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OXYGEN DIFFUSION NEAR THE HEME BINDING SITE OF HORSERADISH PEROXIDASE

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The quenching by molecular oxygen of the fluorescence of several probes complexed to apohorseradish peroxidase has been studied by intensity and time-resolved fluorescence methods. The probes utilized include 1-anilino-8-naphthalene sulfonic acid, 4,4'-bis (1-anilino-8-naphthalene sulfonic acid), and 2-p-toluidinylnaphthalene-6-sulfonic acid. These results are contrasted to those obtained using apohorseradish peroxidase complexed with protoporphyrin IX. The resistance of these complexes to denaturation by guanidine hydrochloride was also determined. The results demonstrate a dramatic increase in oxygen accessibility to the naphthalene probes compared to protoporphyrin IX, which can be correlated to the increased stability of the protein-protoporphyrin IX complex.

The facile diffusion of oxygen through the interior of a number of proteins has been verified by the methods of fluorescence and phosphorescence quenching (1-8). A recent study of oxygen diffusion through apohorseradish peroxidase complexed with protoporphyrin IX, HRP(desFe), determined a bimolecular quenching constant near 2 x 10^{+8} M^{-1} s^{-1} which is one of the lowest quenching constants observed in any protein system (9). This poor accessibility to molecular oxygen accounted for the observations of luminescence from the enzymatically

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ABBREVIATIONS: 1,8-ANS, 1-anilino-8-naphathalene sulfonic acid; Bis-ANS, 4,4'bis (1-anilino-8-naphthalene sulfonic acid); 2,6-TNS, 2-p-toluidinylnaphthalene-6-sulfonic acid; PPIX, protoporphyrin IX; apoHRP, apohorseradish peroxidase; HRP, horseradish peroxidase; HRP(desFE) apohorseradish peroxidase-protoporphyrin IX adduct, GuHCl, guanidinium hydrochloride.

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generated acetone triplet state (10-11). It was also shown that HRP was very resistant to denaturation by GuHCl compared to other heme proteins (9). Time resolved fluorescence measurements on PPIX in HRP(desFe) furthermore demonstrated the absence of any local motion of the fluorophore in the heme binding site (12). The low diffusional rate for oxygen to the heme pocket in HRP (desFe) is thus consistent with the fact that the porphyrin moiety is tightly bound in a rigid environment. It then seemed reasonable to speculate on a relationship between the accessibility of oxygen to fluorophores bound in the heme pocket and the stability of those apoHRP fluorophore complexes as judged by their resistance to denaturation by GuHCl. Here we report on oxygen quenching and GuHCl measurements on several apo-HRP-probe complexes; specifically we compare results using the probes 1,8-ANS, Bis-ANS, 2,6-TNS and PPIX.

MATERIALS AND METHODS

Preparation of apo-HRP Conjugates: 1,8-ANS, bis-ANS and 2,6-TNS from Molecular Probes, were used without further purification. Horseradish peroxidase type VI and GuHCl were from Sigma. The protein's heme group was removed using Teale's method of cold acid and butanone extraction (13), 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 20,000 (14). The apoHRP conjugates with the naphthalene derivates were prepared with at least a 20 fold molar excess of protein in phosphate buffer 0.1 M pH 7.4. The HRP(desFe) conjugate was prepared as previously described (12). The oxygen quenching experiments were carried out also as previously described (9), using a frequency of 30 MHz for 1,8-ANS, 60 MHz for 2,6-TNS and 70 MHz for bis-ANS conjugates of HRP.

Fluorescence time-resolved measurements: Lifetime measurements were carried out using either an ISS Greg 200 spectrofluorometer or a homebuilt multi-frequency phase and modulation fluorometer based on the Gratton design as previously described (15-17). Excitation of the naphthalene probes was accomplished using the 364 nm line of an argon ion laser (Spectra Physics Model 2035-3.5S), and the emission was observed through a Schott KV399 cuton filter which passed wavelengths longer than 380 nm.

RESULTS

Figure 1 shows the displacement of 1,8-ANS and of 2,6-TNS bound to the apoHRP by hemin titration, followed by the decrease of the naphthalene probes' fluorescence. The two curves are virtually identical with an inflection point near a 1:1 molar relation between the probe and the apoHRP. The PPIX also forms a 1:1 conjugate with apoHPR as described by Mauk and Girotti (18). GuHCl

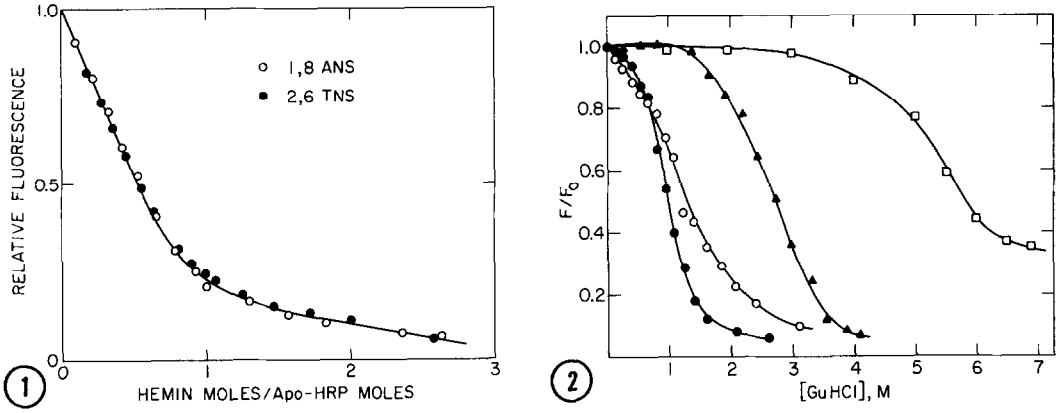

Figure 1. Displacement of 1,8-ANS (-0-) and 2,6-TNS (-●-) from apoHRP by hemin. The excitation wavelengths were 360 nm and 317 nm respectively and the fluorescence was monitored at 475 nm and 432 nm respectively.

