Our data show that, indeed, the polypeptide chain, in the absence of ligands, has direct influence upon the π-electrons of the porphyrin, since under site-selection conditions spectra were obtained which were characteristic of the proteins.

Finally, we note that whereas the emission pattern is characteristic for the protein, the extent of the inhomogeneous broadening was about 400 cm^{-1}, or about 1 kcal/mol, as indicated by the distribution of the 0–0 bands for all the proteins. If the energetics for electron transfer in the native proteins are similar to the energetics to excite an electron from the ground state to a higher state in the porphyrin derivatives, then the midpoint redox potential is similarly broadened. This broadening, which would be about ±30 mV, would have no consequence for equilibrium measurement, but could be significant in kinetic measurements.

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Study of the Active Site of Horseradish Peroxidase Isoenzymes A and C by Luminescence†

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ABSTRACT: Luminescent derivatives of horseradish peroxidase isoenzymes A and C were prepared by replacing the heme with protoporphyrin IX or mesoporphyrin IX. The isoenzymes showed about the same binding affinity as the active enzymes for hydroxamic acid derivatives. The fluorescence and phosphorescence yields and lifetimes of the porphyrin derivatives at room temperature decreased in the presence of substrates. Under site-selection conditions (low temperature and narrow-band excitation), resolution in the emission spectra of the porphyrin derivatives could be obtained, proving that the spectra are inhomogeneously broadened. Addition of substrate resulted in a change in distribution of the 0–0 lines in the resolved spectra. The results are discussed in terms of a distribution of sites which are altered by substrate.

Horseradish peroxidases (HRP)[1] are heme glycoproteins which use hydrogen peroxide to oxidize a wide variety of compounds (Yamazaki & Yokota, 1973). Horseradish contains at least seven isoenzymes, all with protoheme IX as the prosthetic group but with different physicochemical and kinetic properties (Shannon et al., 1966). A basic (C) and an acidic (A) isoenzyme together account for about 75% of the total amount. HRP C has higher enzyme activity and tighter

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[1] Abbreviations: HRP, horseradish peroxidase; HRP A, horseradish isoperoxidase type A2 (acidic); HRP C, horseradish isoperoxidase type C2 (basic); NHA, 2-naphthohydroxamic acid; PP, protoporphyrin IX; MP, mesoporphyrin IX.

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substrate binding than HRP A (Paul, 1958; Marklund et al., 1974; Paul & Ohlsson, 1978).

Joint information from optical, NMR, and EPR spectroscopy suggests that hydrogen donors interact with a hydrophobic site on the apoprotein to alter the spectrum of the heme by perturbing its environment without directly interacting with the heme. Using EPR and NMR, we estimated the distance between substrate and heme to be 3–10 Å (Dunford & Cotton, 1975; Schejter et al., 1976; Rich et al., 1978; Morishima & Ogawa, 1979; LaMar et al., 1980; Gupta et al., 1980). Hydroxamic acid derivatives are substrates of HRP, some with high affinity (Schonbaum, 1973). In this paper, we make use of the fluorescence and phosphorescence of the porphyrin in metal-free derivatives of HRP to examine interactions of hydroxamic acids and porphyrins with the polypeptide chain in HRP C and HRP A.

Materials and Methods

Materials. 2-Naphthohydroxamic acid (NHA) and benzhydroxamic acid were products of Molecular Probes, Inc. (Junction City, OR). Other hydroxamic acid derivatives were gifts of Dr. W. Bonner. Rhodamine 575 dye was purchased from Exciton Chemical Co. (Dayton, OH).

Protoporphyrin IX or mesoporphyrin IX horseradish peroxidase was prepared as described (Vanderkooi et al., 1985).

Measurement of NHA Binding to Horseradish Peroxidase and Protoporphyrin IX Horseradish Peroxidase. The binding of NHA to PP HRP was measured by the difference spectra of absorption using a Perkin-Elmer Hitachi 200 spectrophotometer and by fluorescence quenching using a Perkin-Elmer 650-10S fluorescence spectrophotometer at room temperature.

Fluorescence and Phosphorescence Spectra. Low-resolution steady-state fluorescence spectra of MP HRP and PP HRP were obtained by using an SLM spectrofluorometer (Urbana, IL) equipped with a Hammamatsu 928 photomultiplier. We found a distortion of the emission spectra in the region of 620 nm which was attributed to the inability of the monochromator to transmit parallel and perpendicular components of the light equally. A polarizer in the parallel (i.e., vertical) position was placed on the emission side of the sample to minimize this effect. The spectra were otherwise uncorrected.

High-resolution fluorescence spectra were obtained by using the instrument previously described (Angiolillo et al., 1982). The sample was frozen by dropwise addition to liquid nitrogen (Vanderkooi et al., 1975).

