Site-Selected Fluorescence Spectra of Porphyrin Derivatives of Heme Proteins†

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Received May 9, 1985

ABSTRACT: The emission spectra of the porphyrin in metal-free and Zn cytochrome *c* and in metal-free mesoporphyrin derivatives of horseradish peroxidases A and C, leghemoglobin, and myoglobin were examined as a function of temperature and excitation wavelength. At room temperature, the emission spectra were unresolved and were independent of excitation wavelength. At low temperature (4.2 K), the spectra depended upon excitation wavelength: using narrow-band excitation into the high-energy side of the 0–1 and 0–0 bands gave unresolved emission spectra whereas excitation into the low-energy side produced quasi-line spectra. The resolved spectra were different for the five proteins and further varied with pH, indicating chromophore–protein interactions. The spectra are interpreted in terms of site selection and phonon interactions.

The optical absorption and emission spectra of large biological molecules are characteristically broad and structureless under conventional measuring conditions. Consequently, not much information concerning the vibrational properties of a chromophore or its interaction with surrounding groups can be obtained from absorption and fluorescence spectroscopy. However, for many organic molecules, high-resolution absorption and emission spectra can be obtained in amorphous condensed phases under conditions of site selection, i.e., narrow-band excitation to allow for the selection of a single subset of molecules and low temperature which decreases the density of states by ensuring that the molecule is in the lowest vibrational level and which minimizes interaction with the solvent by preventing a redistribution of sites (Kohler, 1979; Personov et al., 1972; Cunningham et al., 1975; Friedrich & Haarer, 1984).

Recently, site-selection spectroscopy has been applied to the study of chromophores in proteins. Quasi-line fluorescence spectra were obtained for the porphyrin in an iron-free cytochrome *c* derivative (Angiolillo et al., 1982) and for chlorophyll in greening etiolated leaves (Avarmaa et al., 1984). Friedrich et al. (1981) have used hole-burning techniques to obtain resolved spectra of native phycoerythrin.

Spectra taken under site-selection conditions are generally interpreted to be composed of sharp "zero-phonon" lines which arise from vibrational modes in the chromophore and broad "phonon side bands" which occur because the ground and excited states of the chromophore interact differently with the surrounding molecule(s). Both of these are of interest to the study of proteins. The zero-phonon lines yield spectra which are comparable in terms of information to Raman spectra. Phonon interactions are of interest for the study of chromophores in proteins since the "solvent" is the polypeptide chain; low-frequency vibrations are predicted to play a role in the catalytic functions of enzymes (Gurd & Rothgeb, 1979; Karplus & McCammon, 1981; Chou, 1984).

In this report, we present fluorescence spectra of iron-free heme proteins. Use of metal-free derivatives allows for examination of polypeptide–porphyrin interactions without the complications of metal ligation. The proteins include mesoporphyrin IX derivatives of horseradish peroxidases A and C (MP HRP A and C).[1] These two isoenzymes both catalyze the oxidation of a wide variety of compounds using H_2O_2 but have different polypeptide chains; both contain a single heme which is not covalently bound to the protein. Other proteins examined were mesoporphyrin IX derivatives of myoglobin (MP Mb) and leghemoglobin (MP leg-Hb), both monomeric proteins which reversibly bind oxygen and in which the heme is noncovalently attached to the polypeptide chain. We compare their fluorescence spectra with the metal-free and Zn derivatives of cytochrome *c*, a protein in which the meso-type porphyrin is covalently linked to the polypeptide chain. We interpret the spectra in terms of current theories on spectra in amorphous media, show that details in the emission spectra reveal interactions between the porphyrin and in the polypeptide chain, and discuss possible origins for inhomogeneous broadening in proteins.

