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Time-resolved fluorescence studies on protoporphyrin IX-apohorseradish peroxidase

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The hemin moiety of horseradish peroxidase (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7) was removed and the apoprotein reconstituted with the fluorescent protoporphyrin IX. Steady-state and time-resolved fluorescence properties of the HRP(desFe) adduct were examined; the multifrequency phase and modulation method was utilized for lifetime and dynamic polarization studies. The emission spectrum of HRP(desFe) had maxima at 633 and 696 nm. The lifetime of this emission was characterized by a single exponential decay of 16.87 ns at 22°C. Debye rotational relaxation times for HRP(desFe) were determined using both static (Perrin plot) and dynamic (differential phase and modulation fluorometry) methods; these two approaches gave values of 96 and 86 ns, respectively. A spherical protein of HRP's molecular weight and partial specific volume would be expected to have a Debye rotational relaxation time, at 22°C, in the range of 50 to 60 ns, depending upon the extent of hydration. Hence our results indicate that HRP(desFe) is asymmetric; the global rotational relaxation times observed are consistent with those of a prolate ellipsoid with an axial ratio of 3:1.

Introduction

Horseradish peroxidase (donor: hydrogen-peroxidase oxidoreductase, EC 1.11.1.7) (HRP) is a member of the important group of plant peroxidases which catalyze the oxidation and peroxidation of a variety of organic and inorganic compounds. HRP is a monomeric glycoprotein of molecular weight 44000 containing one noncovalently bound hemin moiety. A number of acidic and basic isoenzymes of HRP have been identified and classified [1–3]. Although the sequence of the HRP-C isoenzyme has been elucidated [4–6], 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 different from these other hemeproteins. One common approach to the study of HRP's active site is the substitution of various hemin analogues [7–12]. Our particular interest concerns dynamic aspects of HRP in solution, including its overall hydrodynamic behavior and nanosecond fluctuations in the protein matrix. Fluorescence spectroscopy is an ideal technique to study these aspects, providing that the system can be rendered fluorescent. Although HRP has a single tryptophan, its emission is strongly quenched by energy transfer to the heme moiety [13,14]. The probability of energy transfer from other exogenous probes (such as FITC or dansyl) to the heme group complicates this approach also. The heme moiety can, however, be removed by various procedures and the protein reconstituted with fluorescent porphyrins such as protoporphyrin IX (PPIX). Several groups have, in fact, reported studies on the iron-free HRP (which we shall designate HRP(desFe)) [7–11]. In this report we discuss results obtained using steady-state and time-re-

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Abbreviations: HRP, horseradish peroxidase; PPIX, protoporphyrin; RZ, reinheitszahl.

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solved fluorescence methods which are directed towards elucidating hydrodynamic aspects of the protein.

Materials and Methods

Preparation of HRP(desFe)

HRP Sigma type VI (lot No. 74F-9525) which contains isoenzyme C (according to Shannon classification [1]), as the major component with a minor isoenzyme B component, or HRP Sigma type IX (lot No. 77F-9515) which contains only isoenzyme C, were utilized without further purification.

The heme group was removed using Teale's method of cold acid and butanone extraction]15], followed by exhaustive dialysis at 4°C against 0.1 M sodium phosphate buffer (pH 7.4). The concentration of apoHRP was determined spectrophotometrically using a molar absorption coefficient at 280 nm of 20000 [12]. A stock solution of Calbiochem PPIX disodium salt was prepared, dissolving, in the dark, 1 mg of PPIX in 10 ml of methanol. After stirring overnight, the PPIX solution was centrifuged for 1 h at $11000 \times g$, and the concentration was quantified spectrophotometrically using an absorption coefficient at 401 nm of 115000 $M^{-1} \cdot cm^{-1}$ (determined in our laboratory).

The HRP(desFe) was prepared by dropwise addition, with gentle stirring, of the stock PPIX solution to the apoHRP solution in 0.1 M phosphate buffer (pH 7.4); the PPIX solution was added until a 20% molar excess of PPIX over apoHRP was achieved. The solution was gently stirred for approx. 5 h at 4°C, centrifuged briefly in an Eppendorf microfuge, and then dialyzed for 24 h (at 4°C) against 0.1 M phosphate buffer (pH 7.4). Finally, the solution was filtered through a 0.2 μm Millipore filter. The final product had an Rz (A_{406}/A_{280}) of 3.2.

