission and unnormalized acceptor absorbance (J), we anticipate that its contribution to energy transfer in TbAP will be small given the negligible absorbance of terbium. To verify this we have estimated the energy transfer rate using the Förster equation (36), $k_{\text{ET}}^{\text{dd}} = 8.785 \times 10^{23} \kappa^2 \eta^{-4} k_{\text{RAD}} R^{-6} J \text{ s}^{-1}$, where η is the refractive index of the medium (taken as 1.36), k_{RAD} is the radiative decay rate of the unquenched tryptophan triplet state (0.087 s^{-1} (37)), and the donor-acceptor distance (R) is taken to be 9.55 Å, an average of the two nearest metal-binding sites, M2 and M3. The orientation factor, κ^2 , is assumed to be 2/3. The overlap integral (J) was determined from the phosphorescence spectrum of AP and the absorbance spectrum of a standard TbCl_3 solution. With $J = 1.84 \times 10^{-20} \text{ M}^{-1} \text{ cm}^3$, we determine $k_{\text{ET}}^{\text{dd}} = 3.6 \times 10^{-4} \text{ s}^{-1}$, which is more than six orders of magnitude less than the experimental result. As anticipated, the triplet state is not significantly quenched via a dipole-dipole process, and therefore an exchange mechanism is indicated.

In electron exchange the rate of energy transfer is given by $k_{\text{ET}}^{\text{exc}} = (2\pi/\hbar) Z^2 J'$, where J' is the spectral overlap integral normalized with respect to both donor emission and acceptor extinction and Z is the exchange integral (38). Assuming hydrogenic wave functions, Dexter arrived at the approximation $k_{\text{ET}}^{\text{exc}} = K J' \exp(-2R/L)$, where K is a constant not related to experimental parameters and L is the average orbital radius involved in the initial and final states for the donor-acceptor pair separated by distance R .

As suggested above the exponential distance dependence of exchange is consistent with the observation of two donor lifetimes in TbAP if terbium ions in sites M2 and M3 act as independent quenchers. For example, assuming that K, J' , and

L are of similar magnitude for both sites and taking L as one (39), the difference in distance between the two sites to Trp^{109} (0.3 Å) translates into an expected ratio in rate of energy transfer of 2. This is in reasonable agreement with the experimental ratios of k_{ET} 360 and 120 s^{-1} (ratio = 3) and k_{ET} and 470 and 130 s^{-1} (ratio = 4) for deoxygenated and oxygenated samples, respectively. We note that such an analysis is inconsistent if the most distant site M1 and either M2 (ratio = 2400) or M3 (ratio = 4400) are considered. Thus if distance is the main determinant of k_{ET} , the contribution of terbium in M1 would not be observed in the present experiment, being instead overwhelmed by terbium in site(s) M2 and/or M3. Furthermore the above interpretation suggests that terbium is partitioned between sites such that the likely occupancy is TbAP (M1, M2) and TbAP (M1, M3), the binding of metal in M1 being a prerequisite for phosphorescence (35).

An alternative explanation for the multiexponential donor decay based on the presence of a single terbium acceptor is that small conformational heterogeneity exists in the TbAP sample. Given the distance sensitivity of the exchange mechanism, the postulated conformers would therefore show differences in Trp^{109} -terbium distance of 0.3–0.4 Å. It clearly will be necessary to conduct a more detailed study to determine the exact terbium binding-site distribution in this system.

It is interesting to compare our results with those obtained for the terbium-elastase system (17) in which energy transfer was assigned to an exchange process with a rate constant of 8300 s^{-1} , 17–70 times larger than that found for TbAP. From the atomic coordinates for porcine pancreatic elastase (40), we find a distance of 8.4 Å from the single metal binding site to the center of the putative energy donor Trp^{141} . Assuming, as above, that K and J' are of similar magnitude and $L = 1 \text{ Å}$ for both systems and using average donor-acceptor distances of 8.4 and 9.65 Å for terbium-elastase and TbAP, respectively, we calculate the rate of energy transfer in the terbium-elastase sample should be 12-fold larger than that in the TbAP sample. All things considered this is in good agreement with the experimental results. An additional consideration that may modulate the rate of electron exchange is the geometric orientation of the donor $^3(\pi\pi^*)$ wave functions, orthogonal to and electron-deficient in the indole plane. In AP both metal-binding sites M2 and M3 are elevated 7 and 10° , respectively, with reference to the center of the indole plane, whereas in elastase this angle to the calcium site is 35° . Thus a contributing factor to the different energy transfer rates observed may be an increase in favorable donor-acceptor orbital interaction in terbium-elastase relative to TbAP. Previous studies of exchange interactions in bichromophoric molecules (41) have demonstrated the importance of orientation of the interacting orbitals on k_{ET} .

Finally we note that Kirk and co-workers (16) have suggested that through-bond energy transfer with an exponential dependence on the number of interconnecting bonds is important in terbium-indolyl-EDTA complexes. Because the through-bond pathway between donor and acceptor is smaller in TbAP than in terbium-elastase, the above mechanism is not applicable, rather the energy transfer is best understood in terms of a through-space exchange mechanism.

We anticipate that the contribution of protein triplet states to enhanced terbium luminescence is a general phenomenon. Many proteins show RTP from buried tryptophan residues with lifetimes greater than $\sim 0.5 \text{ ms}$ (42), whereas those in which phosphorescence is not easily observed probably have triplet state lifetimes on the order of $20 \mu\text{s}$, the value observed for free

tryptophan in deoxygenated solution (43). Although tyrosine RTP has yet to be observed, the increased terbium emission in model tyrosine-containing peptides upon sample deoxygenation (4) suggests that its triplet state may also contribute to the enhanced luminescence process. Given the common occurrence of protein triplet states following photoexcitation, we reiterate the previously stated caution (2, 17) to investigators employing the Förster equation for the determination of intra-protein distances in terbium-substituted systems. An experiment comparing the enhanced emission of deoxygenated and air-equilibrated samples would in principle allow the researcher to gauge the contribution of protein triplet states to the enhanced luminescence.

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