Spectral Properties of Environmentally Sensitive Probes Associated with Horseradish Peroxidase[†]

Mauricio Lasagna,[‡] Victor Vargas,[§] David M. Jameson,[||] and Juan E. Brunet*,[‡]

Instituto de Química, Universidad Católica de Valparaiso, Casilla 4059, Valparaiso, Chile, Departamento de Química, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile, and Department of Biochemistry and Biophysics, University of Hawaii, 1960 East-West Road, Honolulu, Hawaii 96822

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ABSTRACT: The environmentally sensitive fluorescent probes 6-propionyl-2-(*N*,*N*-dimethylamino)naphthalene (PRODAN) and 2′-(*N*,*N*-dimethylamino)-6-naphthoyl-4-*trans*-cyclohexanioc acid (DANCA) form complexes with the heme binding site of apohorseradish peroxidase. The dissociation constants of the PRODAN and DANCA complexes were determined from anisotropy titration data to be approximately 8.7×10^{-5} and 3.3×10^{-4} M, respectively. From comparison of the steady state fluorescence spectra of PRODAN and DANCA in solvents of varying dielectric constants, and in the apohorseradish peroxidase complex, we conclude that the heme binding site of horseradish peroxidase is relatively polar. The lifetimes of PRODAN and DANCA in organic solvents of varying polarities can be fit to single exponential decays. However, the lifetimes of PRODAN and DANCA associated with apohorseradish peroxidase, determined using a background subtraction method to correct for the non-negligible fluorescence of unbound probe, fit best to a distribution of lifetime values. We attribute these lifetime distributions to microenvironmental heterogeneity which is also consistent with the observed dependence of the emission maxima of PRODAN−apohorseradish peroxidase upon the excitation wavelength. In neither the PRODAN nor the DANCA case was evidence found in the time-resolved data for relaxation of the protein matrix around the excited state probe dipole.

Horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) (HRP)[1] is a member of the important group of peroxidases, found in plants, animals, and microbes, which catalyze the oxidation and peroxidation of a variety of organic and inorganic compounds. A number of acidic, neutral, and basic isoenzymes of HRP have been identified and classified (Shannon et al., 1966; Paul & Stigbrand, 1970; Delincee & Radola, 1970; Bartonek-Roxå et al., 1991). HRP-C is a monomeric glycoprotein with a molecular mass of 44 000 Da (approximately 18% of the molecular mass is due to eight covalently linked carbohydrate chains) which contains one noncovalently bound hemin moiety linked to the protein matrix through hydrophobic and electrostatic interactions among the propionic groups and the basic amino acids of the active site (Yonetani et al., 1972; Yoeman & Hager, 1980). The fifth position of the iron is linked to the imidazole group of His 170, while the sixth position is unliganded (Dunford, 1982, 1993; Smulevich et al., 1991). Although the sequence of HRP-C has been elucidated (Welinder, 1976, 1979, 1985) and this isoenzyme (Smith et al., 1990; Hartmann & Ortiz de Montellano, 1992) and HRP-*n* (Bartonek-Roxå & Eriksson, 1994) have been recently cloned, crystal structures have not yet been obtained. The active sites of HRP, hemoglobin, and myoglobin all utilize the same hemin moiety, yet the chemical reactivity of the heme group in HRP is quite distinct from the reactivities of these other hemeproteins. One common approach to the study of HRP's active site is the substitution of various hemin analogues (Tamura et al., 1972; Mauk & Girotti, 1974; Ugarova et al., 1979, 1981; Aviram, 1981; Horie et al., 1985). For example, Ugarova et al. (1981) studied the shift in the Soret absorption of PPIX complexed with apoHRP and observed that the pK_a for protonation of the protoporphyrin pyrrolic ring was decreased by 4 units compared to that of free PPIX, from which they concluded that the dielectric constant of the heme binding site was near 20. More recently, Smulevich et al. (1994) investigated the heme active site of HRP-C using resonance Raman spectroscopy on a recombinant protein and several mutants, prepared by site-directed mutagenesis methodologies, and concluded that the distal heme pocket architecture differed significantly between these proteins and comparable cytochrome *c* peroxidase mutants.

