Reactions of excited triplet states of metal substituted myoglobin with dioxygen and quinone

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ABSTRACT The triplet state absorption and phosphorescence of Zn and Pd derivatives of myoglobin were compared. Both metal derivatives exhibit long triplet state lifetimes at room temperature, but whereas the Pd derivative showed exponential decay and an isosbestic point in the transient absorption spectra, the decay of the Zn derivative was nonsingle exponential and the transient absorption spectra showed evidence of more than one excited state species. No difference was seen in triplet quenching by oxygen for either derivative, indicating that differences in the polypeptide chain between the two derivatives are not large enough to affect oxygen penetrability. Quenching was also observed by anthraquinone sulfonate. In this case, the possibility of long-range transfer by an exchange mechanism is considered.

INTRODUCTION

Myoglobin, a monomeric globular protein of mol wt 18,800 with the function to transport oxygen, is particularly well suited to serve as a model to study the intramolecular motions of proteins. Its structure is known, thereby permitting structural and functional correlations to be made. In addition, two optical methods are available to study protein dynamics: flash photolysis of the CO complex (Frauenfelder et al., 1979; Janes et al., 1988) and relaxation of excited states of luminescent derivatives (Jameson et al., 1984; Albani and Alpert, 1987; Austin and Chan, 1978).

Based upon these optical studies, it was proposed that oxygen and carbon monoxide can enter myoglobin with little barrier offered by the protein matrix. Whether large molecules can penetrate so easily is more problematic. The quenching of Zn-protoporphyrin triplet state with anthraquinone sulfonate (AQS) was suggested as a measure of diffusion through the structure of myoglobin by Barboy and Feitelson (1987).

In this work the triplet state properties of Zn and Pd derivatives of myoglobin and their interactions with quenchers are studied. Both metal derivatives exhibit long triplet state lifetimes at room temperature, but the Zn derivative of myoglobin is five-coordinated and out-of-plane (as is deoxy myoglobin), whereas Pd porphyrin is planar (as is oxy myoglobin). Therefore, quenching reactions of excited-state analogues of the two functional conformations of myoglobin can be studied. The high-emission yield and rapid intersystem crossing of Pd porphyrin luminescence allows us to compare steady-state intensities and time-resolved decay in the time scale from 100 ps to 1 ms.

MATERIALS AND METHODS

Materials

Mesoporphyrin, Pd-mesoporphyrin, Pd-coproporphyrin, and Zn-protoporphyrin IX were obtained from Porphyrin Products Inc. (Logan, UT). Myoglobin type I from horse skeletal muscle, catalase from bovine liver, glucose oxidase from *Aspergillus niger*, glucose, and glycerol were purchased from Sigma Chemical Co. (St. Louis, MO). AQS and Zn-acetate were obtained from Fisher Scientific Co. (Pittsburgh, PA). AQS was recrystallized from water before use. All other chemicals were the best analytical grade commercially available, used without further purification.

Preparation of samples

Zn mesoporphyrin was prepared by addition of Zn-acetate (0.5 M in water) to mesoporphyrin (10 mM in dimethylformamide), the mixture was kept at 60°C for 6 h, then the excess Zn-acetate was removed by adding water, and the precipitated Zn-MP was centrifuged, washed with water, and dissolved in dimethylformamide. Heme was removed from myoglobin by the method of Teale (1959) with minor modifications and was replaced either by Pd-mesoporphyrin, Zn-protoporphyrin, or Zn-mesoporphyrin. The porphyrin in dimethylformamide was added to the apoprotein dropwise with stirring followed by incubation for 45 min on ice. The ratio of porphyrin to protein was monitored by recording the protein absorption peak at 278 nm to the Soret peak. To insure that no aggregation of the porphyrin occurs and that all porphyrin is protein-bound, the porphyrin was added in substoichiometric ratio to the protein.

The metal-substituted samples, Pd-mesoporphyrin-myoglobin (Pd-MP-myoglobin), Zn-mesoporphyrin-myoglobin (Zn-MP-myoglobin), and Zn-protoporphyrin-myoglobin (Zn-PP-myoglobin), were stored in liquid N_2 until use. The samples were thawed, centrifuged, and passed through a small Sephadex G-25 column to remove possible denatured protein or free porphyrin. Because of the reported instability of the Zn-PP-myoglobin (Andres and Atassi, 1970), this derivative was stored no longer than 2 wk before use.

Oxygen was removed from the samples for transient absorption and phosphorescence measurements as follows. The protein was dissolved in a buffer containing 0.1 M NaCl, 50 mM Tris-HCl, pH 7.0 and 0.3% glucose. This buffer was initially degassed under an aspiration and

bubbled with argon. A small volume of solution containing glucose oxidase and catalase was added to the samples in the cuvette at a final concentration of 80 and 16 nM, respectively. All these procedures were performed under constant flow of argon to exclude air, the cuvettes were then closed with a quartz stopper and sealed with parafilm (American Can Co., Greenwich, CT). Stock solution of AQS was deoxygenated in the same manner. The samples were protected from exposure to light throughout these manipulations.

Instrumentation and analysis

Absorption spectra were obtained with a model 200 Perkin-Elmer Corp., Pomona, CA) and a model DMS-300 (Varian Associates, Inc., Palo Alto, CA) spectrophotometer. Fluorescence and phosphorescence spectra were recorded on a Perkin-Elmer LS-5 luminescence spectrometer. Delayed fluorescence and phosphorescence intensities were obtained with a Perkin-Elmer 650-10 S fluorescence spectrophotometer. Steady-state phosphorescence intensity was measured at 77 K with this instrument using a liquid nitrogen cold finger dewar (H. S. Martin, Vineland, NJ) that fit into the cell compartment.