Fluorescence measurements

(1) Steady-state measurements. Steady-state fluorescence measurements were made using an ISS GREG 200 spectrofluorometer (Champaign, IL). Perrin plots were obtained maintaining the temperature at 22°C with a thermostated circulating bath to within ± 0.1 degree. The viscosity of the sample was varied by the addition of sucrose aliquots from a 60% stock solution. Solvent viscosities as a function of sucrose concentration were obtained from the Handbook of Biochemistry and Molecular Biology [16]. For the polarization measurements the conjugate was excited at 514 nm with a 4 nm bandwidth and the emission was observed through a Corion LL-600 cuton filter. Polarization was calculated according to the equation:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities corresponding to the parallel and perpendicular polarized components, respectively, of the emission.

(2) Time-resolved measurements. Lifetime and dynamic polarization measurements were carried out using either the ISS Greg 200 spectrofluorometer or a homebuilt multifrequency phase and modulation fluorometer based on the Gratton design [17,18]. Excitation of the HRP(desFe) was accomplished using the 514.5 nm line of an argon ion laser (Spectra Physics Model 2035-3.5S), and the emission was observed through an OG 570 cuton filter (Schott) which passes wavelengths longer than 560 nm. For lifetime measurements the exciting light was polarized parallel to the vertical laboratory axis while the emission was viewed through a polarizer oriented at 55°. In the multifrequency phase and modulation technique the intensity of the exciting light is modulated and the phase shift and relative modulation of the emitted light, with respect to the excitation, are determined. Phase and modulation lifetimes (τ^P and τ^M) are then calculated according to the equations:

$$\tan[P] = \omega\tau^P \tag{2}$$

$$M = [1 + (\omega\tau^M)^2]^{-1/2} \tag{3}$$

where P is the phase shift, M the relative modulation, and ω the angular modulation frequency [19]. An emitting system characterized by a single exponential decay will yield identical phase and modulation lifetimes irrespective of the modulation frequency [20].

In the dynamic polarization measurements the sample is illuminated by light polarized parallel to the vertical laboratory axis with intensity modulated at variable frequencies. The phase delay, $\Delta\Phi$, between the perpendicular and parallel polarization components of the emission can then be directly determined as well as the ratio of their AC components, Y. For an isotropic rotation one obtains the expressions [21].

$$\Delta\Phi = \tan^{-1}\{(18\omega r_0 R) / [((k^2+\omega^2)(1+r_0-2r_0^2)) + 6R(6R+2k-kr_0)]\} \tag{4}$$

$$Y^2 = \{[(1-r_0)k+6R]^2 + (1-r_0)^2\omega^2\} / \{[(1+2r_0)k+6R]^2 + (1+2r_0)^2\omega^2\} \tag{5}$$

where r_0 is the limiting anisotropy ($r = 2P/3 - P$), R the rotational diffusion coefficient, and k the radiative rate constant ($= 1/\tau$).

Results

The results obtained with either the type VI or type IX starting materials were indistinguishable. The