Fluorescence Decay Measurement. Fluorescence decay measurement was carried out by using an Ortec photon-counting fluorescence lifetime instrument (Oak Ridge, TN) equipped with a thyrotron-gated lamp filled with nitrogen gas (Products for Research, Ontario, Canada). A band-pass filter which transmitted the nitrogen line at 337 nm was used to isolate the excitation light, and a Schott RG570 cut-off filter was placed on the emission side. The decay was analyzed by Laplace transform for deconvolution of the lamp function as previously described (Dixit et al., 1982). The decay curves were fit to one or two components, or one or two components with scatter. The χ^2 values were calculated. When the χ^2 value was not different between one or two components, the single-component fit was taken. χ^2 maximum counts were typically <0.02.

Phosphorescence Emission and Absorption of Porphyrin. Phosphorescence emission and the absorption of the excited triplet state were measured as described (Dixit et al., 1982). A Phase-R DL-1000 pulse dye laser was used for excitation. The lasing dye solution was 50 μM rhodamine 575 in ethanol. A tungsten lamp was the light source for absorption. The emission was isolated by using a Spex minimate monochromator and the light detected with a Hammamatsu 986R photomultiplier. The signal from the PM tube was amplified by a Johnson Foundation photometer amplifier and digitized with a 2805 Gould Biomation transient recorder which was interfaced with an Apple II desk computer for analysis. Typically, the measurements of phosphorescence emission and triplet transient absorption were averaged from more than 10 decay profiles to improve the signal to noise ratio. For the phosphorescence measurement, the sample was deoxygenated by bubbling with Ultrapure argon (Airco, Philadelphia, PA) for 20 min in a glovebag.

Results

Effect of Hydroxamic Acid Derivatives on Fluorescence Spectra of Porphyrins in Iron-Free Enzymes and Determination of Binding Constants. Steady-state fluorescence spectra of PP HRP A and C are shown in Figures 1 and 2. It is apparent that there are subtle but significant differences between them. Addition of naphthalenehydroxamic acid to either of the two HRP isoenzymes results in a shift in the emission maxima and a decrease in fluorescence yield (Figures 1 and 2). The quenching and shift in the emission spectrum were more pronounced for HRP C than for HRP A. The excitation spectra of porphyrin in PP HRP also showed shifts in the presence of bound NHA; similar changes were also evident in the visible absorption spectra (not shown). The changes in the absorption spectra were smaller than the shifts in the emission spectra.

The fluorescence yields of the protoporphyrin enzymes were quenched by NHA to a limiting value at high concentrations (Figure 3). It was therefore possible to calculate the apparent dissociation constant (K_d) according to the following equation, assuming that the enzyme–NHA complex is stoichiometric (1:1) and the concentration of the complex (HRP·NHA) small compared to the total concentration of NHA. Under these conditions

$$\frac{1}{\Delta F} = 1 + K_d \frac{1}{[\mathrm{NHA}]_T}$$

The change in fluorescence intensity produced by substrate

$$\Delta F = \frac{F_0 - F}{F_0 - F_\infty}$$

is related to K_d according to

$$K_d = \frac{([\mathrm{HRP}]_T - [\mathrm{HRP{\cdot}NHA}])([\mathrm{NHA}]_T - [\mathrm{HRP{\cdot}NHA}])}{[\mathrm{HRP{\cdot}NHA}]}$$

where F_0 is the observed fluorescence intensity of protoporphyrin IX HRP without NHA, F is the observed fluorescence intensity with NHA, and F_∞ is the observed fluorescence intensity with excess NHA. $[\mathrm{HRP}]_T$ is the total concentration of HRP. We obtained an apparent dissociation constant of 24 μM for PP HRP A. NHA binds much tighter to PP HRP C than PP HRP A. The apparent dissociation constants for a series of hydroxamic acid derivatives to HRP C are proportional to those reported by Schonbaum (1973) for the native enzyme and only slightly less (Table I), which was taken to mean that the binding site of the protein was essentially intact.

Characterization of Porphyrin Fluorescence Emission. We further defined the fluorescence emission of porphyrins by comparing the spectra and lifetimes in solvents and in the HRP isoenzymes. The results are summarized in Table II.