Phosphorescence lifetimes were measured using the instrument described by Vanderkooi et al. (1987*a*). The phosphorescence lifetime values were obtained from decay profiles by analysis for exponential decay using the ASYSTANT$^+$ program (Macmillan Software Co., New York, NY). The transient absorption decay data were fitted to a model of multiexponential decay using an iterative fitting based on the method of minimizing residuals from a convolution of the instrument function with the decay model.

The triplet state absorption spectra were acquired using a transient absorption method. A Q-switched Nd:YAG laser (DCR-1A, Quanta Ray, Mountain View, CA) supplied the actinic light pulse, nominally 8 ns in duration and 7–8 mm in diameter. The actinic light (355 nm in our experiments) was focused onto the sample cuvette with a cylindrical lens, forming an ellipse 2 mm (H) by 7–8 mm (W). The probe light was generated by a Xe arc lamp (model L2359, Hamamatsu Corp., Middlesex, NJ) and was collimated and focused by a pair of off-axis paraboloidal mirrors. This light then hit a bifurcated randomized quartz fiber bundle (Fiberguide PCS fiber; 2 mm (H) × 1.5 mm (W) common end, 2 mm diameter bifurcated ends) producing two equal intensity probe beams which were directed onto the sample cuvette normal to that of the actinic beam. Each beam was separately collected by a set of quartz fibers (Fiberguide PCS; 2 mm diameter receiving ends, 2 × (1.2 mm (W) × 1.5 mm (H)): 1.5 mm separation) and imaged onto the entrance slits of a triple monochromator/flat-field spectrograph (Triplemate 1877, Spex Industries Inc., Edison, NJ). The probe light was detected with an intensified dual diode array system (DIDA-512 array, ST-120 controller, FG-100 fast pulser, Princeton Instruments Co., Trenton, NJ) which has a gate duration of 5 ns. The spectrograph normally employed 150 g/mm gratings, producing a simultaneously detected spectral window ~140 nm wide. The timing between the laser probe light and the intensifier gate was controlled with a four-channel digital delay generator (model 9650, Precision Instruments Co., Knoxville, TN) which has a GPIB link to the controlling computer (AT 386). The data from the ST-120 controller were directly accessed by the AT computer memory and stored on the hard disk. The raw data were then ratioed and converted to absorbance; the software also allows for spectral calibration and hard copy output.

Alternatively, the transient absorption spectrometer was converted into a time (rather than wavelength) based instrument with two simple modifications: the pulsed lamp assembly was replaced with a cw/pulsed lamp assembly (Cermax 300-W lamp (ILC Technology Inc., Sunnyvale, CA) and PS 300-1 power supply) and a side port on the spectrograph (normally used for alignment purposes) was activated with a swing-out mirror. The mirror reflected the probe beams onto a fast response dual photomultiplier assembly consisting of an adjustable exit slit, a 45° silvered wedge (which spatially separates the two probe beams) and a matched pair of fast response photomultipliers (model R1547, Hamamatsu Corp.). The photomultiplier current was directly converted into voltage through selected terminating resistors and the signal was monitored with a 400 Ms/s digital scope (model 4072, Gould, Inc., Cleveland, OH). The scope was controlled through a GPIB interface and the raw data are converted into an absorbance-time format with the software routine.

Subnanosecond and nanosecond decay parameters were measured using a time-correlated single photon counting instrument described by Holtom et al. (1986). Excitation was achieved using a mode-locked Ar ion laser, which pumped a dye laser. Frequency doubling was achieved through a KDP crystal.

RESULTS

Optical properties of metal-substituted myoglobins

On Fig. 1 the absorption (*A*), emission (*B*), and the transient absorption spectra of Zn-PP-myoglobin (*C*) are presented. The absorption peaks for Zn-PP-myoglobin are at 596, 553, 428, 356, and 277 nm, where the 277-nm peak represents the protein absorption. The emission peaks, measured in coincidence with the lamp flash, are at 595 and 647 nm and are identified as "prompt" fluorescence (Fig. 1 *B*). The long-lived luminescence of Zn-PP-myoglobin, measured at discrete times after excitation, shows E-type "delayed" fluorescence characterized by the same spectrum as the prompt fluorescence. Its intensity is ~2–3% relative to that of the prompt fluorescence. Phosphorescence emission is represented with a broad band centered around 735 nm (Fig 1 *B*, *solid line*). The absorption (Fig. 1 *A*) and the fluorescence emission (Fig. 1 *B*, *dotted line*) spectra are similar to those published by Albani and Alpert (1987).

The microsecond-to-millisecond transient absorption spectra after excitation are shown for Zn-PP-myoglobin (Fig. 1 *C*). The features of the transient absorption spectra are a depletion of the Soret band absorption, with a new absorption band at 460 nm. Likewise, the corresponding visible bands disappear, and a new absorption occurs around 600 nm (not shown). The spectra did not exhibit an isosbestic point. Furthermore, the decay kinetics to the ground state depended upon the wavelength of measurement. The decay profiles of the transient absorption at 460 nm (Fig. 2 *A*) and the recovery of the photobleaching at 428 nm (Fig. 2 *B*) show nonexponential behavior. The decay profiles could be fit by a double-exponential function with good correlation, as shown on Fig. 2 *C*.