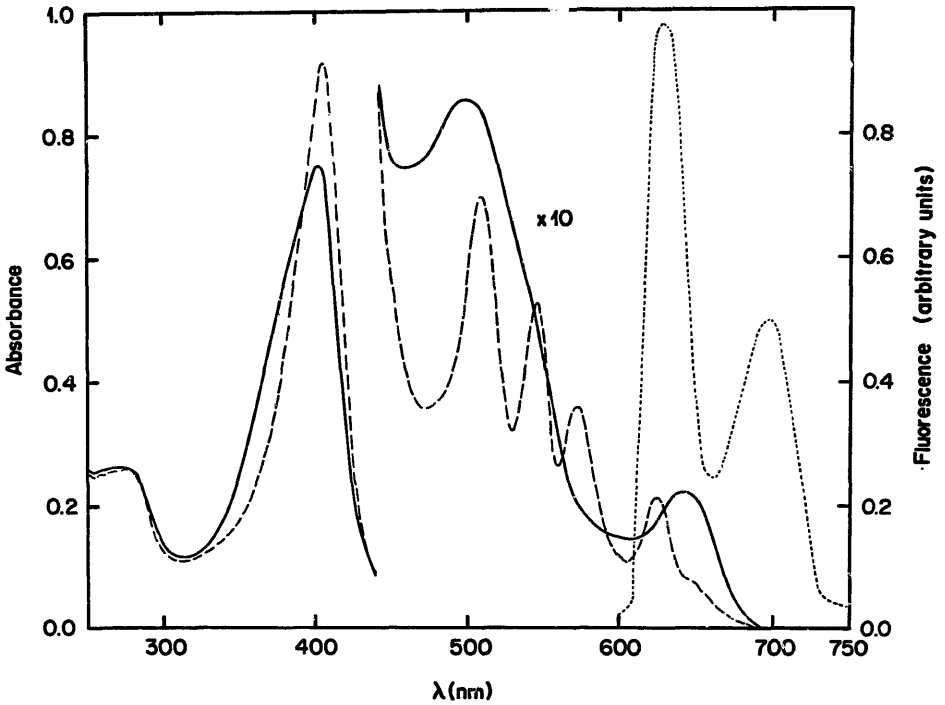

Fig. 1. Absorption spectra of HRP (solid line) and HRP(desFe) (dashed line) and corrected emission spectrum of HRP(desFe) (dotted line) upon 514 nm excitation.

absorption spectra of HRP and HRP(desFe) and the emission spectrum of HRP(desFe) are shown in Fig. 1; the buffer utilized was 0.1 M phosphate (pH 7.4). The emission spectrum, obtained by excitation at 514 nm and corrected for the detector response function, shows maxima at 633 and 696 nm.

Lifetime results on the HRP(desFe) are shown in Fig. 2. The phase and modulation data points corresponds to the closed and open symbols, respectively, while the solid lines correspond to the calculated curves for a single exponential decay of 16.87 ns. The χ^2 for these data was 1.51.

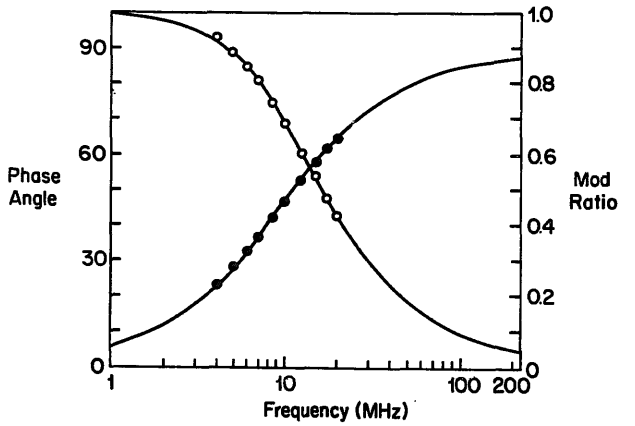

Fig. 2. Multifrequency phase (●) and modulation (○) lifetime data for HRP(desFe) in 0.1 M phosphate buffer (pH 7.4). Solid curves correspond to a single exponential decay of 16.87 ns. Excitation wavelength was 514.5 nm; emission was observed through a Schott OG 570 cuton filter which passes λ > 560 nm.

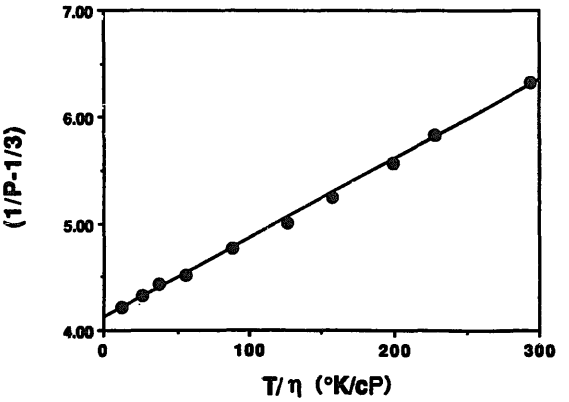

Fig. 3. Perrin plot for HRP(desFe) at 22°C; viscosity was varied by sucrose addition. Excitation wavelength was 514 nm; emission was observed through a Corion LL 600 cuton filter which passed λ > 600 nm.