Materials and Methods

"Porphyrin" (iron-free) cytochrome *c* was prepared from horse cytochrome *c* as described in Vanderkooi & Erecinska (1975). Zn cytochrome *c* was prepared from the iron-free derivative as reported by Vanderkooi et al. (1976). Sperm whale myoglobin was purchased (Sigma Chemical Co., St. Louis, MO) and purified, the largest fraction being used (Rothgeb & Gurd, 1978). HRP A and C (Paul & Stigbrand, 1970a,b) and leg-Hb (Appleby et al., 1975) were isolated. They were split into heme and protein (Teale, 1959), and the latter was dialyzed against several changes of water to remove 2-butanone. Mesoporphyrin, prepared from hydrolysis of its chromatographed and twice-crystallized methyl ester by 6 M HCl, was dissolved in 4 equiv of NaOH and suitably diluted with Tris-HCl, pH 8. Apoprotein was allowed to react with 25% excess of MP for 1 h at pH 8, 4 °C, then dialyzed against the appropriate buffer, and chromatographed. Some denatured protein and excessive or damaged MP had to be re-

† This work was carried with support from National Science Foundation Grant PCM 84-0844 and Swedish Medical Research Council Grant 3X-7130.
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[1] Abbreviations: MP, mesoporphyrin IX; HRP C, horseradish isoperoxidase type C2 (basic); HRP A, horseradish isoperoxidase type A2 (acidic); Mb, myoglobin; leg-Hb, leghemoglobin; DEAE, diethylaminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-7931$01.50/0

moved. Gel filtration did not yield good protein–MP preparations as judged from the visible spectrum. Separation was achieved by means of ion-exchange CM-52 chromatography in 50 mM sodium phosphate, pH 6.5, for MP Mb and MP HRP C, and by DEAE-52 chromatography in 50 mM sodium acetate for leg-Hb (pH 5.2) and HRP A (pH 5.8). Porphyrins are spontaneously released from apoproteins much faster (k = 0.005–0.1 min^{-1}) than hemes,[2] and therefore, DEAE is competitive for MP. This procedure ensured that no unbound porphyrin was in our samples. (For our experiments, it did not matter that the porphyrin:protein ratio was less than 1 since we were examining the emission only from the porphyrin.)

Fluorescence spectra were obtained by using the equipment described previously (Angiolillo et al., 1982). Excitation was with a Spectra Physics 171 krypton ion laser or a Coherent 599 dye laser which was pumped by the krypton laser. Power at the sample was typically 100–200 mW. We saw no dependency in the emission spectrum upon the power of the exciting light under these conditions. The emission wavelengths were isolated with a 1-m JY Ramanor HG2S double monochromator. The effective instrumental resolution was 1 cm^{-1}.

Samples were frozen in two ways. In one, dropwise addition of the sample (which included 50% propanediol) was made to liquid nitrogen. The resulting pellets were immersed in liquid helium or nitrogen in an optical Dewar (H. S. Martin, Vineland, NJ). In this arrangement, the pellets were in direct contact with the liquid. In the second, the sample in 50% glycerol/water in a plastic cuvette was immersed into liquid nitrogen and then placed into the liquid helium. This procedure gave optically clear samples.

Spectra taken with two or more separate preparations of the proteins and with the two freezing techniques gave reproducible results.

Results

Emission Spectra from Porphyrin Cytochrome c. Figure 1 shows the emission spectra of porphyrin cytochrome c at 293, 77, and 4.2 K. At 293 K, the spectra are broad with maxima at 16 200 and 14 700 cm^{-1}; the spectra follow "Kasha's rule" in that the emission spectra do not change with excitation wavelength (Kasha, 1950). At 77 and 4.2 K, the emission spectra show broad bands when excited at 520.8 and 530.9 nm, although the bands are somewhat more narrow than those at room temperature. In contrast, considerable fine structure is evident when excitation at 568.2 nm is used (peaks at 16 065, 16 149, 16 191, 16 255, 16 280, and 16 381 cm^{-1}). These peaks are evident at both 77 and 4.2 K; however, the bandwidths are about 3 times narrower at the lower temperature.