In Fig. 3 the absorption (*A*), emission (*B*), and the transient absorption spectra (*C*) of Zn-MP-myoglobin

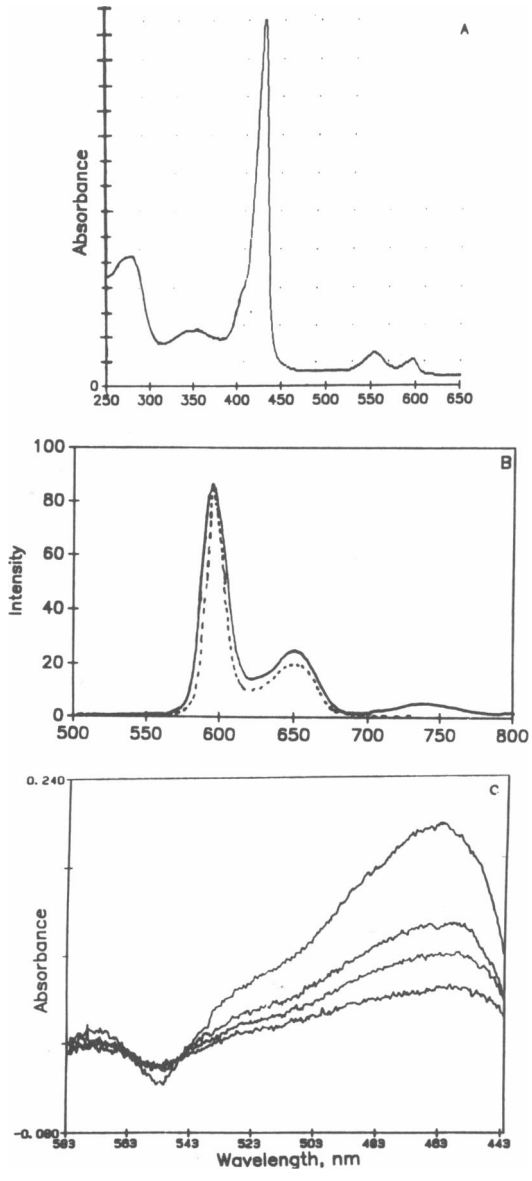

FIGURE 1 Optical properties of Zn-PP-myoglobin at room temperature. (*A*) Absorbance, (*B*) emission (Fluorescence [---], phosphorescence [—]) spectra, for both emission spectra the excitation wavelength was 428 nm. (*C*) Time-resolved transient absorption spectra of Zn-PP-myoglobin, the delay times after illumination at 355 nm were 0.1 μs, 500 μs, 1 ms, 2 ms, respectively. The samples were prepared as described in Methods and the measurements were performed as indicated in Instrumentation.

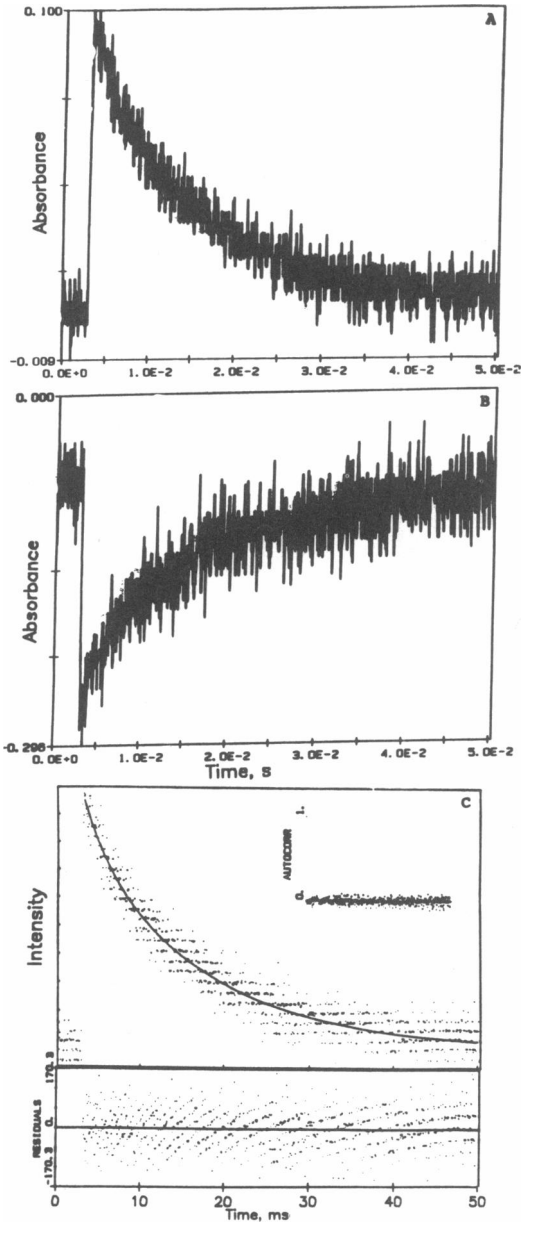

FIGURE 2 Zn-PP-myoglobin transient absorption decay at room temperature. (*A*) Decay of transient absorption at 460 nm, (*B*) recovery of the photobleaching at 428 nm, (*C*) a typical double exponential fit of the decay of transient absorption of Zn-PP-myoglobin at 460 nm. The calculated lifetime values are $\tau_1 = 2.4$ ms, $\tau_2 = 14.4$ ms and the amplitudes are $A_1 = 0.08$, $A_2 = 0.29$, respectively. The samples were prepared as described in Methods and the measurements were performed as indicated in Instrumentation.

myoglobin are presented. The absorption peaks for Zn-MP-myoglobin are at 583, 544, 416, 354, and 275 nm, where the 275-nm peak represents the protein absorption. The emission peaks, measured in coincidence with the lamp flash, are at 585 and 636 nm and are identified as prompt fluorescence (Fig. 3 *B*) showing a characteristic blue shift compared to the protoporphyrin derivative (Fig. 1 *B*).

