Reactions of Excited-State Cytochrome *c* Derivatives. Delayed Fluorescence and Phosphorescence of Zinc, Tin, and Metal-Free Cytochrome *c* at Room Temperature†

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ABSTRACT: The decay mechanisms of the excited triplet state of porphyrin in porphyrin (metal-free) cytochrome *c*, zinc cytochrome *c*, and tin cytochrome *c* have been characterized by time-resolved emission spectra and decay kinetics. Delayed fluorescence provides a significant radiative pathway for triplet decay at room temperature for the cytochrome *c* derivatives and permits a sensitive measure of the triplet state. The lifetime of delayed fluorescence is identical with that of the phosphorescence, and the decay follows first-order kinetics. The intensity of delayed fluorescence is directly proportional to the laser flash intensity. These results indicate that the cytochrome derivatives exhibit E-type delayed fluorescence, which results from the repopulation of the excited singlet state by thermal activation of the triplet molecules. The activation energies of the delayed fluorescence are 2573 cm^{-1} or 7.35 kcal/mol for porphyrin, 2560 cm^{-1} or 7.32 kcal/mol for zinc, and 2213 cm^{-1} or 6.33 kcal/mol for tin cytochrome *c* derivatives. These values are less than those predicted by the observed energy differences between the singlet and the triplet states from the spectra, and this discrepancy may be due to the inability to estimate the true 0–0 energy gap. Exchange of hydrogens in porphyrin cytochrome *c* by deuterium resulted in an increase in its triplet lifetime from 9 to 14 ms at 77 K. This result has been interpreted to be due to the existence of vibrational coupling between the T and the S_0 states, which provides a route for deactivation of the porphyrin triplet state.

In native cytochrome *c* the excited states of the porphyrin are extremely short-lived due to effective coupling to the iron. Removal of the iron from the protein results in a derivative in which the covalently bound porphyrin exhibits luminescence; this luminescence is dramatically influenced by the core of the porphyrin, whether consisting of two protons or a metal cation. The luminescence can, therefore, provide an unique intrinsic optical probe for studies of electron transfer.

In this paper we examine reactions which the triplet state of iron-depleted derivatives of cytochrome *c* can undergo at room temperature. The excited triplet state is an interesting tool for the study of proteins because the lifetime of spin-forbidden processes are often longer than milliseconds; triplet states can, therefore, provide a spectroscopic probe on the millisecond time scale. Furthermore, the interactions that excited triplet and singlet states undergo with surrounding molecules have different probabilities because of the different spin multiplicity. For instance, in the singlet state dipolar interaction is spin allowed and highly probable, whereas electron exchange interactions become important for the triplet state (Turro, 1978). Consequently, different information concerning the environment can be obtained by using singlet and triplet excited states.

We have previously described the preparation and luminescence properties of metal-free and some metal derivatives of cytochrome *c* and their use in characterizing the interactions of cytochrome *c* with proteins and membranes (Vanderkooi & Erecinska, 1975; Vanderkooi et al., 1976, 1980; Glatz et al., 1979). The triplet state of zinc cytochrome *c* can be detected optically at room temperature (Dixit et al., 1981), and the anisotropy of phosphorescence of the porphyrin has been used to measure the motion of membrane-bound cytochrome *c* (Dixit et al., 1982).

In order to study reactions involving the triplet state, it is necessary to determine the factors affecting its formation and decay. Here we characterize long-lived emission from porphyrin (i.e., metal-free) cytochrome *c*, zinc cytochrome *c*, and tin cytochrome *c* in terms of delayed fluorescence and phosphorescence.

Materials and Methods

Type III and type VI horse heart cytochrome *c*, glucose, glucose oxidase (type VII), and catalase (bovine liver) were products of Sigma Chemical Co. (St. Louis, MO). Matheson Coleman & Bell (Norwood, OH) supplied 1,2-propanediol. Deuterium oxide was obtained from Wilmad Glass Co. (Buena, NJ). Airco Co. (Philadelphia, PA) supplied ultrapure argon. Deionized and distilled water was used for all buffers.