The excitation absorption spectra (Figures 1 and 2) of the two isoenzymes are nearly the same. The emission spectra

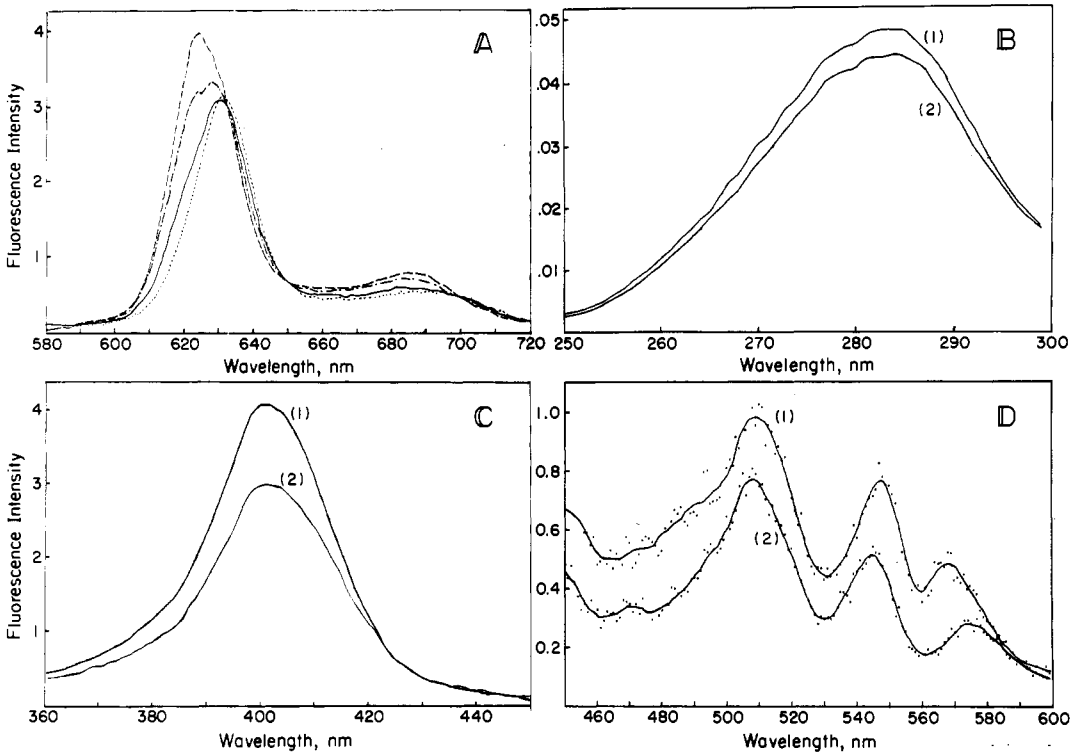

FIGURE 1: Uncorrected excitation and emission spectra of PP HRP C. Protein concentration was 9.3 μM. Medium, 10 mM potassium phosphate buffer, pH 6.0 at room temperature. (A) Emission spectra of PP HRP C (--) without NHA, (-·-) with 0.5 μM NHA, (—) with 2.5 μM NHA, and (···) with 9.5 μM NHA. Excitation wavelength, 400 nm. (B–D) Excitation spectra of PP HRPA (1) without NHA and (2) with 200 μM NHA. Emission wavelength, 630 nm.