Naphthalene-based fluorescence probes have often been utilized to estimate the polarity of biological environments such as micelles, membranes, and specific regions of proteins. Studies on the heme binding site of myoglobin using anilinonaphthalene derivatives such as 1-anilino-8-

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* To whom correspondence should be addressed.
[‡] Universidad Católica de Valparaiso.
[§] Universidad de Chile.
[||] University of Hawaii.

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[1] Abbreviations: PRODAN, 6-propionyl-2-(*N*,*N*-dimethylamino)-naphthalene; DANCA, 2′-(*N*,*N*-dimethylamino)-6-naphthoyl-4-*trans*-cyclohexanioc acid; LAURDAN, 6-dodecanoyl-2-(*N*,*N*-dimethylamino)-naphthalene; ACRYLODAN, 6-acryol-2-(dimethylamino)naphthalene; ANS, 1-anilino-8-naphthalenesulfonic acid; bis-ANS, 4,4′-bis(1-anilino-8-naphthalenesulfonic acid); TNS, 2-*p*-toluidinylnaphthalene-6-sulfonic acid; PROMEN, 6-propionyl-2-methoxynaphthalene; HRP, horseradish peroxidase; apoHRP, apohorseradish peroxidase; PPIX, protoporphyrin IX; DMF, dimethylformamide.

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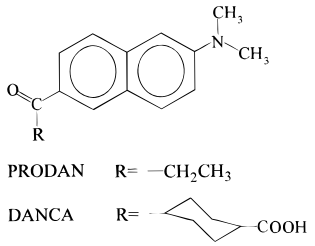

FIGURE 1: Structures of PRODAN and DANCA.

naphthalene sulfonate (ANS) or 2-*p*-toluidinyl-6-naphthalene sulfonate (TNS) are well-known examples of the use of environmentally sensitive probes (Stryer, 1965; Gafni et al., 1977; Dodluk et al., 1979; Bismuto et al., 1987b). Another group of environmentally sensitive naphthalene-based probes was synthesized by Weber (Weber & Farris, 1979). These probes, 6-propionyl 2-(*N*,*N*-dimethylamino)naphthalene (PRODAN) (Figure 1), 2′-(*N*,*N*-dimethylamino)-6-naphthoyl-4-*trans*-cyclohexanioc acid (DANCA) (Figure 1), and 6-dodecanoyl-2-(*N*,*N*-dimethylamino)naphthalene (LAURDAN) were designed to possess large and well-defined excited state dipole moments, with respect to the nuclear framework. Prendergast et al. (1983) also synthesized 6-acryol-2-(dimethylamino)naphthalene (ACRYLODAN), a sulfhydryl reactive derivative of PRODAN. The excited state dipole of PRODAN, originally estimated at 18−20 D (Weber & Farris, 1979), has more recently been estimated as 7−8 D (Balter et al., 1988; Catalan et al., 1991; Bunker et al., 1993). Macgregor and Weber (1986) utilized the absorption and fluorescence properties of DANCA to study the polarity of the heme binding site of sperm whale myoglobin. DANCA was specifically synthesized with the rationale that the additional carboxylic acid moiety would improve its binding to apomyoglobin with respect to PRODAN which bound poorly. DANCA did, in fact, bind more strongly to apomyoglobin than PRODAN, and the dissociation constant of the DANCA−apomyoglobin complex was found to be 1.2×10^{-5} M. On the basis of the spectrum of the bound probe, Macgregor and Weber concluded that the heme binding site of sperm whale myoglobin was strongly polar, contrary to previous assessments (Stryer, 1965). This polarity was accounted for semiquantitatively by consideration of the electrostatic interaction energies of the peptide bond dipoles, near the heme binding site, with the bound probe. Macgregor and Weber (1986) furthermore pointed out that the virtual insolubility of ANS in truly nonpolar solvents excluded the possibility of establishing a reliable polarity scale for this probe.