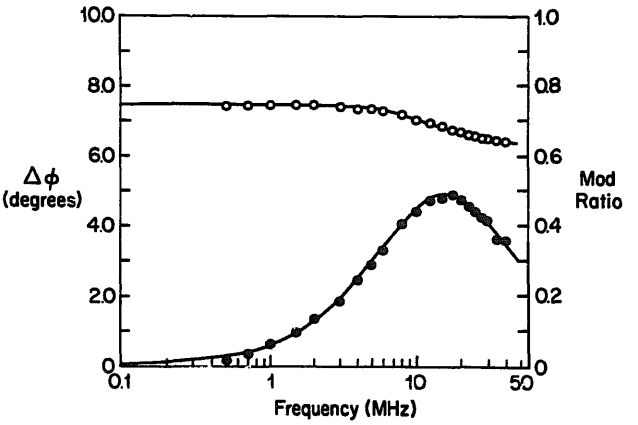

Fig. 4. Differential phase (●) and modulation (○) data for HRP(desFe); experimental conditions the same as in Fig. 3. Solid curves correspond to values obtained for the best-fit analysis of phase and modulation data; $\rho_1 = 86$ ns, $\rho_2 = 12$ ns, $a_1 = 0.163$, $a_2 = 0.005$.

A Perrin plot corresponding to sucrose addition at 22°C is shown in Fig. 3. A limiting polarization value of 0.225 ± 0.002 was obtained by extrapolation of $T/\eta \rightarrow 0$. An initial approach to calculating rotational diffusion rates from polarization data utilizes the Perrin equation:

$$1/P - 1/3 = (1/P_0 - 1/3)(1 + 3\tau/\rho_h) \quad (6)$$

where P is the observed polarization, τ is the lifetime, P_0 the intrinsic or limiting polarization and ρ_h the harmonic mean of the Debye rotational relaxation times about the principle rotational axes. From our results the rotational relaxation time of HRP(desFe) was calculated as 96 ns (±3 ns). We should also note here that sucrose had no significant effect on the lifetime (data not shown).

Dynamic polarization results are shown in Fig. 4. The data were analyzed according to both single and double rotator models. The fit to the single rotator model gave ρ_h equal to 83 ns with P_0 of 0.231 and a χ^2 value of 0.242. The χ^2 was improved slightly, to 0.193, by incorporation of a fast component with a small associated amplitude; the terms from this analysis were $\rho_1 = 86$ ns, $\rho_2 = 12$ ns with associated amplitudes (anisotropies) of 0.163 and 0.005, respectively. Dynamic polarization and lifetime measurements were also carried out at several temperatures; the global rotational relaxation times as a function of T/η are shown in Fig. 5.

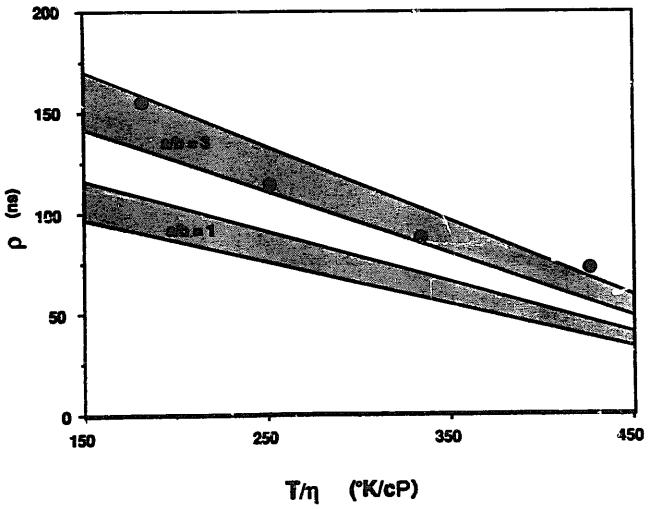

Fig. 5. Calculated rotational relaxation times, as a function of temperature/viscosity, for a spherical protein ($a/b = 1$) and a prolate ellipsoid of axial ratio 3:1 ($a/b = 3$). Details of the calculations are given in the text. The shaded regions correspond to hydration ranges of 0.2–0.4 ml/g. Solid circles correspond to global rotational relaxation times obtained for HRP(desFe) using the dynamic polarization method.