Metal-free cytochrome *c* and zinc cytochrome *c* were prepared by the reported procedures (Vanderkooi & Erecinska, 1975; Vanderkooi et al., 1976). To exchange the pyrrole hydrogens of porphyrin cytochrome *c* with deuterium, porphyrin cytochrome *c* was lyophilized and redissolved in deuterium oxide.

Emission from the triplet state was detected following laser flash excitation using the instrument described previously (Dixit et al., 1982). A Phase-R DL 1000 dye laser was used for excitation; lasing dye was 0.1 mM Rhodamine 575 (Exciton Chemical Co., Dayton, OH) in ethanol. The excitation maximum was 577 nm, and the excitation pulse length was about 1 μs. Temperature control of the sample was achieved by circulating water through the cell block. For measurement at 77 K, a cold-finger-type liquid nitrogen Dewar was used. Time-resolved emission spectra were plotted by taking the intensity of emission at a given time after the laser flash on the decay curve at every wavelength.

The detection of delayed fluorescence and phosphorescence from the porphyrin derivatives depends critically upon removing oxygen from the sample. This was accomplished essentially as described previously (Horie & Vanderkooi, 1981). Cuvettes with screw top caps were used. The sample was

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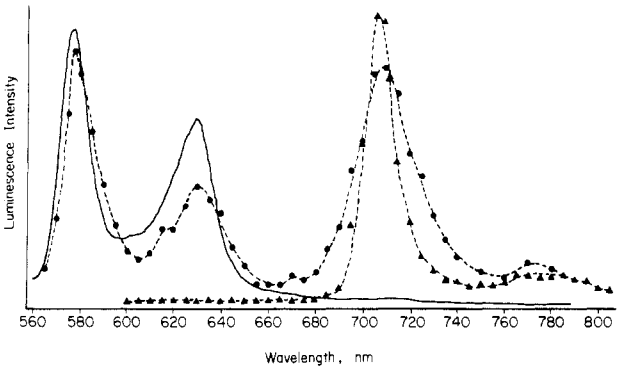

FIGURE 1: Prompt emission (–) and time-resolved delayed emission (---) of tin cytochrome *c* (25 μM) in 20 mM ammonium acetate, pH 7.0, at 20 °C (●) and at 77 K in the presence of 50% 1,2-propanediol (▲). Delayed emission spectrum were recorded 4.6 ms after the laser flash. The half-maximal band path was 2.5 nm. At 20 °C the sample also contained glucose oxidase and catalase for the purpose of removing the oxygen as described under Materials and Methods. For prompt emission spectrum, excitation wavelength 550 nm and half-maximal band-pass 2 nm. In this case oxygen was not removed from the sample.

bubbled with argon gas at a rate of 1 bubble/s for 30 min to 1 h in a glove bag. After bubbling, a coupled enzyme system was added in these final concentrations: glucose oxidase (75 μg/mL), catalase (12.5 μg/mL), and 0.3% glucose. The sample was bubbled with argon again for 10 min. The cuvette was then capped, and the junction was wrapped with multilayers of parafilm as a final precaution.

Fluorescence spectra were obtained with a Perkin-Elmer 650 spectrofluorometer equipped with a Hammamatsu 928 photomultiplier. For prompt fluorescence measurements, oxygen was not removed from the sample. Fluorescence lifetimes were obtained by using a single-photon counting lifetime instrument as previously described (Dixit et al., 1983). A Schott RG590 cutoff filter was used to isolate the emitted light from the excitation light.