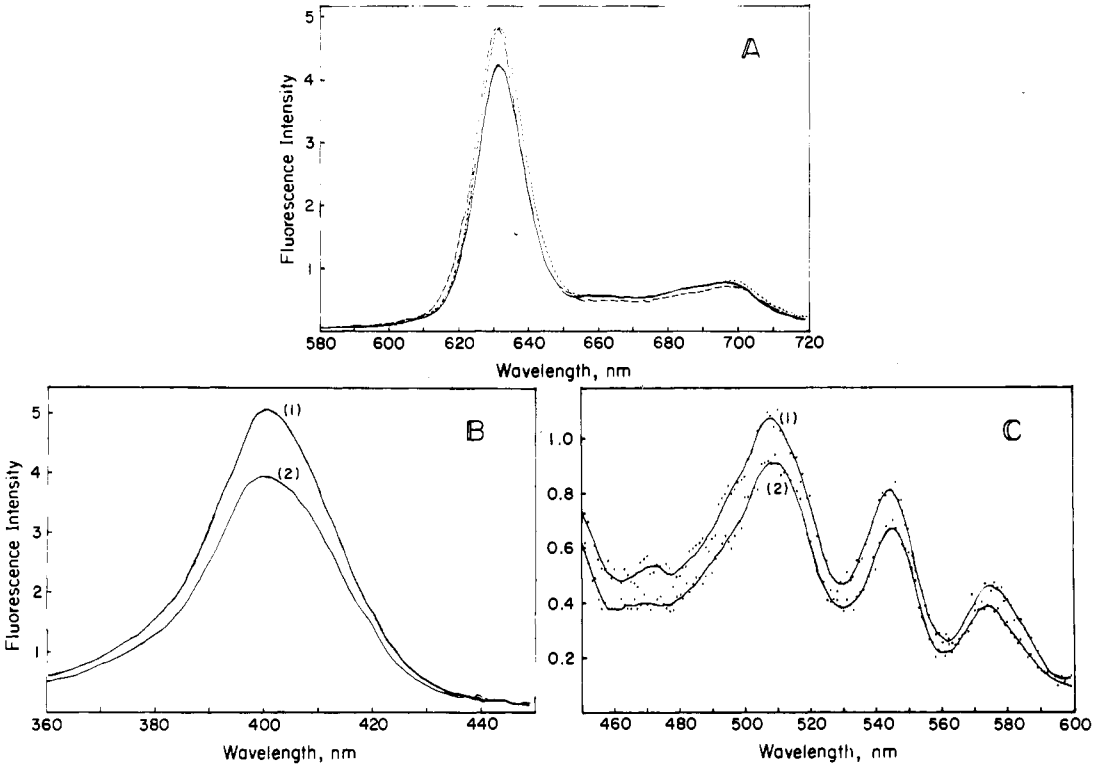

FIGURE 2: Uncorrected excitation and emission spectra of PP HRP A. Conditions are given in Figure 1. (A) Emission spectra of PP HRP without (--), with 10 μM NHA (---), and with 200 μM NHA (—). (B and C) Excitation spectra of PP HRP A (1) without NHA and (2) with 200 μM NHA. Emission wavelength, 630 nm.

are more noticeably different with maxima at 631 nm for the A isoenzyme and 624 nm for the C isoenzyme. The fluorescence decay curves of the PP and MP derivatives were satisfactorily described by a single-exponential function. Addition of NHA decreased the fluorescence lifetime, consistent with the decrease in fluorescence yield which can be observed in Figures 1 and 2. The decay curve in the presence of NHA could also be described by an exponential. An example of the fluorescence decay curves of PP HRP is given in Figure 4.

To further define the emission properties of porphyrins, the fluorescence lifetimes of protoporphyrin IX and mesoporphyrin

Table I: Apparent Dissociation Constants (K_d) of Protoporphyrin IX HRP C–Hydroxamic Acid Complexes and Fluorescence Quenching[a]

	K_d	fluorescence quenching (%)
2-naphthohydroxamic acid	<1 (0.2)[b]	47
	24[c]	12[c]
p-hydroxybenzohydroxamic acid	36 (9.0)[b]	17
o-hydroxybenzohydroxamic acid	118 (7.8)[b]	13
p-chlorobenzohydroxamic acid	17 (6.0)[b]	9
o-chlorobenzohydroxamic acid	158	21
benzohydroxamic acid	21 (2.4,[b] 65)[d]	14
	133[e]	36[e]

[a] Fluorescence quenching was compared at an excitation wavelength of 400 nm and an emission wavelength of 625 nm. Medium conditions: 0.02 M phosphate buffer, pH 6.0. Protein concentration was 0.2 μM. [b] These values were quoted by Schonbaum (1973) for native HRP from Worthington Biochemical Corp. [c] These values were obtained against protoporphyrin IX HRP A. [d] This value was quoted by Aviram (1981) for protoporphyrin IX HRP from Sigma Chemical Co. [e] These values were obtained against mesoporphyrin IX HRP C.

IX were measured in different solvents (Table II). At a given partial pressure, oxygen quenched the lifetime of protoporphyrin to a much lesser extent in the enzyme than in solvents.

Triplet Absorption and Emission of the Porphyrin Enzyme. Because the binding of NHA affected the fluorescence emission properties of iron-free HRP, it was of interest to examine the effect of NHA on the phosphorescence of HRP. The transient absorption and emission profiles of the triplet-state protoporphyrin are given in Figure 5. The lifetimes in the absence of NHA are 4.8 and 5.5 ms for HRP A and C,

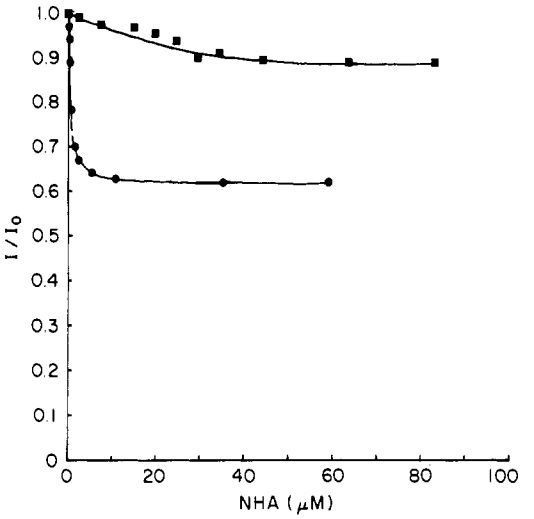

FIGURE 3: Fluorescence quenching of PP HRP A (■) and C (●) by NHA. Protein concentration was 5.5 μM. Medium, 20 mM potassium phosphate buffer, pH 6.0 at room temperature. Excitation wavelength, 410 nm; emission wavelength, 625 nm. I, fluorescence intensity; I_0, fluorescence intensity without NHA.

respectively. Addition of NHA decreased the lifetime to a limiting value, and the apparent biphasic curves in Figure 5 for the basic enzyme could be accounted for by emission from two species: free enzyme and enzyme containing bound NHA. At saturating concentrations of NHA, the lifetimes were 2.8 ms for PP HRP A and <1 ms for PP HRP C. No long-lived intermediates were detected since the absorption and emission decay profiles were the same.