The heme binding site of horseradish peroxidase (HRP) has not been studied by fluorescence methods as extensively as that of myoglobin. Some studies on HRP have been carried out using ANS, TNS, bis-ANS, and protoporphyrin IX (Tao, 1969; Rosen, 1970; Ugarova et al., 1981; Jullian et al., 1989; Brunet et al., 1994). The dynamics of the HRP heme binding site have been studied by measuring its accessibility to molecular oxygen (Brunet et al., 1990; Vargas et al., 1991) and by determining the mobility of fluorescent probes associated with this site (Brunet & Pulgar, 1993; Brunet et al., 1994). The exceptional sensitivity of PRODAN and DANCA to their electrostatic environments and the intriguing results of Macgregor and Weber on the DANCA−apomyoglobin system prompted us to apply similar methodologies to the HRP system. Our initial goal was, specifically, to ascertain if environmentally sensitive probes such as PRODAN and DANCA would associate with the heme binding site of horseradish peroxidase and illuminate aspects of the the heme environment such as general polarity and relaxation processes. These studies were also motivated, in part, by our previous demonstration that the heme binding site of HRP was very inaccessible to oxygen which implied that the protein matrix surrounding the bound heme moiety was fairly rigid (Vargas et al., 1991). In our initial experiments, however, we ascertained that PRODAN binds significantly better to apoHRP than does DANCA, in contradistinction to the myoglobin system. We report here on steady state and time-resolved fluorescence studies of PRODAN and DANCA, in solvents of varying polarities and associated with the heme binding site of HRP.

MATERIALS AND METHODS

ApoHRP was prepared from Sigma Type VI HRP by extraction of the heme group using Teale's method of cold acid and butanone extraction (Teale, 1959), followed by exhaustive dialysis at 4 °C against 0.1 M pH 7.4 sodium phosphate buffer. The concentration of apoHRP was determined spectrophotometrically using a molar absorption coefficient at 280 nm of 20 000 M^{-1} cm^{-1} (Morishima et al., 1977). PRODAN was purchased from Molecular Probes, Inc. (Junction City, OR). Bunker et al. (1993) reported a fluorescent contaminant in samples of PRODAN from Molecular Probes, Inc., which was revealed by the appearance of an emission near 430 nm upon 280 nm excitation. Considering its spectral properties, this contaminant is probably 6-propionyl-2-methoxynaphthalene (PROMEN), one of the intermediates in the synthetic pathway to PRODAN (Weber & Farris, 1979). We noted this contaminant in some of our PRODAN samples but not in others; when present, the contaminant was extracted from a saturated water solution with *n*-hexane according to the procedure of Bunker et al. (1993). DANCA was a generous gift from Professor Gregorio Weber and was used without further purification. The PRODAN−apoHRP and DANCA−apoHRP adducts were prepared (unless otherwise specified) by addition of an appropriate amount of a solution of PRODAN or DANCA in water to 2×10^{-4} M solutions of apoHRP; the final ratio of apoHRP to probe depended upon the experiment and was usually in the range of 300. The concentrations of the probes were estimated using molar extinction coefficients at 360 nm of 14 500 M^{-1} cm^{-1} for aqueous solutions and 18 300 M^{-1} cm^{-1} for ethanol solutions (Weber & Farris, 1979; Macgregor & Weber, 1986). Absorption measurements were performed on a Varian Cary 219 spectrophotometer, while fluorescence spectra were obtained on a SPEX Fluorolog spectrofluorometer interfaced to an AT&T 6300 minicomputer utilizing software from ISS, Inc. (Champaign, IL). Anisotropy titration measurements on the PRODAN and DANCA complexes with apoHRP were done using an ISS Greg 200 spectrofluorometer with excitation at 360 nm and observation of the emission at wavelengths greater than 380 nm through a Schott KV399 cut-on filter.

Time-resolved measurements were obtained using an ISS Greg 200 multifrequency phase and modulation fluorometer. Excitation was accomplished using the 325 nm line of a helium−cadmium laser (Liconix Model 4240NB, Sunnyvale,

CA), and emission was observed through a Schott KV399 cut-on filter. The exciting light was vertically polarized, and emission was observed through a polarizer oriented at an angle of 55° with respect to the vertical axis to eliminate polarization effects on the lifetime values (Spencer & Weber, 1970). In the multifrequency phase and modulation technique, the intensity of the exciting light is modulated, the phase shift and relative modulation of the emitted light, with respect to the excitation, are determined, and lifetimes are then calculated according to well-known equations (Spencer & Weber, 1969; Weber, 1981). The measured phase and modulation values may be analyzed as a sum of exponentials by using a nonlinear least squares procedure wherein the goodness of the fit to a particular model (for example, single or multiple exponentials) is judged by the value of the reduced chi-square (χ^2) (Jameson et al., 1984). The data can also be fit using a continuous distribution (Lorentzian) of lifetime values characterized by a center value and width as described by Alcala et al. (1987a).