Results

Delayed emission of tin cytochrome *c*, zinc cytochrome *c*, and free base porphyrin cytochrome *c*. Figure 1 compares prompt fluorescence and the emission spectra following several milliseconds after excitation of tin cytochrome *c* in aqueous liquid solution. The peak of long-lived emission at 710 nm corresponds to the expected 0–0 transition of the phosphorescence for tin cytochrome *c* (Vanderkooi et al., 1976). The spectrum of the delayed emission at lower wavelengths corresponds to that of the prompt fluorescence. Therefore, the delayed emission in these wavelength regions is identified as delayed fluorescence. In Figure 2, similar results are shown for zinc cytochrome *c*, where emission at 730 nm is identified as phosphorescence and emission with maxima at 595 and 650 nm is identified as delayed fluorescence. The delayed emission spectrum is shown at three temperatures. It can be noted that the ratio of delayed fluorescence to phosphorescence increases as the temperature increases. In Figure 3, the emission of porphyrin cytochrome *c* in deuterium oxide is shown. An attempt was made to measure the long-lived emission of porphyrin cytochrome *c* in water. The yield was low, and the lifetime was short, but both increased when deuterium oxide was substituted for water. (We discuss the mechanism for this later.) The small peak at 770 nm can be identified as phosphorescence and emission with maxima at 618 and 680 nm as delayed fluorescence.

The relative yield of delayed fluorescence to prompt fluorescence in the metalloporphyrin derivatives was estimated by measuring the fluorescence intensity before and after removal of oxygen. The intensity increased 7–10% for zinc cytochrome *c* and 4–8% for tin cytochrome *c*.

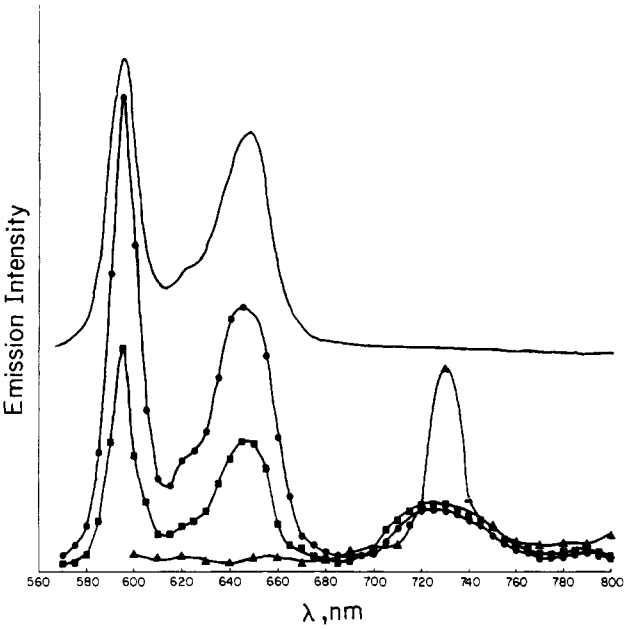

FIGURE 2: Prompt emission spectrum (—) and time-resolved delayed emission spectrum of zinc cytochrome *c* (40 μM) in 20 mM ammonium acetate, pH 7.0, at 20 °C (●), 5 °C (■), and 77 K in the presence of 50% 1,2-propanediol (▲). For prompt fluorescence, excitation wavelength 550 nm and half-maximal band pass 2 nm. For delayed fluorescence intensities were recorded 3 (▲) and 1.7 ms (●, ■) after the laser flash. In this case, the sample also contained the enzyme system described under Materials and Methods for removal of oxygen.

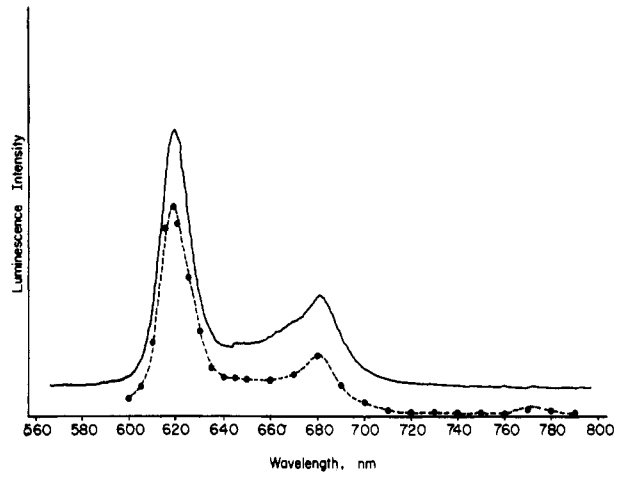

FIGURE 3: Time-resolved delayed emission spectrum (●) and prompt emission spectrum (—) of porphyrin cytochrome *c* (25 μM) in deuterium oxide at 20 °C. For prompt emission, excitation wavelength 540 nm and half-maximal slit width 2 nm. Delayed fluorescence represents intensity 7.6 ms after the laser flash. Half-maximal band path 2.5 nm. The sample also contained the enzyme system for removal of oxygen.