The emission of porphyrin cytochrome c was further characterized by varying the excitation wavelengths through the lower energy side of the 0–1 band (Figure 2). When the sample is progressively excited at higher wavelength throughout the 0–1 band, the spectra simplify until sharp peaks are observed. The arrows indicate peaks which are shifted 730 cm^{-1} from the excitation frequency. These peaks "appear" and "disappear" over an excitation range of about 400 cm^{-1}. In the case of excitation at 588.7 nm, a single line could be seen with an associated "wing" of about 100-cm^{-1} width. When one excites into the high-energy side of the 0–0 band at 605.3 nm, only broad emission can be observed. We discuss this later in terms of phonon interactions.

The effect of pH on the emission spectra was measured over a range where side groups on the protein undergo protonation

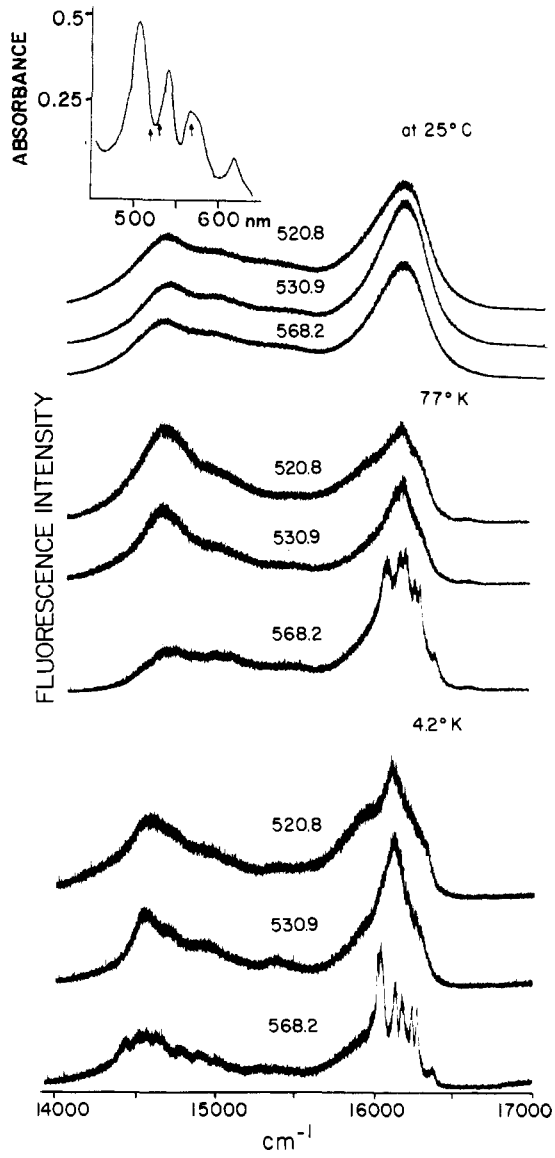

FIGURE 1: Fluorescence emission spectra of porphyrin cytochrome c at 25 °C, 77 K, and 4.2 K. At 77 and 4.2 K, porphyrin cytochrome c (0.05 mM) was dissolved in 1:1 propanediol/water (v/v). At 25 °C, the sample was dissolved in 10 mM phosphate buffer, pH 7. The inserted absorption spectrum indicates the excitation frequencies. Excitation wavelengths and temperatures are indicated on the figure.

but the porphyrin ring does not (Figure 3). At extreme pHs, the relative intensity of the fine-structured bands changes but their absolute positions do not.

Emission from Zn Cytochrome c. In order to further characterize the spectra obtained under site-selection conditions, we examined the emission of the zinc derivative of cytochrome c; this derivative was used because we could excite into the 0–0 absorption band using our equipment. The results are shown in Figure 4. Excitation at 568.2 nm, on the low-energy side of the 0–1 absorption band, gave sharp emission bands superimposed upon unstructured emission (Figure 4A). Excitation at 580.1 nm, at the peak of the room temperature absorption, gave only unstructured emission. Emission using excitation at 582.8 and 588 nm gave sharp lines; in the latter case, an essentially phononless spectrum was obtained (Figure 4D).