Table II: Fluorescence Lifetimes of Protoporphyrin IX and Mesoporphyrin IX[a]

porphyrins in solvent	gas	lifetime (ns) ± SD	absorption: Soret (λ_{max}, nm)	absorption: visible (λ_{max}, nm)	emission (λ_{max}, nm)
protoporphyrin IX					
ethyl acetate/methanol (99:1 v/v)	N_2	15.3 (n = 1)	402	503, 237, 578, 630	632
	air	10.2 ± 0.25 (n = 6)			
ethyl acetate/methanol (1:1 v/v)	N_2	15.2 (n = 1)			
	air	10.5 ± 0.43 (n = 5)	402	503, 537, 575, 630	632
	O_2	4.55 (n = 2)			
methanol	N_2	14.9 (n = 1)			
	air	10.1 ± 0.16 (n = 5)	402	503, 537, 570, 630	630
	O_2	5.13 (n = 2)			
ethanol	N_2	15.1 ± 0.16 (n = 3)			
	air	10.9 ± 0.14 (n = 5)	402	504, 539, 576, 627	632
	O_2	5.26 (n = 2)			
ethanol/distilled water (1:1 v/v)	N_2	15.6 ± 0.43 (n = 8)			
	air	14.8 ± 0.11 (n = 6)	402	503, 539, 572, 624	628
	O_2	12.1 (n = 2)			
protoporphyrin HRP C					
20 mM phosphate buffer, pH 6.0	N_2	16.2 (n = 2)			
	air	16.1 (n = 2)	401	509, 548, 568, 622	624
	O_2	15.9 (n = 2)			
with 20 μM NHA in 20 mM phosphate buffer, pH 6.0	air	14.7 (n = 2)	401	508, 545, 574, 630	632
protoporphyrin HRP A					
20 mM phosphate buffer, pH 6.0	N_2	15.0 (n = 2)			
	air	15.0 ± 0.20 (n = 5)	400	508, 545, 575, 629	631
	O_2	14.4 (n = 1)			
with 100 μM NHA in 20 mM phosphate buffer, pH 6.0	air	14.7 (n = 2)	400	509, 545, 574, 630	632
mesoporphyrin IX					
ethyl acetate/ethanol (99:1 v/v)	air	9.2 (n = 2)	395	496, 526, 567, 620	620
ethanol	air	10.1 (n = 2)	395	497, 529, 565, 620	620
mesoporphyrin HRP C					
20 mM phosphate buffer, pH 6.0	air	18.4 (n = 2)	398	500, 538, 558, 612	615
mesoporphyrin HRP A					
20 mM phosphate buffer, pH 6.0	air	16.3 (n = 2)	396	498, 535, 565, 618	620

[a] Temperature, 20 °C. Other conditions given under Materials and Methods.

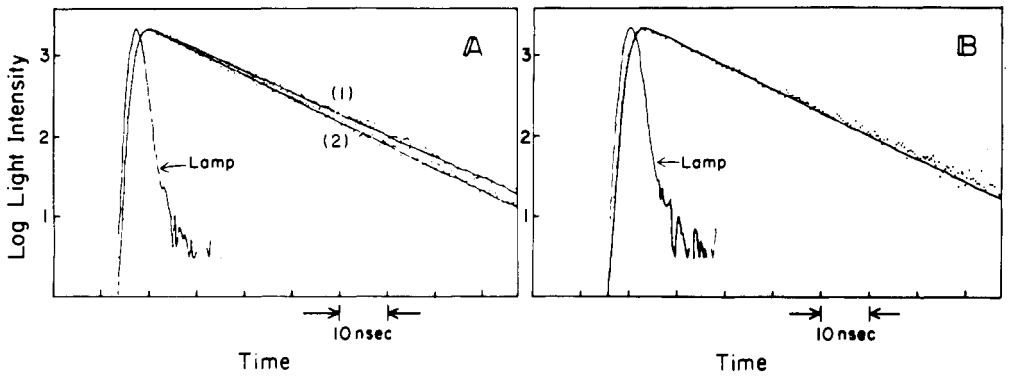

FIGURE 4: Fluorescence decay of PP HRP. Protein concentration was 10 μM. Medium, 20 mM potassium phosphate buffer, pH 6.0 at room temperature. (A) (1) PP HRP C; (2) PP HRP C + 20 μM NHA. (B) PP HRP A. Solid lines represent the best fit to a single exponential. Values given in Table II.