The lifetime data on the PRODAN−apoHRP and DANCA−apoHRP complexes were derived using a background subtraction method (Reinhart et al., 1992). The method involves the measurement of phase and modulation values of a "blank" which determines the background phasor. This value is then subtracted from the sample phasor, and the true phase and modulation values for the sample are calculated. The solvent blank used in these cases was the dialysate solution obtained by dialysis of 2.6 mL of 3.1×10^{-5} M apoHRP and 0.94×10^{-6} M PRODAN (or 0.98×10^{-6} M DANCA) against 20 mL of buffer.

RESULTS

The dissociation constants for the PRODAN−apoHRP and DANCA−apoHRP complexes were estimated by an anisotropy titration method (Jameson & Sawyer, 1995) based on the original observations of Weber (1952) on the additivity of polarization values. Specifically, the anisotropies of PRODAN or DANCA solutions, in the absence and presence of apoHRP, were determined and the fraction of PRODAN and DANCA bound as a function of apoHRP concentration was estimated using the additivity of the anisotropy function, according to the equation

$$f_b = (r_{obs} - r_f)/(r_b - r_f) \quad (1)$$

where r_{obs} is the observed anisotropy and r_f and r_b are the anisotropies of free and bound probe, respectively. In this treatment, the relative quantum yields of free and bound probe must also be taken into account. The data are shown in Figure 2; the best fit curves corresponded to r_b equal to 0.30 in both cases and dissociation constants of approximately 8.7×10^{-5} and 3.3×10^{-4} M for PRODAN and DANCA, respectively. The r_b value of 0.30 was also determined directly by measurement of the anisotropy of apoHRP−probe complexes, at high protein−probe ratios, through a Corion 436 nm interference filter which isolates the emission primarily from the bound probe. These r_b values, as compared to the r_0 value of PRODAN or DANCA of 0.356 at 360 nm excitation determined in glycerol at 0 °C, are reasonable for both probe−protein cases, given the known rotational relaxation time for HRP of near 90 ns (Jullian et al., 1989; Brunet et al., 1994) and the short

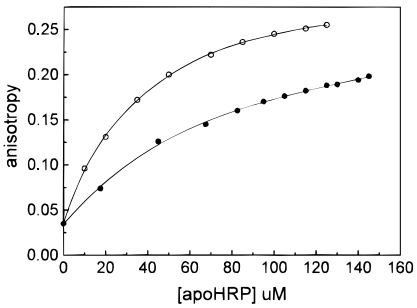

FIGURE 2: Anisotropy as a function of apoHRP concentration for the DANCA−(●) and PRODAN−apoHRP (○) complexes. Experimental points are fit with a K_d of 3.3×10^{-4} and 8.7×10^{-5} M, respectively.

lifetimes of the bound probes (*vide infra*). The anisotropies indicate that each probe experiences some degree of local motion in the heme binding site, as opposed to PPIX which is virtually immobile when associated with apoHRP (Brunet & Pulgar, 1993; Brunet et al., 1994). Specifically, if the rotational motions of the probes are modeled as isotropic, then the half-cone angles calculated for the local motions of PRODAN or DANCA associated with HRP are in the range of 13−14° while that of PPIX is on the order of 3°. The relative quantum yield of bound to free PRODAN was estimated to be 3.0 under the precise experimental conditions (excitation wavelength and emission filter) used in the anisotropy titrations (in the DANCA−apomyoglobin system, Macgregor and Weber found a 4-fold increase in the quantum yield upon binding).