The spectra obtained when exciting into the 0–0 absorption band are arranged in Figure 4 as the change in frequency from the laser excitation line and are aligned with the 0–0 line obtained by using 568.2-nm excitation. The sharp lines occur at the same place (Figure 4 A,C,D), and we can identify lines

[2] K.-G. Paul, M. L. Smith, R. J. Ohlsson, and K. Hjortsberg, personal communication.

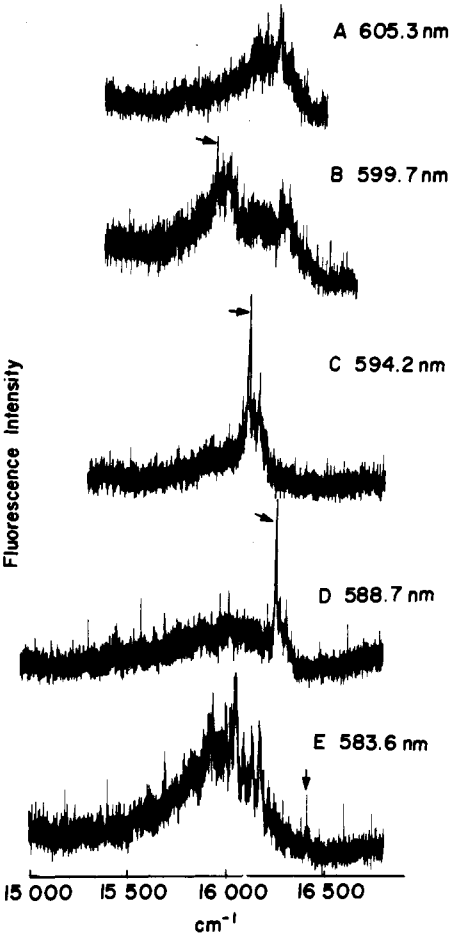

FIGURE 2: Site-selected spectra of porphyrin cytochrome *c* in 1:1 propanediol/water (v/v) at 4.2 K. The excitation wavelengths are given in the figure. Conditions are given in Figure 1.

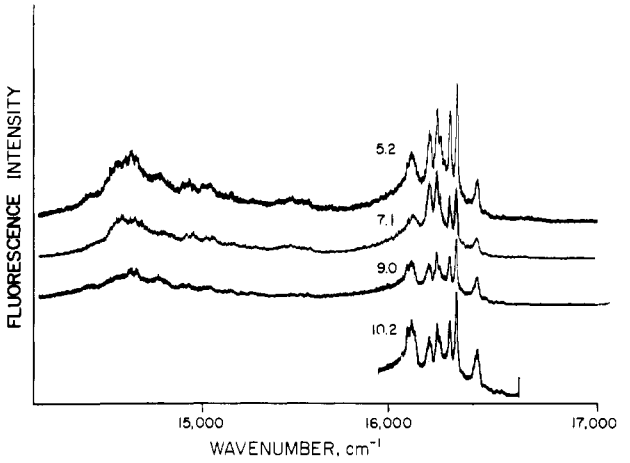

FIGURE 3: pH dependency of emission of porphyrin cytochrome *c*. Excitation wavelength, 568.2 nm; temperature, 4.2 K. Porphyrin cytochrome *c* was dissolved in 10 mM phosphate buffer at the indicated pH and in 50% glycerol. Temperature, 4.2 K.

at 755, 798, 971, 1012, 1150, 1220, 1320, 1357, 1398, 1428, 1551, and 1606 cm^{-1}.