The transient absorption spectra of Zn-MP-myoglobin has a maximum around 445 nm and photobleaching at

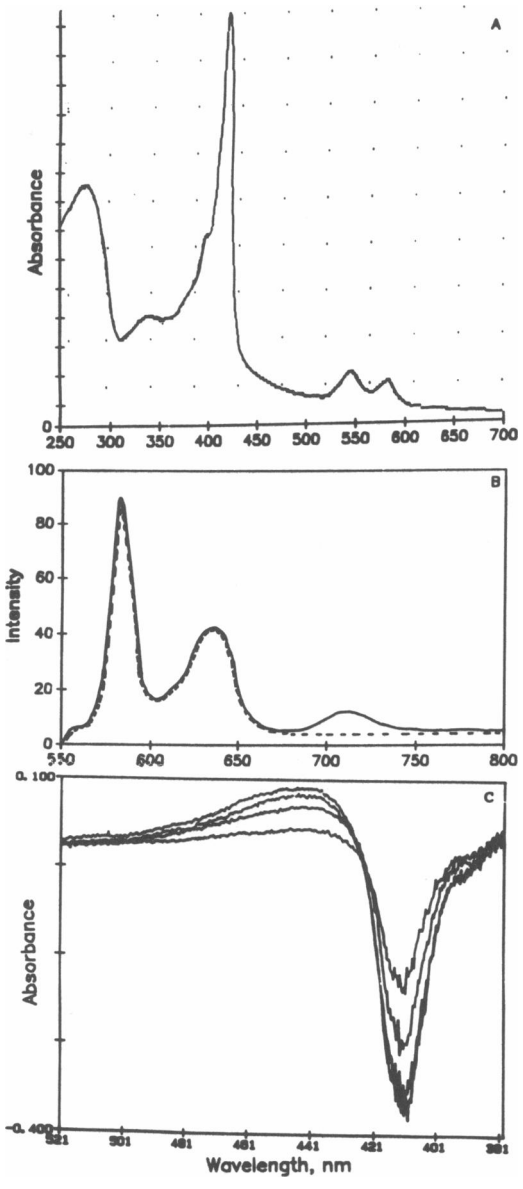

FIGURE 3 Optical properties of Zn-MP-myoglobin at room temperature. (*A*) Absorbance; (*B*) emission (fluorescence [---], phosphorescence (—) spectra. For both emission spectra the excitation wavelength was 405 nm. (*C*) Time-resolved transient absorption spectra of Zn-MP-myoglobin, the delay times after illumination at 355 nm were 100 μs, 500 μs, 4 ms, 20 ms, respectively. The samples were prepared as described in Methods and the measurements were performed as indicated in Instrumentation.

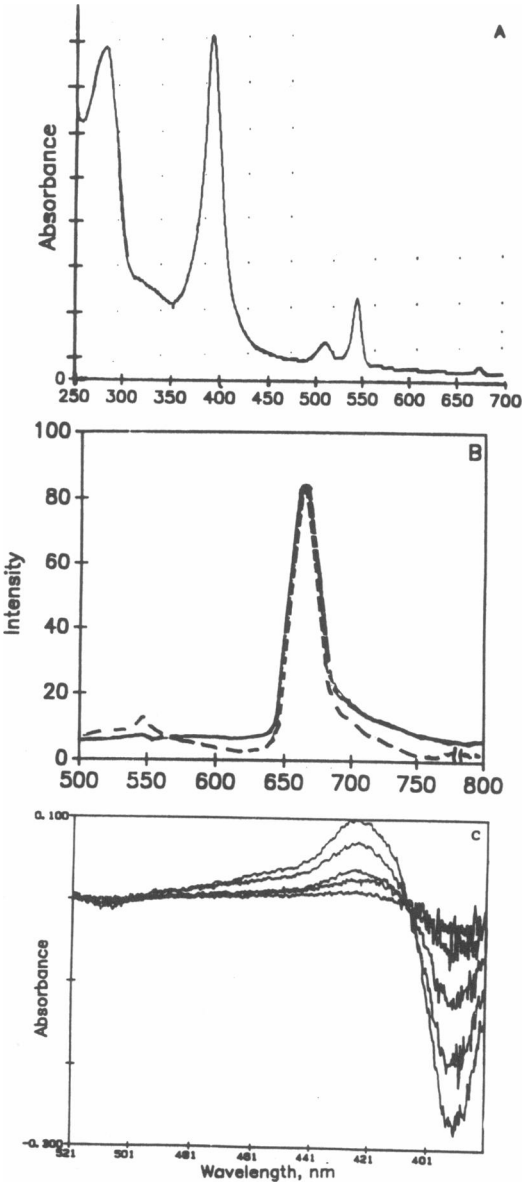

FIGURE 4 Optical properties of Pd-MP-myoglobin at room temperature. (*A*) Absorbance, (*B*) emission (fluorescence[---], phosphorescence [—]) spectra. For both emission spectra the excitation wavelength was 392 nm. (*C*) Time-resolved transient absorption spectra of Pd-MP-myoglobin, the delay times after illumination at 355 nm were 1 μs, 100 μs, 500 μs, 1 ms, 2 ms, respectively. The samples were prepared as described in Methods and the measurements were performed as indicated in Instrumentation.

the Soret band (415 nm) as shown in Fig. 3 *C*. The decay kinetics of the transient absorption and the recovery of the photobleaching are nonexponential and the spectra does not have an isosbestic point. Both the absorption and the emission features of the Zn-MP-myobglobin are very similar to the Zn-PP-myoglobin with two major differences. The overall spectra is shifted to the blue by ~10 nm and the triplet state is longer lived.