Delayed fluorescence can arise from several mechanisms. E type, originally found in eosin (Parker & Hatchard, 1961), results from the repopulation of the excited singlet state from the first triplet state by thermal activation:

$$T_1 \rightarrow S_1$$

Under thermal equilibrium, the decay profiles of the delayed fluorescence and phosphorescence are identical and exponential, and lifetimes are independent of concentration and excitation light intensity (Parker, 1967).

Other mechanisms for repopulation of the singlet state are possible. For instance, P type arises from a reaction between

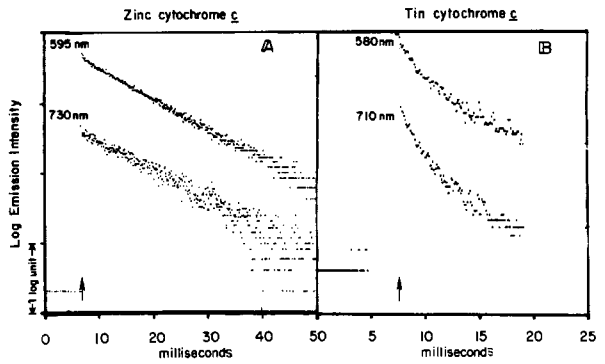

FIGURE 4: Decay of delayed fluorescence and phosphorescence of zinc cytochrome *c* (A) at 595 and 730 nm, respectively, and tin cytochrome *c* (B) at 580 and 710 nm, respectively, in 20 mM ammonium acetate, pH 7.0 at 20 °C. The arrows indicate the time of the laser flash.

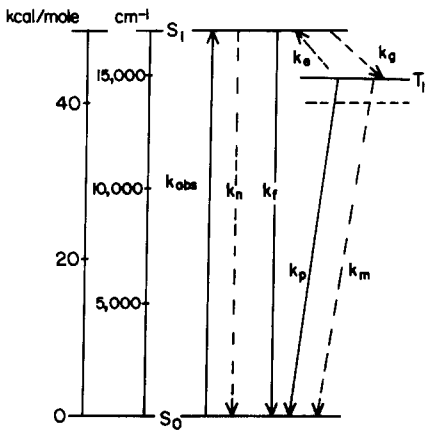

FIGURE 5: Energy level diagram: decay processes for the singlet and triplet states. Dashed lines represent nonradiative transitions, and solid lines represent radiative transitions.

two triplet states leading to one excited singlet and one ground-state singlet molecule:

$$T_1 + T_1 \rightarrow S_0 + S_1$$

In this case and other cases involving bimolecular mechanisms, the decay of delayed fluorescence and phosphorescence are not the same and are complex functions of concentration and light intensity.

We conclude that the delayed fluorescence observed from the cytochrome *c* derivatives arises solely from a thermal mechanism under the conditions of our measurement. This can be seen from the identical and first-order decay of the delayed fluorescence and phosphorescence for zinc and tin porphyrin derivatives (Figure 4). The lifetimes were independent of concentration of the cytochrome derivatives in the range 1–100 μM and were unchanged when the excitation intensity was reduced by a factor of 10 through use of a neutral density filter. Furthermore, the yield of emission was proportional to light intensity.