Emission Spectra from Mesoporphyrin Derivatives of Proteins. The emission spectra of MP HRP C, MP HRP A, MP leg-Hb, and MP Mb were unresolved at room temperature and, like porphyrin cytochrome *c*, follow Kasha's rule (not shown). At 4.2 K, the emission spectra of all these proteins showed some resolution using 520.8 and 530.2 nm for excitation; however, with excitation at 568.2 nm, quasi-line emission spectra were obtained (Figures 5–8). The positions

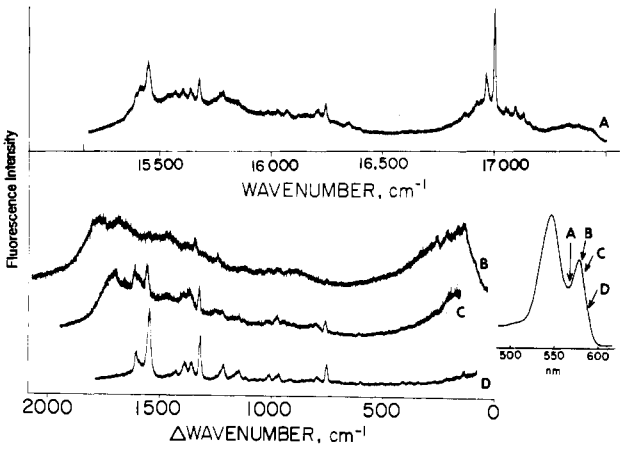

FIGURE 4: Emission from Zn cytochrome *c* at 4.2 K. Zn cytochrome *c* (1 μM) in 10 mM phosphate buffer, pH 7, and 50% glycerol. Excitation: (A) 568.2 nm; (B) 580.1 nm; (C) 582.8 nm; (D) 588.0 nm. Insert: Absorption spectra of Zn cytochrome *c* at room temperature.

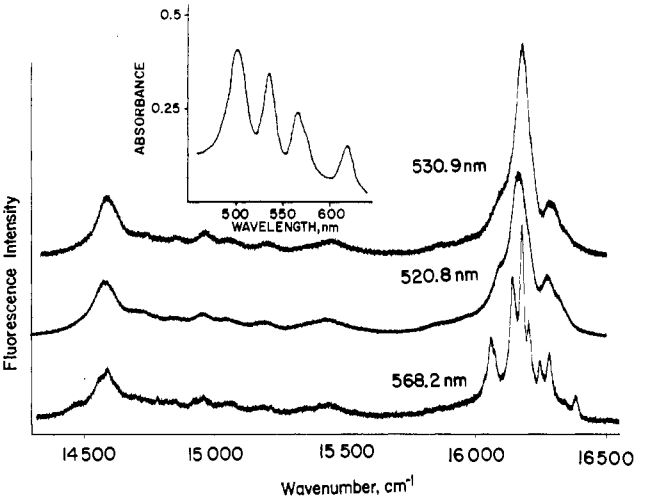

FIGURE 5: Emission spectra of MP HRP A (10 μM) in 1:1 propanediol/50 mM phosphate buffer, pH 6 (v/v), at 4.2 K. The inserted spectrum shows the absorption at room temperature. Excitation frequencies are indicated on the figure.

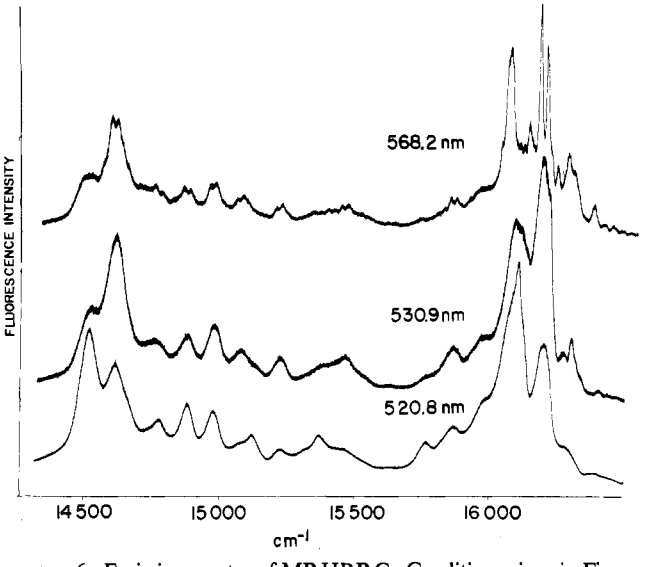

FIGURE 6: Emission spectra of MP HRP C. Conditions given in Figure 5.