The demonstration that PRODAN and DANCA are associated with the heme binding site of HRP is not as straightforward as it is for several other naphthalene-based probes (Vargas et al., 1991) due to the lower binding affinity which necessitates the use of higher apoHRP concentrations to facilitate binding and, hence, higher levels of hemin for displacement. These considerations suggested that the displacement of probe by hemin was best followed by anisotropy, which is relatively insensitive to the inner filter effects at the excitation and emission wavelengths caused by the hemin absorption. The decrease in anisotropy of the probe−apoHRP systems (data not shown), taking into account the relative quantum yields of bound and free probes, demonstrated that hemin displaced the probes stoichiometrically, indicating that the fluorophores are associated with the heme binding site as are other naphthalene-based probes (Vargas et al., 1991). It was also observed that the emission properties (maximum emission wavelength and band width) of micromolar solutions of PRODAN were not altered by addition of 2×10^{-5} M HRP, although the observed intensity was reduced due to inner filter effects, indicating the lack of interaction between PRODAN and the holoprotein.

To obtain the true emission spectra associated with PRODAN and DANCA bound to apoHRP, a symmetric-Gaussian fitting procedure (Origin from MicroCal Software, Inc., Northhampton, MA) similar to that described by Torgerson et al. (1979) was utilized. In these fits, the shapes of the emission spectra of free and bound probes are assumed to be Gaussian in the wavenumber domain, and since the curves associated with free probes are accurately known, the spectra corresponding to bound probes can be recovered from the composite spectra as shown in Figure 3. The corrected emission spectra of PRODAN in various solvents and the

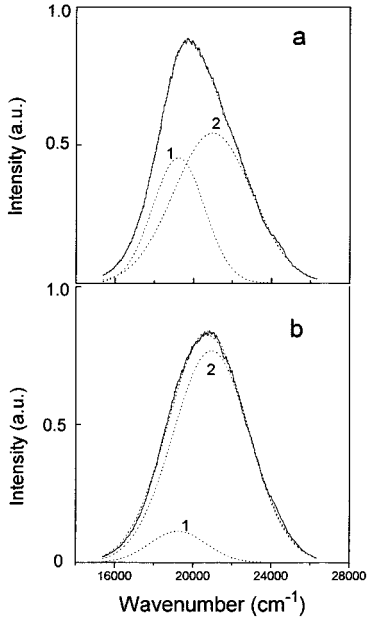

FIGURE 3: Fluorescence spectra of the probe−apoHRP conjugates in 0.1 M pH 7.4 phosphate buffer and symmetric-gaussian fits for (a) DANCA−apoHRP and (b) PRODAN−apoHRP. In both cases, the curves labeled 1 correspond to free probe while those labeled 2 are the resultant fit assigned to bound probe.

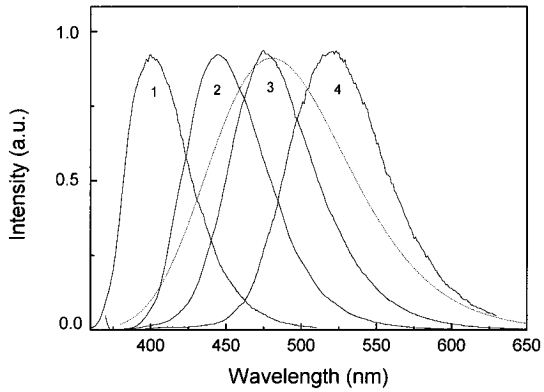

FIGURE 4: Corrected fluorescence spectra for PRODAN in (1) cyclohexane, (2) acetone, (3) 2-propanol, and (4) 0.1 M pH 7.4 phosphate buffer. The spectrum obtained from the Gaussian fit for PRODAN-apoHRP is shown by the dotted line. Excitation wavelength is 360 nm.

true emission spectra of the probe associated with apoHRP are shown in Figure 4. One notes that the emission maxima associated with PRODAN and DANCA bound to apoHRP are 478 nm (20 921 cm^{-1}) and 477 nm (20 964 cm^{-1}), respectively, indicating, in both cases, blue shifts relative to water (520 nm, 19 231 cm^{-1}). These results are tabulated in Table 1 along with the emission maximum observed by Macgregor and Weber (1986) for the DANCA−apomyoglobin system. The excitation wavelength dependence of the emission maximum of PRODAN in solvents of varying polarity and associated with apoHRP is shown in Figure 5. The values for the PRODAN−apoHRP complexes were obtained using the symmetric-Gaussian fit procedure described above.