Discussion

Our procedure for the preparation of HRP(desFe) differed from previous reports in that the disodium salt of PPIX was dissolved in methanol and added directly to neutral solutions of the apoprotein. The resulting product was very stable (vide infra) and the observed lifetime, 16.87 ns, was in the general range observed by Horie et al. [9]. We had previously observed that a photoproduct of PPIX could form in the case of Hb(desFe) [22] and in organic solutions of PPIX [23]. This photoproduct, which corresponds to photoprotoporphyrin, has a shorter lifetime than PPIX and an emission maximum near 670 nm [23]. The presence of photoproduct can be readily checked by comparing the emission spectrum of the sample upon excitation at 440 nm (which preferentially excites the photoproduct) to the emission spectrum obtained upon excitation at 514 nm (which preferentially excites the PPIX). Our HRP(desFe) samples were periodically checked over the course of weeks and did not show evidence of photoprotoporphyrin formation.

The excitation polarization spectrum of PPIX is quite complex (data not shown). The limiting polarization, at 514 nm excitation, obtained for HRP(desFe) from the Perrin plot (0.225) and from the dynamic polarization measurements (0.231) were in good agreement. The dynamic polarization method allows us to separate the global motion of the protein from fast, local motion of the probe and, in the HRP(desFe) case, directly demonstrated that the extent of local motion of PPIX in the heme binding site was very limited as one would expect for a tightly bound ligand.

The rotational relaxation time attributed to the global motion of the protein was found to be 96 ns from the Perrin plot and 86 ns from the dynamic polarization study. Using an ANS adduct of HRP, Tao [24] reported a rotational relaxation time of 94 ns. The rotational relaxation time of a rigid, spherical protein may be approximated by [25]:

$$\rho_0 = \frac{3\eta V}{RT} = \frac{3M\eta(\bar{v} + h)}{RT} \quad (7)$$

where V is the protein's molar volume, η the solvent viscosity, R the gas constant, T the absolute temperature, M the molecular weight, $\bar{v}$ the partial specific volume, and h the degree of hydration. Glycoproteins generally have a lower partial specific volume than other proteins due to the low partial specific volume of the sugar residues compared to that of amino acid residues; the degree of hydration of glycoproteins may also be greater than other proteins [26]. For HRP with a molecular weight of 44000 in aqueous buffer (one centipoise at 25°C) and assuming a partial specific volume of 0.70 ml/g [27] and hydration in the range of

0.2 ml/g to 0.4 ml/g, we calculate a rotational relaxation time in the range of 50 to 60 ns. A macromolecule is generally not an isotropic rotator, however, and rotational relaxation times about the various axes will affect the actual observed rotational diffusion parameters, and these rates will be dependent upon the relative orientation of the absorption and emission dipoles of the fluorophore with respect to the macromolecular axes (see for example Refs. 29–31). Our range of 86–96 ns (determined by dynamic and steady-state polarization methods), however, suggests that the protein deviates significantly from spherical symmetry. An estimation of the extent of asymmetry may be obtained from the rotational relaxation times calculated for various ellipsoids of revolution [30]. In Fig. 5 we show the curves calculated, as a function of temperature/viscosity, for a spherical protein and for a prolate ellipsoid with an axial ratio of 3 : 1. The data points correspond to the measured rotational relaxation times, obtained at different temperatures, for HRP(desFe) using the dynamic polarization method; the shaded areas show the effect of hydration on the calculated rotational relaxation times (the lower and upper lines of each shaded region correspond to 0.2 ml/g and 0.4 ml/g hydration, respectively). These results and simulations suggest that HRP(desFe) models reasonably well as a prolate ellipsoid with an axial ratio in the range of 3 : 1. Sedimentation velocity and diffusion experiments [27] also indicated that HRP was nonspherical having a f/f_0 value of 1.36. The actual assignment of true macromolecular axes to specific axes of hydrodynamically equivalent ellipsoids is not necessarily warranted, however, and a comparison of our results and predictions with a crystal structure is desirable. Attempts to extract two rotational relaxation times from the dynamic polarization data were equivocal, i.e., these results did not statistically improve the data fit. However, the newer Global Analysis approach [32,33], to fitting anisotropy data, which links data obtained from several probes oriented differently with respect to the macromolecular rotational axes, should allow progress in this direction.

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