of the emission peaks and the relative intensities are different for each of the mesoporphyrin proteins. For example, for MP

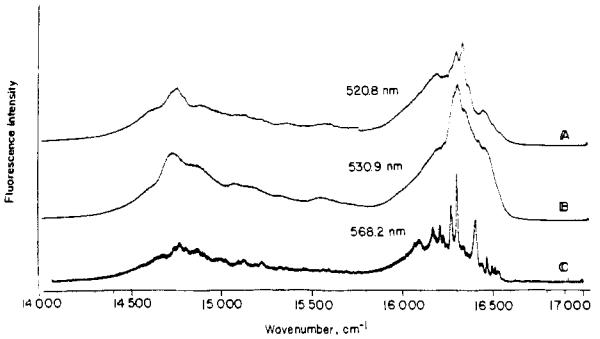

FIGURE 7: Emission of MP leg-Hb. Conditions given in Figure 5.

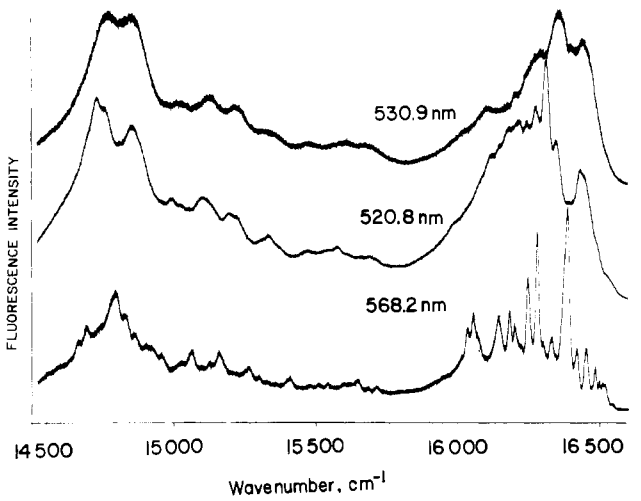

FIGURE 8: Emission of MP Mb. Conditions given in Figure 5.

HRP C, a partially resolved "doublet" at 16 070 cm^{-1} is the predominant feature (Figure 6); this feature is seen in the spectra of the other proteins but is relatively much smaller. Since the chromophore is the same in all these examples, the difference in relative intensity of the emission peaks is an indication of chromophore–protein interactions.

Discussion

Our data show that resolved spectra can be obtained for porphyrins in iron-free heme proteins. In order to interpret the results, we first discuss the cases where resolved fluorescence spectra are observed and then consider reasons for broadening.

Thousands of degrees of freedom are possible for proteins in solution, although many conformations are unlikely. Realistically, however, there are perhaps hundreds of species of molecules in the inhomogeneous mixture, all of which are excited by using broad-band excitation. With narrow-band excitation, only those molecules with transitions which are isoenergetic with the excitation frequency will be excited. When excitation is into the low-energy side of the 0–1 or 0–0 bands, and the interconversion of conformations is prevented by low temperature, simple emission spectra are obtained (Figures 2 and 4D); these spectra shift with the excitation frequency as predicted from the selection of subsets of molecules with corresponding deviations in the electronic transition energy. Although it is not ruled out that some of the resolved lines are from Raman emission, this is unlikely for the major features of the spectra, as concluded from two lines of evidence. First, expected internal conversion rates are very fast compared with the measured fluorescence lifetimes—15 ns for the free base porphyrins and 3 ns for Zn porphyrin cytochrome *c* (Dixit et al., 1982). Hence, it is very unlikely that unrelaxed excited states will be observed. Second, direct experimental evidence that emission occurs from the same state regardless of excitation is shown in Figure 4A,D. Excitation into the 0–1 band of Zn cytochrome *c* gives an intense band at 17 003 cm^{-1}, at 596 cm^{-1} removed from the excitation frequency. Excitation into the 0–0 band gives identical vibrational structure as excitation into the 0–1 band with no feature at 596 cm^{-1} removed from the excitation frequency. Therefore, the spectra appear to be due to fluorescence.