In Fig. 4 the absorption (*A*), the emission (*B*), and the transient absorption spectra (*C*) of Pd-MP-myoglobin are presented. The absorption peaks for Pd-MP-myoglobin are at 545, 511, 392, and 280 nm, where the 280-nm peak

TABLE 1. **Optical properties of Zn and Pd substituted myoglobins**

Myoglobin derivative	Absorption maxima	Fluorescence maxima	Triplet absorption maximum	Phosphorescence emission	τ_p
	nm	*nm*	*nm*	*nm*	*ms*
Zn PP	277, 356, 428, 553, 595	595, 647	460	735	14.6
Zn MP	275, 354, 416, 544, 583	585, 636	445	710	20
Pd MP	280, 392, 511, 545	550 (weak)	418	660	1.2

Measurements were carried out at 22°C as described in Materials and Methods. τ_p is the phosphorescence lifetime in the absence of oxygen.

represents the protein absorption (Fig. 4 *A*). These values are identical to the recently reported values for the the same derivative (Cowan and Gray, 1989). A small emission at ~550 nm represents fluorescence. Both the prompt and delayed emission spectra show a single peak around 660 nm, which is attributed to long-lived luminescence of the Pd porphyrin (Fig. 4 *B*). The transient absorption maximum is at 418 nm (Fig. 4 *C*). An isosbestic point was observed in the emission and the decay profile could be fit with a single exponential function. The values of the phosphorescence lifetimes for both derivatives are in good agreement with the data coming from the analysis of the deacy of the transient absorptions.

The phosphorescence lifetimes were measured for all derivatives in the absence of oxygen; values of 14.6 ms for Zn-PP-myoglobin, 20 ms for Zn-MP-mesoporphyrin, and 1.2 ms for Pd-MP-myoglobin at 20°C represent the longest-lived component. For the Zn derivatives the decay profiles of delayed fluorescence and phosphorescence were equivalent and independent of excitation wavelength.

The optical properties are summarized in Table 1.

Oxygen quenching of phosphorescence

The central metal in myoglobin is expected to subtly affect the polypeptide chain, and therefore there may be differences in diffusion through the chain in the Pd and Zn derivatives. Phosphorescence quenching occurs at low oxygen concentration, allowing for a measure of oxygen diffusion with negligible contribution oxygen molecules inside the protein at the beginning of the reaction. Oxygen at these concentrations also does not quench the shorter-lived fluorescence. The Stern-Volmer quenching constant of Zn-PP-myoglobin for oxygen was determined using Pd-coproporphyrin as an internal standard to measure oxygen concentration according to Vanderkooi et al. (1987*b*). The quenching rate constant for oxygen at 20°C was found to be 9.7×10^7 M^{-1} s^{-1} obtained from the quenching curve shown on Fig. 5 *A*; this value is in good agreement with earlier published values (Zemel and Hoffman, 1981; Barboy and Feitelson, 1987, 1989). The determination of the quenching constant of Pd-MP-

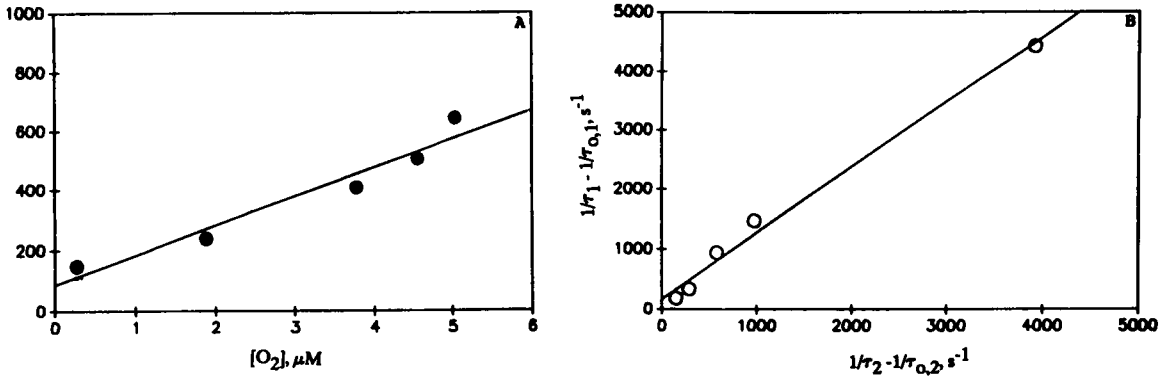

FIGURE 5 Phosphorescence quenching of Zn-PP-myoglobin and Pd-MP-myoglobin by oxygen at 20°C. (*A*) Stern-Volmer plot of phosphorescence quenching of Zn-PP-myoglobin by oxygen, where the phosphorescence lifetime in the absence of oxygen is τ_0 and in the presence is τ. (*B*) Comparison of phosphorescence quenching Pd-MP-myoglobin and Zn-PP-myoglobin by oxygen. Where $\tau_{0,1}$, $\tau_{0,2}$ the phosphorescence lifetimes in the absence of oxygen and τ_1, τ_2 the phosphorescence lifetimes measured at the same oxygen concentration of Pd-MP-myoglobin and Zn-PP-myoglobin, respectively. The oxygen concentrations were determined according to Vanderkooi et al. (1987) and the samples were prepared as described in Methods.

myoglobin was not possible using Pd-coproporphyrin as an internal standard because of its similarity in spectra and lifetime with Pd-myoglobin. However, the lifetime values of the Zn and Pd derivatives of myoglobin can be determined simultaneously in the presence of the same oxygen concentration. After a simple rearrangement of the well known Stern-Volmer (1919) equation:

$$\tau_o/\tau = 1 + k_q\tau_o[Q], \qquad (1)$$

where τ and τ_o refers to the lifetimes in the absence of quencher and at quencher concentration $[Q]$, the ratio of their quenching constants k_q for two dyes will be

$$1/\tau_2 - 1/\tau_{o,2} = k_{q,2}/k_{q,1}(1/\tau_1 - 1/\tau_{o,1}) \qquad (2)$$

where the subscripts 1 and 2 refer to Pd-MP-myoglobin and Zn-MP-myoglobin, respectively. One can obtain the ratio of the quenching rate constants of the two derivatives for oxygen by plotting $(1/\tau_2 - 1/\tau_{o,2})$ vs. $(1/\tau_1 - 1/\tau_{o,1})$. Fig. 5 *B* shows the comparison of the two quenching constants; $k_{q,2}/k_{q,1}$ is 1.1 ± 0.1. This result suggests that there is no significant difference between the oxygen penetrability of the Zn and Pd porphyrin myoglobins.