The lifetime data for PRODAN and DANCA in several solvents is shown in Table 2; in all cases, the data could be fit to single exponential decays. The phase and modulation data for DANCA in glycerol [from Bismuto et al. (1987a)] and for PRODAN associated with apoHRP are given in Table 3. The lifetime data for PRODAN and DANCA in phosphate buffer and associated with apoHRP are summarized in Table 4. In these cases, the data fit best to a two-component model consisting of one fixed, discrete, very short component (attributed to scattered light) and one Lorentzian component.

Table 1: Emission Maxima of Corrected Fluorescence Spectra of PRODAN and DANCA in Various Solvents and Associated with apoHRP (20 °C)[a]

	PRODAN		DANCA	
	λ (nm)	ν (cm^{-1})	λ (nm)	ν (cm^{-1})
cyclohexane	400	25 000	insoluble	
acetone	445	22 472	445	22 472
DMF	455	21 978	455	21 978
2-propanol	477	20 964	474	21 097
ethanol	488	20 492	483	20 704
buffer[b]	520	19 231	520	19 231
apoHRP[c]	478	20 921	477	20 964
apomyoglobin[d]	−	−	459	21 786

[a] Excitation at 360 nm. [b] 0.1 M phosphate buffer (pH 7.4). [c] From the symmetric-Gaussian fit. [d] From Macgregor and Weber (1986).

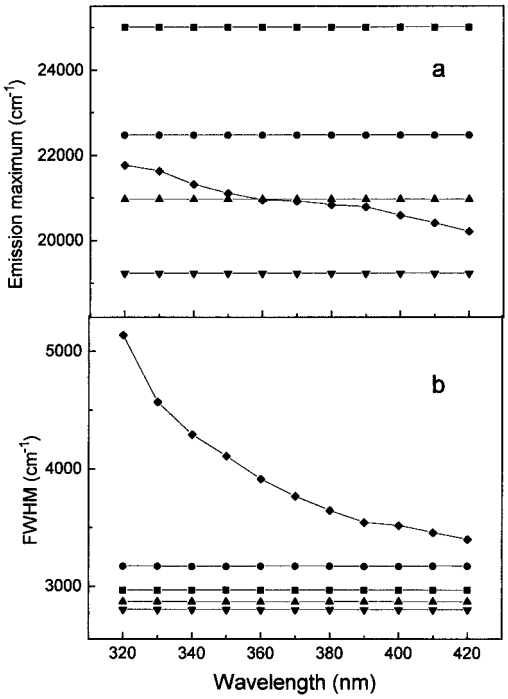

FIGURE 5: Excitation wavelength dependence of the emission of PRODAN in solvents of different polarity and associated with apoHRP. (a) Average energy of the fluorescence against excitation wavelength in cyclohexane (■), acetone (●), 2-propanol (▲), and 0.1 M pH 7.4 phosphate buffer (▼) and bound to apoHRP (◆). (b) Emission band width at half-maximum for the same solvents as above.

Table 2: Lifetimes of PRODAN and DANCA in Various Solvents (20 °C)[a]

	PRODAN		DANCA	
	τ (ns)	χ^2	τ (ns)	χ^2
cyclohexane	0.24	7.3	insoluble	
dioxane	3.18	1.4	2.63	3.2
acetone[b]	3.35	2.0	3.17	4.1
DMF	3.65	3.0	3.49	3.5
ethanol	3.37	1.4	3.40	3.0

[a] Excitation at 325 nm. [b] Excited at 360 nm with a Xenon lamp.

Table 3: Time-Resolved Data for DANCA in Glycerol and PRODAN Bound to apoHRP at 20 °C[a]

frequency (MHz)	phase	modulation	τ^P (ns)	τ^M (ns)
	(a) DANCA/Glycerol[b]			
10.00	16.00	0.967	4.56	4.19
20.00	33.30	0.883	5.23	4.23
30.00	41.80	0.781	4.74	4.24
40.00	50.80	0.683	4.88	4.26
60.00	64.70	0.523	5.61	4.32
90.00	80.20	0.374	10.24	4.38
120.00	88.90	0.273	69.07	4.67
150.00	97.50	0.217	−8.06	4.77
	(b) PRODAN/apoHRP			
15.00	18.06	0.886	3.46	5.55
19.00	21.29	0.853	3.26	5.12
24.00	25.12	0.810	3.11	4.80
30.40	28.83	0.757	2.88	4.52
38.50	32.55	0.715	2.64	4.04
48.70	37.14	0.645	2.47	3.87
61.60	40.80	0.588	2.23	3.55
78.00	45.10	0.520	2.05	3.35
98.70	49.75	0.450	1.91	3.20
124.90	52.75	0.388	1.68	2.03
158.00	55.71	0.334	1.48	2.84
200.00	58.89	0.280	1.32	2.73

[a] τ^P and τ^M represent phase and modulation lifetimes, respectively. [b] From Bismuto et al. (1987a).