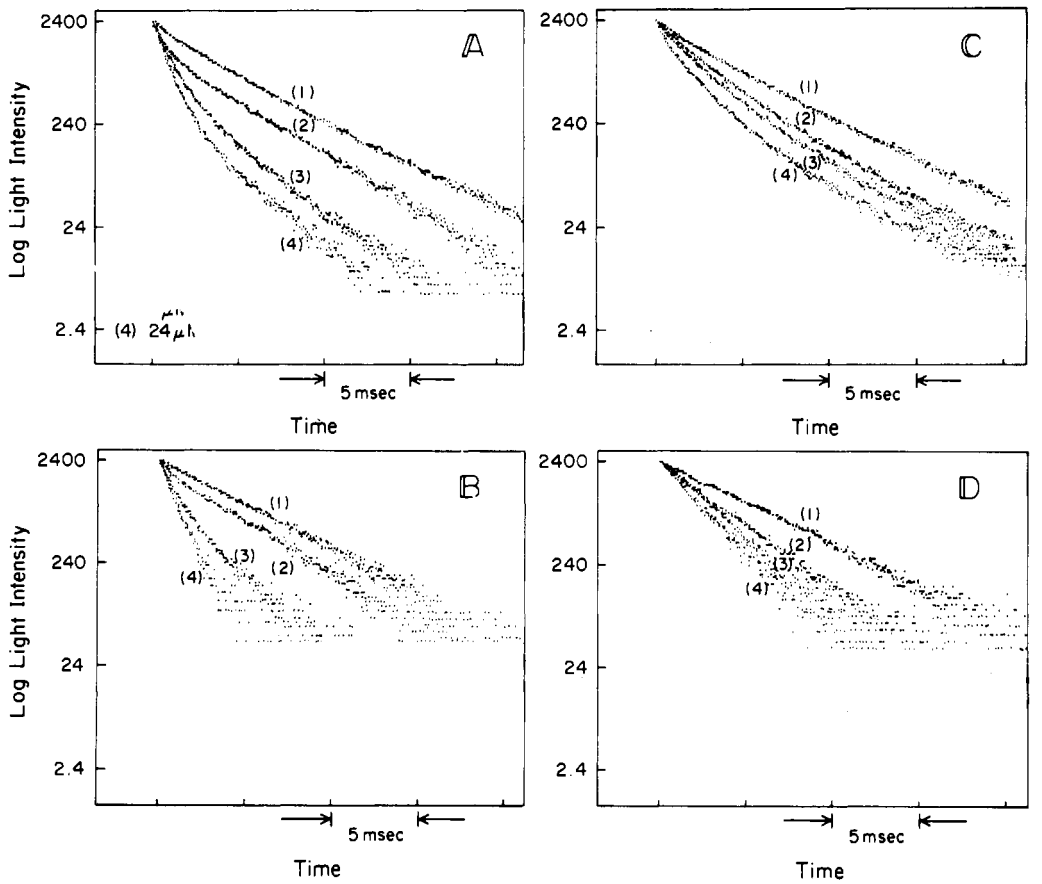

FIGURE 5: Semilogarithmic plots of phosphorescence emission and triplet absorption decay of PP HRP, titrated by NHA. Protein concentration was 5.5 μM. Medium, 10 mM potassium phosphate buffer, pH 6.0 Temperature, 20 °C. The deoxygenated sample was excited by a laser pulse at 575 nm, and the emitted phosphorescence or the transient absorption was monitored as described under Materials and Methods. Emission (A, 720 nm) and absorption (B, 460 nm) of PP HRP C. [NHA]: (1) 0, (2) 1, (3) 3, and (4) 24 μM. Emission (C, 720 nm) and absorption (D, 460 nm) of PP HRPA. [NHA]: (1) 0, (2) 8, (3) 16, and (4) 24 μM.

Resolved Fluorescence Spectroscopy of Porphyrins. Under normal illumination conditions and temperatures, the fluorescence spectra of porphyrins are broad (Figures 1 and 2), and the emission spectra do not vary with excitation wavelength. We have previously shown that under site-selection conditions (i.e., narrow-band excitation and low temperature), resolved spectra for porphyrin in proteins can be obtained which can be interpreted in terms of the vibrational structure of the ground and excited states (Angiolillo et al., 1982; Vanderkooi et al., 1985).

In Figure 6, the emission spectra of MP HRP C and A at 4.2 K are presented. Excitation at 569.2 nm, at the low-energy side of the second absorption band, yielded resolved spectra. The multiple bands in the region of 16 000–16 400 cm^{-1} are attributed to 0–0 transitions of various molecules shifted in energy in the ground state; the frequency difference between the 0–0 bands reflects the vibrational levels of the excited state (Vanderkooi et al., 1985). Taking the spectra in Figure 6A,C to be the sum of spectra from individual "sites", we notice that individual spectra of MP HRP C and MP HRP A are nearly the same but that the relative contributions from each site are different. The porphyrin in HRP C has most of its emission centering around 16 100 cm^{-1}, while the distribution of 0–0 lines of HRP A is greater and there is a greater concentration of lines closer in energy to the excitation wavelength.