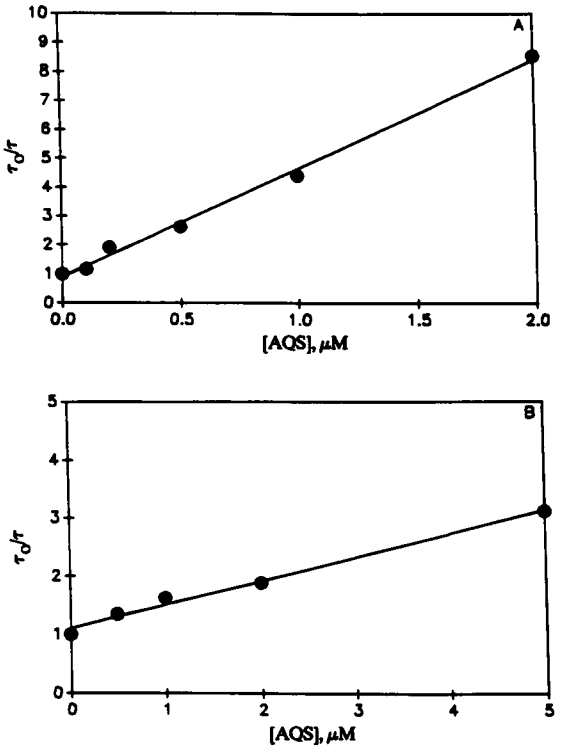

FIGURE 6 Phosphorescence quenching of Zn-PP-myoglobin and Pd-MP-myoglobin by AQS at 20°C. Stern-Volmer plot of phosphorescence quenching of Zn-PP-myoglobin (*A*) and Pd-MP-myoglobin (B) by AQS, measured by decrease in lifetime of phosphorescence emission at 20°C. Where τ_o and τ are the phosphorescence lifetimes in the absence and in the presence of the quencher, respectively. The excitation wavelength was 545 nm to avoid the optical problems coming from the contribution of AQS absorption at the Soret region. The samples were prepared as described in Methods.

Excited triplet state reaction with quinone

Quinones are known to be effective quenchers of porphyrin triplet states, interacting by electron exchange or transfer (Pileni and Gratzel, 1980; Nahor et al., 1981; Lindsey et al., 1983) or by the formation of exciplexes (Roy et al., 1974). Barboy and Feitelson have recorded the reaction of AQS with Zn porphyrin (Barboy and Feitelson, 1986) or with Zn-PP-myoglobin (Barboy and Feitelson, 1987). Quenching by AQS was reexamined here.

In the time range of 100 μs to 1 ms, we saw no evidence for a transient photoreduced product (Figs. 1 *C* and 4 *C*). The Stern-Volmer plots for AQS quenching of Zn-PP-myoglobin (Fig. 6 *A*) and Pd-MP-myoglobin (Fig. 6 *B*) based upon long-lived luminescence are linear. The quenching rate constants for AQS were $2.6 \times 10^8\ M^{-1}\ s^{-1}$ and $3.4 \times 10^8\ M^{-1}\ s^{-1}$ of the Zn and the Pd derivative, respectively. The value found for AQS quenching constant of Zn-PP-myoglobin confirms the value of Barboy and Feitelson (1987).

At low viscosity and steady-state conditions, the intensity and lifetime quenching profiles of Pd-MP-myoglobin coincided. But, as seen in Fig. 7, in high-viscosity medium, there is a deviation between the intensity and lifetime measurement, with the effect being more pronounced at low temperature. Under all these conditions, there was no effect on the ground state absorption spectrum of the Pd-MP-myoglobin, indicating no evidence of a ground state complex.

Because the integrated luminescence intensity shows more quenching than the transient measurement under conditions that diffusion is slow we could expect that there could be a fast quenching term. We therefore examined the decay of Pd-MP-myoglobin on the nanosecond time scale (Fig. 8 *A*). There is a fast component, with lifetime of ~100 ps. The intensity of this component increased in the range of 550 nm, and is therefore likely to be due to Pd porphyrin fluorescence. For the unquenched sample, no further decay was seen in this time range. Addition of AQS reduced the intensity of long-lived component (Fig. 8 *B*). A fast component at times <0.5 ns could be attributed to a background contribution of fluorescence from the AQS and from the Pd porphyrin. No other decay was observed except the long lived phosphorescence which, on the time scale of this experiment, is essentially constant (Fig. 8).