Table 4: Lifetime Distribution (Lorentzian) Analysis of PRODAN and DANCA in Phosphate Buffer (0.1 M, pH 7.4) and Associated with apoHRP (20 °C)[a]

sample	center (ns)	width	f[c]	χ^2
PRODAN/buffer	1.29	1.07	0.931	3.0
PRODAN/apoHRP[b]	2.63	3.59	0.985	2.5
DANCA/buffer	1.54	0.74	0.987	2.5
DANCA/apoHRP[b]	2.91	1.95	1.000	3.3

[a] Excitation at 325 nm. [b] Data obtained using background substraction method. [c] In cases where f is less than unity, a small fractional component of 1 ps, attributed to scattered light, was used in the data fit.

DISCUSSION

The dissociation constants observed for the PRODAN−apoHRP and DANCA−apoHRP complexes, 8.7×10^{-5} and 3.3×10^{-4} M, respectively, indicate that at the concentrations of apoHRP utilized for steady state spectra (2×10^{-4} M) even a 300-fold excess of protein over probe still results in only 69 and 38% binding of PRODAN and DANCA, respectively. The 3-fold increase in quantum yield of the fluorescence probes upon binding means that, in these cases, approximately 87 and 65% of the total fluorescence is due to bound PRODAN and DANCA, respectively. The signal from free probe is thus non-negligible in both cases, and the true spectra and lifetimes associated with bound probes must be resolved from the mixture. By using the symmetric-Gaussian fit approach, we were able to estimate the spectral parameters of bound PRODAN and DANCA (Figure 3). The emission maxima of these probes, 478 and 477 nm, respectively, were quite red-shifted compared to the DANCA−apomyoglobin system which had an emission maximum of 459 nm (Macgregor & Weber, 1986) and indicate that the HRP heme binding site is, generally, more polar than that of myoglobin. PRODAN emits maximally at 477 nm in 2-propanol (Catalan et al., 1991), which has a dielectric constant of 18.3 (*Handbook of Chemistry and Physics*, 1988−1989). As pointed out by Macgregor and Weber (1986) and Valeur (1991), however, comparisons of emission properties of probes in bulk solvents and biological systems, such as protein interiors, are not straightforward. In fact, the details and dynamic aspects of the probe's microenvironment, including the location and disposition of charges and the presence of water molecules, will affect the emission characteristics. The three-dimensional structure of HRP is not yet known, and hence, we cannot carry out calculations on the electrostatic interactions of the peptide bond dipoles of apoHRP with PRODAN as was done in the DANCA−apomyoglobin case (Macgregor & Weber, 1986).