Addition of substrate to both isoenzyme derivatives results in a change in the distribution of the 0–0 emission lines. The spectra of the substrate complexes of the A and C types re-

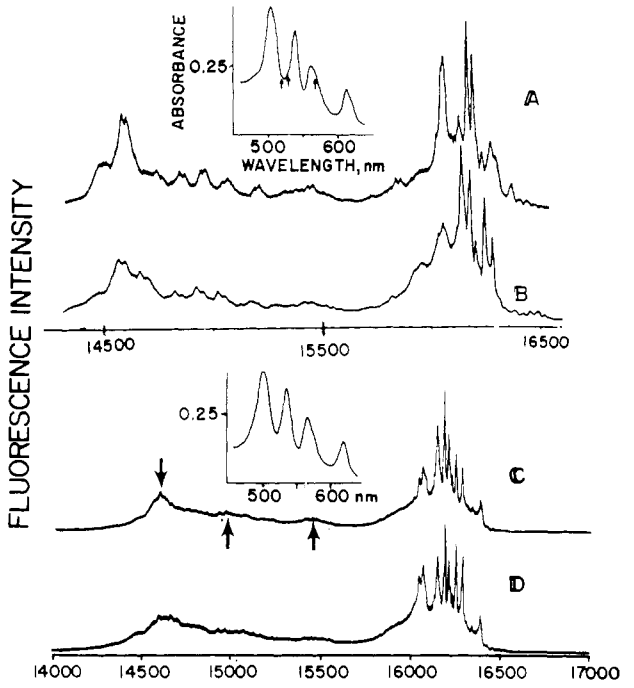

FIGURE 6: Spectra of MP HRP C and A at 4.2 K. (A) The sample contained 10 μM MP HRP C in 10 mM phosphate, pH 6, and 50% propylene glycol. (B) Same as (A) plus 100 μM NHA. Excitation, 568.2 nm.

semble each other in terms of distribution of sites.

Additional bands, due to vibrations, are evident in a fluorescence emission spectrum. For MP HRP A, these vibrational bands can be identified, since these bands show the same intensity distribution as the 0–0 bands. These bands, which are indicated by arrows in Figure 6C, are separated by 702, 1222, and 1572 cm^{-1} from the 0–0 bands. For MP HRP C, identification of the vibrational bands is more difficult because of lower resolution, but a band at 1547 cm^{-1} is evident. Addition of substrate appears to split this band.

Discussion

The two isoenzymes HRP A and C catalyze very much the same reactions although differing profoundly in essential properties: the amino acid composition and p*I*'s (4.0 and 8.8, respectively, A first), the electromotive force, at pH 6 (−146 and −208 mV), the rate constant for the formation of compound I with H_2O_2 (2×10^6 and 2×10^7 M^{-1} s^{-1}), and the affinity for aromatic donors (K_d for HRP A being on the average 3 times the K_d for HRP C) (Shannon et al., 1966; Paul, 1958; Paul & Stigbrand, 1970; Paul & Ohlsson, 1978; Marklund et al., 1974). In the present study, we examine whether further differences between these isoenzymes can be accounted for by different interactions between the polypeptide chain and the heme. To characterize the active site, fluorescent derivatives of the enzymes were prepared by replacing protoheme IX with protoporphyrin IX (Aviram, 1981; Ugarova et al., 1981) or mesoporphyrin IX. The reconstituted enzymes showed about the same binding affinity as the active enzymes for aromatic donors (Paul & Ohlsson, 1978) and hydroxamic acid derivatives (Table I). Furthermore, the native HRP C and PP HRP C show similar tryptophan fluorescence properties and circular dichroism spectra (Ugarova et al., 1981). Therefore, porphyrin HRP derivatives appear to be valid models for native HRP.

The two isoenzymes of HRP showed marked differences in the fluorescence emission spectra (Figures 1 and 2 and Table II). Several explanations for the different emission spectra were considered. Porphyrin emission is affected by solvents, as illustrated by the difference in emission maxima and lifetimes in ethanol/water and ethyl acetate. Leonard et al. (1974) suggested that the red shift in the fluorescence maximum of protoporphyrin IX upon binding to apohemoglobin was probably due to the decreased polarity in the protein cavity. The polarity of the peroxidase heme pocket is reported to be low (Tao, 1968; Rosen, 1970; Rosen & Nilsen, 1971). However, since fluorescence lifetimes and emission spectra of MP and PP depend only little upon solvent polarity (Table II), the large blue shifts of PP HRP C (624 nm) and MP HRP C (615 nm) relative to those of PP and MP in solvents (632 and 620 nm, respectively) indicate the existence of additional effects.