We can calculate the upper limit for a bimolecular rate constant for zinc cytochrome *c* in the case of the P-type mechanism. On the basis of the transient absorption at 460 nm after laser excitation, we estimate that we form about 5 μM triplet state out of 60 μM ground state. Since a 10% decrease in lifetime is measurable, we can estimate that the rate constant would be less than 2×10^6 L mol^{-1} s^{-1}. We note that this value is less than 10^{10} L mol^{-1} s^{-1} which was reported by Feitelson & Mauzerall (1982) for the bimolecular rate constant for the reaction between excited triplet octaethylporphyrin molecules in organic solvent. A likely reason for the absence of P-type interactions in the cytochrome *c* derivatives is that the porphyrin is surrounded by the polypeptide chain and contact between the porphyrins is thereby prevented.

Table I: Quantum Efficiencies, Lifetimes, Rate Constants, and Frequency Factors for the Processes in Zinc and Tin Cytochrome *c* at 20 °C[a]

	zinc cytochrome *c*	tin cytochrome *c*
Φ_f	0.055	0.012
Φ_t	0.9	0.95
Φ_p(77 K)	0.011	0.012
Φ_p(293 K)	0.0044	0.0043
Φ_e	0.0044	0.0014
τ_p(77 K)	0.035	0.014
τ_p(293 K)	0.014	0.005
k_f	3×10^8 s^{-1}	10^9 s^{-1}
k_p	71.4 s^{-1}	200 s^{-1}
k_e	1298 s^{-1}	5426 s^{-1}
k_g	4.9×10^9 s^{-1}	79.13×10^9 s^{-1}
k_n	2.46×10^8 s^{-1}	3.2×10^9 s^{-1}
k_m	13 200 s^{-1}	38 600 s^{-1}
A	9.98×10^8 s^{-1} [b]	3.88×10^8 s^{-1} [b]
	1.63×10^9 s^{-1} [c]	2.6×10^9 s^{-1} [c]

[a] Abbreviations: Φ_f = quantum yield of fluorescence; Φ_t = efficiency of triplet formation; Φ_p = quantum yield of phosphorescence; Φ_e = quantum yield of delayed fluorescence; τ_p = lifetime of phosphorescence; A = frequency factor. [b] Calculated from eq 10. [c] Calculated from $A = k_g/3$.

Kinetic Parameters for Delayed Fluorescence and Phosphorescence. The radiative and nonradiative transitions involving singlet and triplet states, when thermal repopulation of the excited singlet state is taken into account, are summarized in Figure 5, which defines all the rate constants. Following Parker (1967) and Feitelson & Mauzerall (1982), we define quantum efficiency of prompt fluorescence, Φ_f, as

$$\Phi_f = \frac{k_f}{k_f + k_n + k_g} \quad (1)$$

The quantum efficiency of triplet formation, Φ_t, is given by

$$\Phi_t = \frac{k_g}{k_f + k_n + k_g} \quad (2)$$

The quantum efficiency of phosphorescence, Φ_p, is

$$\Phi_p = \frac{k_p \Phi_t}{k_p + k_m + k_e} \quad (3)$$

and the quantum efficiency of delayed fluorescence, Φ_e, is

$$\Phi_e = \frac{k_e \Phi_f \Phi_t}{k_p + k_m + k_e} \quad (4)$$

The values of k_g and k_n can be determined from eq 1 and 2 and k_e and k_m from eq 3 and 4. The parameters used for the calculations are listed in Table I. Φ_f was determined from the relative fluorescence intensities in the emission spectra of the derivatives and the fluorescence lifetimes (Vanderkooi et al., 1976). Φ_t was assumed to be close to the experimentally determined value for zinc porphyrin (Gradyushko & Tsvirko, 1971; Dzhagarov et al., 1977) at 77 K. Φ_p at 77 K was determined from the relative intensity of phosphorescence to fluorescence. Φ_p at 20 °C was calculated from the lifetimes and intensities of phosphorescence at 20 °C and 77 K, assuming

$$\tau_{20°C}/\tau_{77K} = \Phi_{20°C}/\Phi_{77K} \quad (5)$$

Φ_e was obtained from the intensity of delayed fluorescence relative to the intensity of phosphorescence. (A correction was made to account for the dependence of photomultiplier response on wavelength.) $K_p(1/\tau_p)$ and $k_f(1/\tau_f)$ are obtained from the measured lifetimes of phosphorescence and fluorescence, respectively. The values of these constants are