Next, we consider why the spectra show so many bands when excited into the 0–1 vibronic band. If there is a distribution of molecules having different transition energies and the excited vibrational levels are closely spaced, then it is possible to excite different subpopulations of molecules by exciting into different excited-state vibrational levels. Multiple 0–0 lines will be observed, one for each of the subpopulations of molecules. The relative intensities of the 0–0 lines when exciting into the higher vibrational levels will be a function of the inhomogeneous broadening and the transition probabilities of the absorption.

Finally, in addition to the inhomogeneous broadening, other factors can broaden the spectra. Homogeneous broadening can arise from lattice vibrations which produce a broad phonon wing associated with each 0–0 transition. Coupling between the phonons of the surrounding molecules and the chromophore occurs because changes in the electronic state of the chromophore upon excitation produce slight changes in the equilibrium position of the surrounding molecules. This coupling produces a broad absorption, called the phonon wing, to the high-energy side of narrow zero-phonon absorption band. Therefore, excitation at the red edge which favors excitation into the phonon bands produces unresolved emission. This has been documented by measuring the dependence of the emission by tuning the excitation of organic molecules in condensed phases (Abram et al., 1975).

The emission pattern obtained for the porphyrin derivatives resembles the general characteristics for organic molecules. The multiple bands in the 16 400-cm^{-1} region which were observed for the free base porphyrin derivatives using 568.2-nm excitation (low-energy side of the 0–1 band) can be interpreted in terms of 0–0 bands arising from excitation of different sites. Excitation at the higher energy side of this band (530.9 nm) produced unresolved emission spectra in all the porphyrin proteins. We note also that excitation at 520.8 nm produced unresolved emission spectra. At 520.8 nm, we are exciting into the low-energy side of another electronic level. The inhomogeneous broadening is expected to be different for each electronic level, and therefore, the lack of resolution may be a combination of both types of broadening.

For heme proteins of the polypeptide chain, the ligands on the iron and the surrounding solvent combine to determine the reactivity of the iron; however, the relative influence of each is not known. For a number of native and artificial (2,4-substituted heme groups) heme proteins, the stretch frequency of the CO infrared absorption responded to changes in the Fe(III)/Fe(II) reduction potential (E_{m7}) in a consistent manner, irrespective of whether such changes were brought about by having protoheme combined with various protein moieties (i.e., native heme proteins) or bringing one apoprotein to combine with various hemes (Smith et al., 1984). It was concluded that the protein moiety can affect the electron availability at the iron atom via the chelating porphyrin. The present exploration of the heme-accommodating pocket in four protohemoproteins, using mesoporphyrin as a probe, was undertaken because in this situation no metal–protein bonds exist.

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.

ACKNOWLEDGMENTS

We gratefully acknowledge the Regional Laser and Biotechnology Laboratory, Department of Chemistry, University of Pennsylvania, for use of equipment. We thank Drs. R. Hochstrasser, J. Leigh, and G. Holtom for helpful discussions.

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

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Received May 9, 1985

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

† This work was supported by National Science Foundation Grant PCM 84-0844 and Swedish Medical Research Council Grants 03X-6522 and 7130.
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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.

0006-2960/85/0424-7935$01.50/0