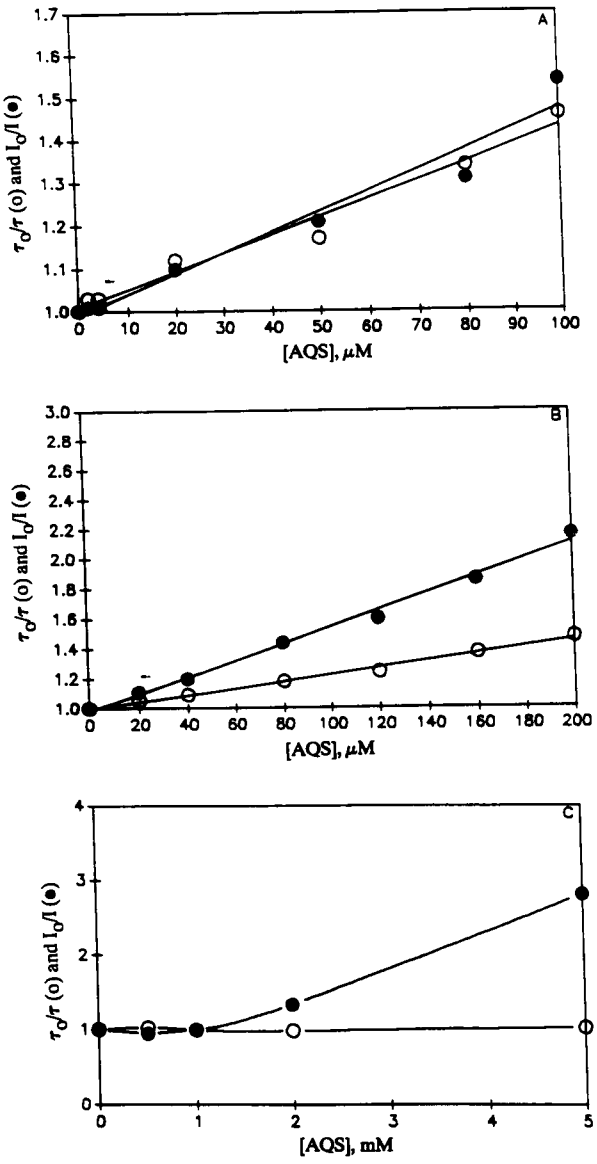

FIGURE 7 Phosphorescence quenching of Pd-MP-myoglobin by AQS at different temperatures. Stern-Volmer plot of phosphorescence quenching of Pd-MP-myoglobin by AQS, measured by decrease both in lifetime (O) and intensity (●) of phosphorescence emission at 298 K (*A*), at 278 K (*B*), and 77 K (*C*), where τ and τ_0 are the phosphorescence lifetimes and I_0 and I are the steady-state phosphorescence intensities in the absence and in the presence of the quencher, respectively. The excitation wavelength was 545 nm to avoid the optical problems coming from the contribution of AQS absorption at the Soret region. The samples contained 85% (wt/wt) glycerol otherwise were prepared as described in Methods.

Finally, if quenching occurs by electron transfer, the quenching reaction might produce ionized intermediates. This possibility was examined by transient absorption methods. In the time range of 100 μs to 1 ms the transient spectra appeared the same as the unquenched samples

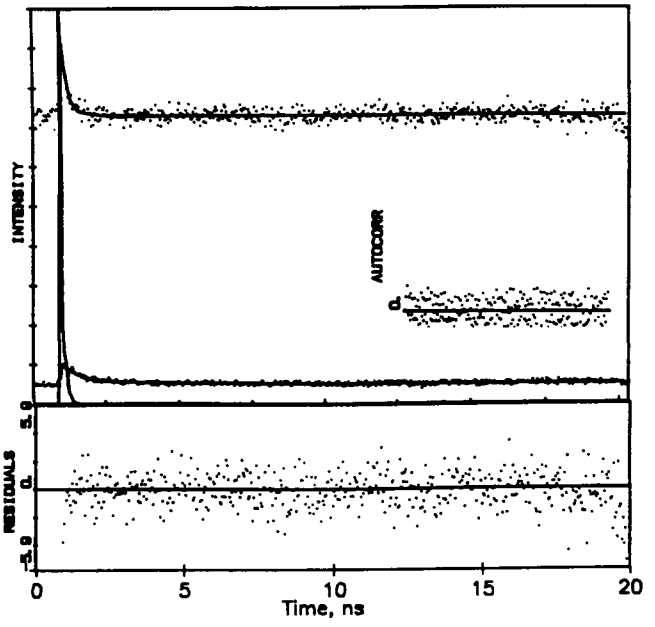

FIGURE 8 Pd-MP-myoglobin fluorescence decay. Fluorescence decay of Pd-MP-myoglobin was measured in the absence (*upper curve*) and in the presence of 200 μM AQS at room temperature using the single photon counting method. The solid lines through the measured points are the computed curves representing the fit to three exponential functions. Excitation and emission wavelengths were 545 and 660 nm, respectively. The samples contained 85% (wt/wt) glycerol otherwise were prepared as described in Methods.

(although the decay was faster). Therefore, no evidence for ionized products was obtained.

DISCUSSION

Replacement of the iron in myoglobin with other metals produces porphyrin derivatives with long fluorescent and phosphorescent lifetimes. Excited states are electronic isomers of the ground state, with both singlet and triplet excited-state molecules being more reactive than the ground-state parent. Specific interactions between excited-state molecules and neighboring molecules include Coulombic interactions and electron-exchange/transfer reactions, reactions that can mimic the electron transfer reactions which native heme proteins undergo. In this way, porphyrin derivatives of heme proteins can provide model reactions for native reactions.

Zn porphyrin has been used previously to study heme proteins and circular dichroic data suggest that the replacement of the Fe produces no serious tertiary or secondary changes in the myoglobin structure (Andres and Atassi, 1970). Like other Zn porphyrins in aqueous solution (Vanderkooi et al., 1987*b*) or in other proteins (Dixit et al., 1984; Koloczek et al., 1987) the long-lived

emission of Zn myoglobins at room temperature exhibits both thermally-activated delayed fluorescence and phosphorescence. In this paper we also introduce the use of Pd porphyrin to study quenching reactions in myoglobin. Pd-MP-myoglobin emits with very strong long-lived luminescence that can be measured under ordinary steady-state illumination. Its emission spectrum resembles the emission of Pd coproporphyrin but the value of lifetime is longer: $\tau_0 = 1.2$ ms for Pd-MP myoglobin (this paper) vs. 0.71 ms for Pd coproporphyrin (Vanderkooi et al., 1987*b*).