Table II: Energetics of Emission of Porphyrin, Zinc, and Tin Cytochrome *c*

	spectral features								kinetic determination	
	$S_1 \rightarrow S_0$			$T_1 \rightarrow S_0$			$S_1 \rightarrow T_1$		$S_1 \rightarrow T_1$	
	nm	cm^{-1}	kcal/mol	nm	cm^{-1}	kcal/mol	cm^{-1}	kcal/mol	cm^{-1}	kcal/mol
porphyrin cytochrome *c*	618	16 180	46.27	770	12 990	37.15	3190	9.12	2573	7.35
zinc cytochrome *c*	595	16 806	46.06	730	13 698	39.17	3108	8.89	2560	7.32
tin cytochrome *c*	580	17 241	49.30	710	14 085	40.28	3156	9.03	2213	6.33

given in Table I.

Parker (1967) described the thermal activation of the triplet as

$$\Phi_e/\Phi_p = \Phi_f \tau_p A e^{-\Delta E/(RT)} \quad (6)$$

where τ_p is the radiative lifetime of the triplet state and A is the frequency factor. For porphyrin cytochrome *c* the phosphorescence intensity is low, and it becomes difficult to measure. We can obtain the energy of activation if we assume that k_g, the rate of S → T intersystem crossing, is not temperature dependent. [In the case of zinc cytochrome *c* this assumption is valid as can be seen from a plot of I_p/τ_p as a function of T (Figure 6, insert).] Then the energy of activation can be obtained from

$$\Phi_e = \Phi_t \Phi_f \tau A e^{-\Delta E/(RT)} \quad (7)$$

We have assumed that τ_p is independent of temperature and experimentally have observed that Φ_f is temperature invariant over the temperature range studied. The reciprocal rate of depletion of the triplet state is defined as τ. Φ_e and Φ_p are proportional to I_e and I_p, respectively, where I_e and I_p are the intensities of delayed fluorescence and phosphorescence, respectively. The energy of activation, ΔE, is obtained from a plot of ln (I_e/I_p) vs. $1/T$ or ln I_e vs. $1/T$ (Figure 6). The values for energies of activation for thermal repopulation are summarized in Table II.

The energies of activation obtained for the porphyrin derivatives are consistently lower than that obtained from the spectra. This can be due in part to the inability to estimate the true 0–0 energies. In fact, the emission peaks of both phosphorescence and fluorescence are broad (Figures 1–3). In addition, the 0–0 bands of absorption and fluorescence are not identical, indicating that the true 0–0 band of emission is shifted.

Under thermal equilibrium the ratio k_e/k_g is determined by the standard free energy difference between the S_1 and T_1 states. Since ΔE is an enthalpy of activation, the preexponent A (frequency factor) in eq 6 contains the entropy term

$$S_1/T_1 = e^{\Delta S/R - \Delta H/(RT)} \quad (8)$$

At thermal equilibrium, the ratio of the populations of the excited singlet and triplet states is

$$S_1/T_1 = k_e/k_g \quad (9)$$

Callis et al. (1977) have assumed that the populations and the difference in entropy between the S_1 and T_1 states arises from the electronic degeneracy of the triplet which is 3 times that of the singlet state. Combining eq 8 and 9 we have

$$k_e/k_g = \tfrac{1}{3} e^{-\Delta E/(RT)} \quad (10)$$

and

$$k_e = \tfrac{1}{3} k_g e^{-\Delta E/(RT)} = A e^{-\Delta E/(RT)} \quad (11)$$

We have calculated the frequency factor, A, by two ways—one by using the experimentally determined energy of activation (eq 10) and the calculated k_e value and the other