The fluorescence lifetimes of protoporphyrin in PP HRP C and A likewise differ from each other (16.2 and 15 ns, respectively) and from protoporphyrin in organic solvents. Oxygen-dependent quenching of the protoporphyrin fluorescence is much more pronounced in organic solvents than in aqueous media (Table II) due to the higher solubility of O_2 in organic solvents. When literature values (International Critical Tables, 1928) for the partitioning of oxygen into the organic solvent are used, the Stern–Volmer (Stern & Volmer, 1919) diffusion coefficient comes out as 2×10^{10} and 1.8×10^{10} for ethyl acetate and methanol, respectively. Oxygen quenched the lifetime of the protoporphyrin enzymes to a much lesser extent. [Note that oxygen in the bulk phase is responsible for the dynamic quenching. Assuming a partition coefficient of between 1 and 4 for O_2 between protein and water (4 for hydrocarbon/water) and a protein diameter of 6 nm, one finds that only from $^1/_{20}$th to $^1/_5$th of the protein molecules would contain oxygen under atmospheric pressure.] The Stern–Volmer constants for the protoporphyrin enzymes could not be accurately determined because the quenching was very small at atmospheric pressure, but they were around (1–5) $\times 10^9$. PP HRP C was somewhat less quenched by oxygen than PP HRP A.

The phosphorescence lifetimes without NHA are 5.5 ms for PP HRP C and 4.8 ms for PP HRP A at room temperature, another indication of a difference between the two derivatives. Since phosphorescence at room temperature can be quenched by torsional strains on the molecule, one reason for the longer lifetime may be that the porphyrin in HRP C is more protected than that in HRP A. Consistent with this is the observation that the heme is spontaneously released easier from HRP A than from HRP C (Smith et al., 1982).

A further clue on the state of the porphyrin derives from the low-temperature spectra. Under site-selection conditions, resolution in the emission spectra of MP HRP A and C can be obtained, proving that the spectra are inhomogeneously broadened (Figure 6). The distribution of 0–0 lines is greater for MP HRP C. Addition of substrate resulted in a change in the distribution of the sites and considerably increased the apparent inhomogeneous broadening for HRP C.

Additional information can be obtained from the vibrational structure in the emission spectra. These are also different for the two derivatives. The last emission bands are 1572 and 1547 cm^{-1} from the 0–0 emission for MP–HRP A and C, respectively. For MP HRP A, the vibrational structure is very resolved, and the same distribution of line seen on the 0–0 emission lines can be seen on the vibrational bands. For MP HRP C, no such structure is evident. Rakshit & Spiro (1974) deduced from Raman spectroscopy that the heme of native HRP C is domed, and Felton et al. (1976) showed that the binding of substrate to HRP made the heme become more planar. We find that binding of NHA shifts the fluorescence

emission spectrum of these vibrational bands. In the case of MP HRP A, the addition of substrate causes very little change in the 0–1 fluorescence. Since the emission spectrum is very resolved, the geometry of the ground and excited states must be similar, suggesting that the porphyrin is planar in HRP A.

The triplet lifetime of protoporphyrin as measured by its emission and absorption was decreased by the addition of NHA to either isoenzyme (Figure 6). At saturating concentrations of NHA, the lifetimes were ≤1 ms for HRP C and 2.8 ms for HRP A. Since the excited triplet state has such a long lifetime in comparison with the excited singlet state, it is not unexpected that the phosphorescence is much more sensitive to environment than fluorescence, based simply on the long lifetime of the triplet state. Although the effect of substrate on the fluorescence and phosphorescence yields at room temperature and the low-temperature spectra can be explained by conformational changes of the polypeptide chain, we point out that there could also be a direct interaction between NHA and porphyrin. Iron to substrate distances of 3.2–10.6 Å (cf. above) may be short enough to permit weak electronic exchange interactions between substrate and porphyrin (Mauzerall, 1978). Some indication of the formation of an exciplex between NHA and porphyrin is seen by the spectra shown in Figure 6. There appears to be an enhanced broad structureless emission in the presence of NHA. The vibrational band at 14 512 cm^{-1} seems to be split. Excitation at various frequencies should help to elucidate whether an exciplex is formed.

In conclusion, porphyrin derivatives of HRP A and C differ in fluorescence and phosphorescence properties. Furthermore, these properties are affected by the presence of substrate. These results provide direct evidence that the porphyrin is affected by the polypeptide chain in the absence of ligands.

Registry No. O_2, 7782-44-7; NHA, 10335-79-2; PP, 553-12-8; MP, 493-90-3; *p*-hydroxybenzohydroxamic acid, 5941-13-9; *o*-hydroxybenzohydroxamic acid, 89-73-6; *p*-chlorobenzohydroxamic acid, 1613-88-3; *o*-chlorobenzohydroxamic acid, 17512-69-5; benzohydroxamic acid, 495-18-1.

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