Previous studies on the photophysical properties of PRODAN (and by inference DANCA) do not reach a consensus on the exact interpretation to be given to the alteration of the various emission properties in different solvents (Rollinson & Drickamer, 1980; Nowak et al., 1986; Ilich & Prendergast, 1989; Balter et al., 1988; Catalan et al., 1991; Bunker et al., 1993; Zurawsky & Scarlata, 1994). For example, the extent of intramolecular charge transfer (IMCT) and twisted intramolecular charge transfer (TICT) character attributed to PRODAN is disputed (Rollinson & Drickamer, 1980; Nowak et al., 1986; Ilich & Prendergast, 1989; Bunker et al., 1993). There is, however, no disagreement over the fact that the excited state dipole moment of PRODAN is significantly larger than that associated with the ground state molecule. As mentioned earlier, one of the questions we wished to address is whether one can observe relaxation processes around this excited state dipole when the probes are located in the heme binding site. Such dynamic processes can manifest themselves by shifts in the observed emission maximum as a function of excitation wavelength (Galley & Purkey, 1970; Demchenko, 1982; Lakowicz & Keating-Nakamoto, 1984; Bismuto et al., 1987b; Chattopadhyay & Mukherjee, 1993) and also apparent increases in the phase lifetime as a function of modulation frequency (Lakowicz & Balter, 1982; Jameson et al., 1984; Lakowicz et al., 1984; Bismuto et al., 1987a,b). For example, time-resolved data indicated charge movements around the excited TNS fluorophore associated with sperm whale myoglobin but not with TNS associated with tuna myoglobin (Bismuto et al., 1987b). The apparent phase lifetime of DANCA in glycerol at 20 °C was shown to be very sensitive to such dipolar relaxation processes, displaying the characteristic increase in the phase lifetime with increasing modulation frequency (Table 3; Bismuto et al., 1987a). The lifetime data for PRODAN−apoHRP (Table 3) and DANCA−apoHRP (data not shown), however, gave no evidence of such relaxation processes which may suggest that the dynamic aspects of the HRP heme binding site differ from those of sperm whale myoglobin. On the other hand, the differences between the lifetimes obtained for the DANCA− and PRODAN−apoHRP complexes, as shown in Table 4, suggest that the probes experience different microenvironments and hence may be oriented slightly differently in the heme binding site. The decreased affinity of DANCA for apoHRP compared to PRODAN suggests that the carboxylic acid moiety on the DANCA presents an unfavorable interaction between this probe and the protein matrix that is not present in the PRODAN case. As already mentioned, this situation is exactly the opposite of the myoglobin case. Also the widths of the lifetime distributions are markedly different, the PRODAN complex having a

broader distribution. This difference in the width of the lifetime distributions is indicative of a difference in the protein matrix sensed by each probe. A narrow distribution is generally attributed to a more homogeneous environment (Alcala et al., 1987b,c). The width of the lifetime distribution associated with intrinsic tryptophan emission has been attributed to the existence of a large number of conformational substates of proteins (Alcala et al., 1987b,c; Silva et al., 1994).

As shown in Figure 5, the emission maxima and band widths of PRODAN dissolved in solvents of varying polarities, and low viscosities, do not display any dependence on the excitation wavelength, whereas such dependence is evident for PRODAN associated with HRP. Red shifts in the wavelength of maximal emission and narrowing of the band width upon shifts in the excitation wavelength toward the red edge of the absorption spectrum are indicative of relaxation processes in the solvent surrounding the fluorophore and are usually only observed in viscous solvents (Chattopadhyay & Mukherjee, 1993; Macgregor & Weber, 1981, 1986; Bismuto et al., 1987a). In the case of PRODAN−apoHRP, given the observed lack of dipolar relaxation effects on the time-resolved data (Table 3), we believe that the observed dependence of the fluorescence spectrum on the excitation wavelength is indicative of a heterogeneous population of PRODAN−apoHRP complexes, as postulated by Macgregor and Weber (1986) for the DANCA−apomyoglobin system. In myoglobin, the heme group is known to be able to bind in at least two orientations (La Mar et al., 1984), but comparable information on the heme orientation in HRP is lacking. Given the fact that the heme group in HRP binds more tightly to the apoprotein than in the myoglobin and hemoglobin cases (Brunet et al., 1990), though, we might expect that such plurality of heme orientations is less likely, but the weaker binding of PRODAN and DANCA to the heme binding site may allow for a plurality of fluorophore orientations. The lifetime distribution data for PRODAN−apoHRP and DANCA−apoHRP (*vide supra*) may also reflect microheterogeneity in the probe−protein population.

To summarize, our results indicate that the heme binding site of HRP-C, like that of myoglobin, has an overall polar character. The observed polarity may be due, in part, to the presence of one or more water molecules. The high-resolution structure of cytochrome *c* peroxidase indicates the presence of a water molecule in the heme binding site which is above but not coordinated to the iron (Finzel et al., 1984), and Smulevich et al. (1994) have speculated that a corresponding water molecule is coordinated with the iron in one of their site-directed mutants of HRP-C. Our results also suggest that the HRP-C heme binding site is more polar than that of myoglobin which may account, in part, for the known difference in the reactivities of the identical hemin moiety in these two hemeproteins.

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