The transient absorption spectra of Zn and Pd derivatives of myoglobin, representing the difference spectra of the singlet ground state and the triplet state, show interesting variations. Within experimental error, the triplet Pd derivative showed a single exponential decay (Fig. 4 *C*) and isosbestic points at 409, 498, and 518 nm in the transient absorption spectra (Fig. 4 *C*). These features usually indicate that a single emitting species is present. In contrast, the Zn derivatives do not have an isosbestic point and a shift in the transient absorption spectra was seen between 100 μs and 10 ms. A nonsingle exponential decay is an indication that more than one species is emitting. The multiple species may exist in the ground state, and/or in the excited state. The Zn porphyrin triplet state is unstable, and the excited-state molecule can undergo distortions in the *x-y* plane. In addition, the Zn can be fifth-liganded and the complex decay behavior may be due to the special properties of the Zn. We note that the optical transient absorption spectrum of CO-dissociated myoglobin does not show shifts between 30 ps and 6.5 ns after photolysis (Janes et al., 1988) and so in this regard, Pd-MP-myoglobin resembles the native protein.

Oxygen quenches the phosphorescence of both Zn and Pd porphyrins in myoglobin. There is no significant difference in the quenching of the two, suggesting that oxygen is equally accessible to both porphyrins. The porphyrin presents a large target for oxygen quenching and subtle differences in the polypeptide chain in the two metal derivatives are likely not to be detected by oxygen quenchability. The value for oxygen quenching that we obtained, $9.7 \times 10^7\ M^{-1}s^{-1}$, is comparable with literature values based upon phosphorescence (Alpert and Lindquist, 1975; Zemel and Hoffman, 1981) and for Fe-free myoglobin fluorescence (Jameson et al., 1984) and is about an order of magnitude less than for free porphyrins in solution (Vanderkooi et al., 1987*b*).

The larger molecule, AQS, is effective in quenching the triplet state of both Pd and Zn porphyrin myoglobin. The quenching constant was about the same for the two derivatives and agrees well with the previously reported value for Zn-PP-myoglobin (Barboy and Feitelson, 1987, 1989). For the Pd porphyrin derivative the steady-state emission from the triplet state can be measured independently. We found that at low viscosity, the steady-state and transient measurements agreed well (Fig. 7 *A*). At higher viscosities and lower temperatures, a positive deviation in the steady-state intensities was observed. Under these conditions no transient quenching was observed at the fastest time that could be measured, around 100 ps (Fig. 8). At 77 K an upward break in the quenching profile occurred around 1 mM, even though there was no change in the emission lifetime.

One model that can be considered to describe the above results, proposed by Barboy and Feitelson (1987), is that AQS needs to penetrate into the protein in order for electron transfer to occur. These same authors have also observed that the quenching does not depend upon the temperature and viscosity in the classic Stokes description, and conclude that diffusion through the polypeptide chain may play a role.

To account for the observation of quenching at low viscosities we would like to consider a model in which quenching occurs by long-range transfer. This model is formally very similar to that proposed by Barboy and Feitelson, but transfer (i.e., electron diffusion) and not the physical diffusion of the molecule from the surface of the protein to the porphyrin, is the rate-limiting step.

Because there is no detectable spectral overlap between the emission of these porphyrins and the absorption of anthraquinone, dipolar energy transfer is unlikely. Therefore, we consider an electron exchange mechanism. Excited state porphyrins are known to be quenched by quinones over large distances (Mauzerall, 1973, 1978). Model studies have indicated that excited-state porphyrins can interact with quinones over separation distances up to 1 nm (Lindsey et al., 1983). We note that at 77 K, where diffusion through both the solvent and the protein should be negligible, steady-state quenching was observed (Fig. 7 *C*). For electron exchange at fixed distances, transfer rates depend exponentially on distance. The quenching radius R for half quenching (ie., $k_{et} = k_{em} = 1/\tau_0$), can be obtained from a plot of ln (I/I_0) vs. acceptor concentration (Strauch et al., 1983; McLendon, 1988) according to $R_0 = [1/(4/3\pi C)]^{1/3}$, where C is the quencher concentration. The value of R corresponds to an average distance of 9.8 Å, assuming random distribution of quenchers around the myoglobin. The absolute value of this distance is somewhat uncertain, however. This is true because, first, it is experimentally difficult to accurately measure intensities at 77 K. Secondly, at high AQS concentrations there may be an association of the AQS with the protein. Nevertheless, we conclude that long-range transfer is possible. We note that the quenching of tryptophan phosphorescence of various proteins by small molecules also appears to occur by a long-range process (Calhoun et al., 1988).

The heme of myoglobin is assymetrically placed in the polypeptide chain. The closest approach to the heme edge is ~3 Å. Therefore, it is likely that transfer can occur from the surface of myoglobin to the porphyrin. Although quinones can form exciplexes with porphyrins, no evidence of its formation was found in the emission spectra. Likewise, no evidence of cation formation was seen in the transient absorption spectrum. The formation of ions is stabilized by an aqueous environment (Nahor et al., 1981), and therefore the lack of ion formation is consistent with the hydrophobic nature of the heme pocket.

Whether long distance transfer also plays a role in oxygen quenching is more difficult to assess. It has been assumed that the quenchability of excited states by oxygen indicate that oxygen can freely penetrate myoglobin (cf. references cited in introduction). Recently, Ghiron et al. (1988) have pointed out that oxygen quenching of tryptophan in proteins could indicate that long-range transfer is possible. Because oxygen diffuses through myoglobin, as is well documented by flash photolysis experiments, we would expect that diffusion and not long-range transfer is the rate determining factor in the phosphorescence quenching reaction, but without additional information we cannot rule out a contribution from long-range interactions.

In summary, subtle differences were seen in the electronic relaxation parameters of Pd and Zn substituted myoglobins in that relaxation of the triplet state Pd derivative showed no evidence of structural rearrangement whereas the Zn porphyrin did. The data on quenching by AQS are consistent with long-range transfer, and therefore are a function of distance between the donor and acceptor. No difference was seen in the interaction with oxygen between the excited triplet state Pd or Zn myoglobins.

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