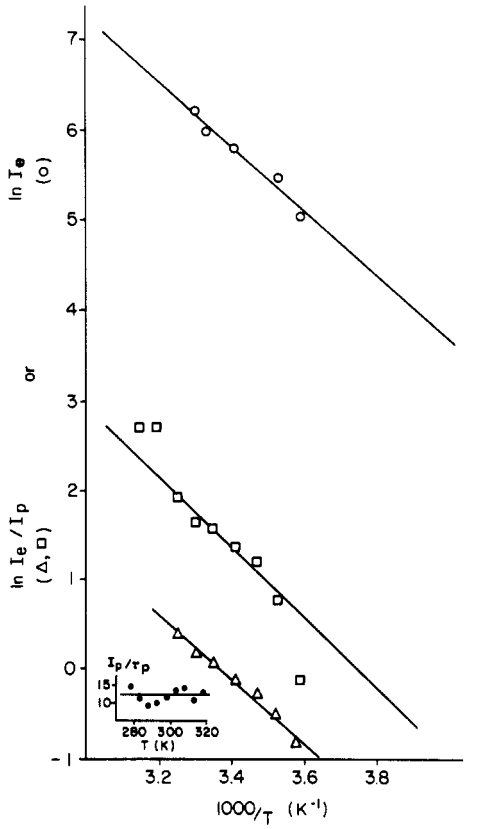

FIGURE 6: Energy of activation. I_e and I_p are the integrated intensities of delayed fluorescence and phosphorescence, respectively, τ is the lifetime of delayed fluorescence (ms), and T is the absolute temperature of 15 μM porphyrin cytochrome *c* (○) (I monitored at 577 nm), 15 μM zinc cytochrome *c* (□) (I at 595 nm and I at 730 nm), and 15 μM tin cytochrome *c* (△) (I_e at 580 nm and I_p at 710 nm) in 20 mM ammonium acetate, pH 7.0. The insert shows the plot of I_p/τ_p as a function of temperature for zinc cytochrome *c* where τ_p is the phosphorescence lifetime.

by using the calculated k_g value and from the relationship

$$A = k_g/3 \quad (12)$$

In the case of zinc cytochrome *c*, the two values (9.98×10^8 s^{-1} and 1.63×10^9 s^{-1}) agree well with each other. However, for the Sn derivative values (3.9×10^8 s^{-1} and 2.6×10^{10} s^{-1}) the difference is larger (Table II). Due to shorter lifetimes for the Sn derivative compared with those of zinc cytochrome *c*, the values of rate constants for the former are less accurately determined. In addition, the tin cytochrome *c* does not appear to exhibit a rigorously exponential decay. [The fast component could not be resolved by our instrument (Figure 4), and therefore the long component was used.]

Isotope Effect of the Emission of Porphyrin Cytochrome c. Hydrogen isotope effects on intersystem crossing rates are generally small for $S_1 \rightarrow T_1$ transitions but are larger for $T_1 \rightarrow S_0$ transitions (Turro, 1978). This is attributed to the small energy gap between S_1 and T_1 states (3000 cm^{-1} in the case of the porphyrin cytochrome *c* derivatives) and hence an already existing high probability of surface crossing. In contrast, there is a large gap between T_1 and S_0 states (13 000 cm^{-1} for