Figure 2. Fluorescence decrease for the 1,8-ANS (-●-), 2,6-TNS (-0-),bis-ANS (-Δ-) and PPIX (-□) conjugates of apoHRP as a function of GuHCl concentration. The fluorescence was measured from the integrated area of the spectra. The excitation wavelength was 364 nm for the naphthalene probes and 514 nm for the HRP(desFe).

unfolding of the HRP conjugates are shown in Figure 2. The naphthalene probes and PPIX show a decrease in their fluorescence upon displacement from the protein as the GuHCl increases due to their low fluorescence yield in buffer.

Stern-Volmer plots of the intensity and lifetime quenching by oxygen of the fluorescence of the apoHRP bound to the naphthalene probes and PPIX are shown in Figures 3-a through 3-d. From the slopes of the intensity quenching, the Ksv constants shown in Table I were obtained. The average lifetimes, obtained from a two component discrete exponential decay analysis and weighted according to the fractional contributions to the intensity, are shown also in Table I.

DISCUSSION

Details of the dynamics of oxygen diffusion through protein interiors remain obscure, but are generally believed to be related to nanosecond fluctuations in the protein matrix. Most of the measurements on oxygen quenching of fluorescence and phosphorescence have been carried out on intrinsic tryptophan residues (1-7). In those studies, the tryptophan residues have been located at various regions in the protein, i.e., interior versus exterior, but all of the tryptophans studied have been accessible to oxygen.

In this report, we have studied one particular region of HRP, the heme binding site, and endeavored to elucidate differences in the protein matrix in

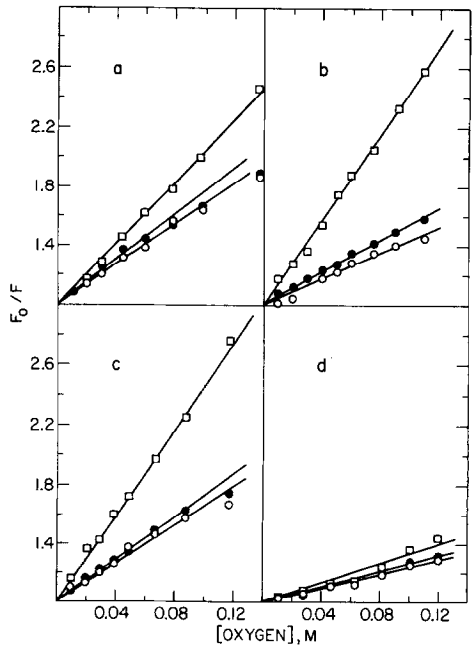

Figure 3. Intensity (-□-), phase lifetime (-●-) and modulation lifetime (-0-) quenching of the emission of 1,8-ANS (a); bis-ANS (b); 2,6-TNS (c) and PPIX (d) conjugates of apoHRP. For the naphthalene probes, the exciting wavelength was 364 nm and the emission was observed through a KV399 cuton filter. The conditions for the HRP(desFe) experiments are the same as described in the text.

response to the presence of different probes. Our results demonstrate that oxygen accessibility to the heme binding site depends upon the probe utilized. As shown in Table I, the naphthalene probes, in general, are significantly more accessible to oxygen than is PPIX. This finding is consistent with the interpretation that the PPIX is very tightly bound ($Kd<10^{-12}M$) and presumably confers a rigidity to the surrounding protein matrix. This conferred rigidity also is manifested in that site's resistance to GuHCl denaturation. The naphthalene probes are bound less tightly though (Kd 10^{-5} - 10^{-6}) (19), and hence the protein matrix comprising the heme

TABLE I

LIFETIMES, STERN VOLMER CONSTANTS AND BIOMOLECULAR QUENCHING CONSTANTS FOR OXYGEN QUENCHING OF HRP CONJUGATES

Probe	$\langle\tau\rangle$(nsec)	Ksv (M^{-1})	Kq(M^{-1} s^{-1})
PPIX	16.9	3.2	$1.9 \times 10^{+8}$
1,8-ANS	17.7	10.2	$5.7 \times 10^{+8}$
2,6-TNS	9.5	15.5	$16.4 \times 10^{+8}$
bis-ANS	8.1	14.2	$17.4 \times 10^{+8}$

binding site should be expected to be less rigid and more susceptible to denaturation by GuHCl.

We may also note the possible relevance of our data on oxygen diffusion in HRP to previous observations on the relative protection to photoinactivations conferred by hemin and 1,8-ANS complexed to HRP (20). Specifically, it was observed that 1,8-ANS, when complexed to HRP, afforded some protection against photoinactivation by ultraviolet light (assessed by recombination of the inactivated protein with hemin and enzymatic assay), but that this protection was much less than that conferred by hemin. This protection was judged to be not simply due to the relative efficiencies of energy transfer between the amino acid residues and hemin or 1,8-ANS. Given our observations of increased oxygen diffusion in the 1,8-ANS complex relative to the PPIX complex, we could now suggest that more facile diffusion of oxygen in the HRP protein matrix could also play a role in the decreased protective role of 1,8-ANS relative to hemin in oxygen requiring photoinactivation processes.

In summary, we have shown that the accessibility of molecular oxygen to the heme binding site of HRP varies depending upon the nature of the molecule occupying that site. This observation suggests that the diffusion of small molecules in protein interiors depends not just on the protein matrix per se, but rather on the overall dynamic aspects of the particular protein-probe system being studied.

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