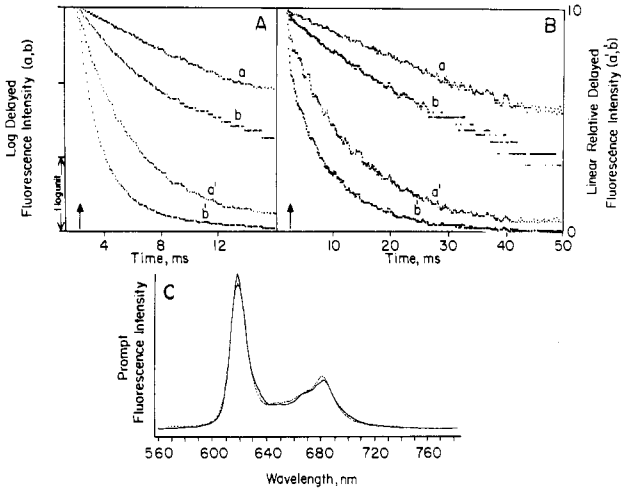

FIGURE 7: Decay of the triplet state and fluorescence emission spectra of porphyrin cytochrome *c*. Porphyrin cytochrome *c* concentration was 25 μM. (A) Medium was 20 mM ammonium acetate, pH 7.0, in water (a) and deuterium oxide (b). Emission wavelength 620 nm. (B) Medium was 20 mM ammonium acetate, pH 7.0, in water (a) and deuterium oxide (b). Emission wavelength 770 nm. Arrows indicate the laser flash. (C) Prompt fluorescence of 25 μM porphyrin cytochrome *c* at 20 °C in 20 mM ammonium acetate, pH 7.0 (—), and in deuterium oxide (---). Excitation wavelength 540 nm; half-maximal band-pass 2 nm.

cytochrome *c* derivatives; Table II), hence a slower rate and greater influence by vibrational coupling. As expected, the phosphorescence lifetime of porphyrin cytochrome *c* is dramatically affected by D_2O. The lifetime of porphyrin cytochrome *c* in water was 2.2 ms at 20 °C and 9 ms at 77 K; this compares with 4.8 ms at 20 °C and 14 ms at 77 K in deuterium oxide (Figure 7). A small difference in the vibrational structure of the porphyrin cytochrome *c* emission spectrum in water and D_2O at room temperature is evident, which is another indication of the coupling of the protons with the electronic transitions (Figure 7C).

No difference in the lifetimes of the triplet Zn and Sn derivatives was observed in D_2O and water. Since these derivatives have no exchangeable protons on the porphyrin ring, and the porphyrin is protected from the solvent by the polypeptide chain, this result is not unexpected.

Discussion

The history of the relationship between delayed fluorescence and phosphorescence goes back to the beginning of this century. Perrin (1929) originally thought that all long-lived emissions were due to thermal population from a metastable state. Jablonski (1935) is credited with recognizing that emission can occur from this state; this emission, phosphorescence, is red shifted from the fluorescence. Jablonski's scheme presented all the essential features of Figure 5. Delayed fluorescence has been observed in compounds similar to the porphyrin of cytochrome *c*, including zinc octaethylporphyrin (Feitelson & Mauzerall, 1982), chlorophyll *a* and *b* (Parker & Joyce, 1967), bacteriochlorophyll (Schenck et al., 1982), and palladium and platinum porphyrins (Callis et al., 1971).

We show here that the long-lived emission observed in the three cytochrome *c* derivatives is due to delayed fluorescence. This assignment is based upon the coincidence of the spectra with those of the prompt fluorescence (Figures 1–3). The decay times for the delayed fluorescence and phosphorescence were identical and independent of ground or excited triplet state concentrations. These results indicate that the delayed fluorescence is due to an E (thermal) rather than a P (collisional) repopulation of the excited singlet state. This conclusion is further supported by the experimentally determined activation energies that agree fairly well with the energy difference determined from the spectra between the S_1 and T_1 states (Table II).

The values of rate constants given in Table I account for various radiative and nonradiative processes in the case of zinc cytochrome *c*. In the case of tin cytochrome *c*, the rate constants are less accurately determined because of the shorter lifetime of its triplet state.

The yield of phosphorescence of porphyrin cytochrome *c* is very low, and therefore, difficult to measure. Deuterium exchange dramatically increases the intensity and lifetime of the triplet porphyrin cytochrome *c*. This observation is attributed to the vibrational coupling with the pyrrole hydrogens of the porphyrin ring; this coupling provides a major mode of deactivation of the triplet state porphyrin.

The observation of delayed fluorescence is significant for several reasons. Delayed fluorescence provides a more sensitive measure of the triplet state than the phosphorescence at room temperature for all three cytochrome *c* derivatives discussed. This is especially true in the case of free base porphyrin derivative where the singlet–triplet energy gap is small and the singlet quantum yield is high. An important conclusion of our work is that in studying the triplet state reactions of porphyrins, one has to consider the possible involvement of the singlet excited state above cryogenic temperatures.

Registry No. Cytochrome *c*, 9007-43-6.

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