Tryptophan in Horseradish Peroxidase

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Fluorescent derivatives of horseradish peroxidase C were prepared by replacing protoheme by protoporphyrin or mesoporphyrin. Calculations according to Förster on energy transfer allowed the determination of the distances of >2.2 nm between tryptophan and porphyrin (heme) and >2 nm between tryptophan and substrate-binding site.
The modification of the single tryptophan with 2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent) did not affect the enzyme's activity towards hydrogen peroxide or ascorbate. Modified and unmodified peroxidase showed the same affinity for aromatic substrates.

The characteristic property of protoheme peroxidases is the rapid reaction with hydrogen peroxide to give oxidized forms of the enzyme such as the green "compound I" of horseradish peroxidase (HRP) and the red "ES" of cytochrome c peroxidase (CCP). Both products have the formal oxidation level of +5 with the iron atom in the ferryl (+4) form. The spectrum of compound I shows a broadened Soret π–π^* transition and an increased absorption in the 620–670 nm range, whereas ES shows a normal Soret band. The missing charge is accounted for by a radical, localized mainly on the porphyrin ring in compound I and the protein moiety in ES.[1-3]

X-ray crystallography of CCP has revealed one tryptophan (Trp) residue out of 12 localized parallel to, and about 0.36 nm apart from the heme.[4] Its indole nucleus has been considered the resort of the radical in this enzyme.[3] Alternatively, it is a prerequisite for the migration of the radical from heme to the nearby methionine-172.[5,6] HRP contains a single Trp, at position 117,[7] but its location has not been settled by X-ray crystallography. The present study examines the effect of a chemical modification of Trp on characteristic properties of HRP. It also explores the heme-Trp distance in HRP by means of fluorescence energy transfer analysis after replacing heme by porphyrin. Native and porphyrin-HRP C show equal affinities for aromatic substrates[8] and similar Trp fluorescence properties and CD spectra.[9] Hence porphyrin-HRP models HRP reasonably well. Meso- and protoporphyrin (MP,PP) were used.

Materials and methods

HRP isoenzyme C was isolated as described[10], split into heme and apoprotein[11], and the protein moiety dialyzed against many changes of water to remove completely 2-butanone, which may phosphoresce.[12] For recombination the apoprotein was mixed at 0 °C with a 50 % excess of heme or porphyrin at pH 8 in 10 mM TRIS·HCl. After one hour the excess, together with possibly denatured protein, was removed on a column of DEAE-52 in 50 mM sodium phosphate, pH 6.5. Porphyrins are sufficiently tightly bound to apo-HRP C to withstand the attraction of the anion exchanger. Gel filtration on various materials yielded only products with poor peak-to-trough values in the visible spectrum. Concentrations of porphyrin-HRP C were determined on the basis of absorbancies in HCl.[13] The Soret band absorp-

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tivities of MP- and PP-HRP C at pH 6.5 were found to be 138 and 125 cm^{-1} mM^{-1} with a_{Soret}/A_{280} = 7.7 and 6.7, respectively. Porphyrins were obtained by KOH-MeOH saponification of the recrystallized esters. 2-Hydroxy-5-nitrobenzyl bromide (Ar'Br, Sigma), 2-naphthohydroxamic acid[14] (NHA, Molecular Probes Inc., Junction City, OR), and protoheme (Koch-Light) were used as purchased.

Instrumentation and techniques for registering steady state fluorescence have previously been described.[15] The distance between porphyrin and Trp was calculated according to Förster:[16]

$$R_0^6 = 8.8\times10^{-25}K^2\varnothing_D\eta^4J$$
$$R = R_0(E^{-1} - 1)^6$$

where R is the donor-acceptor distance sought for, R_0 is the distance characteristic for 50 % transfer, E is the efficiency of energy transfer, and K^2 is the orientation factor. Because of the symmetry of the chromophores the value $K^2 = \frac{2}{3}$ was used.[17] $\varnothing$ is the quantum yield of the donor in the absence of acceptor. The value = 0.02 for Trp in the apoprotein was estimated from a comparison of the fluorescence emission intensities of apo-HRP and free tryptophan.[18] η is the refractive index of the medium between donor and acceptor. We used $\eta = 1.4$. J is the overall spectral overlap of donor emission and acceptor absorption:

$$J = \int_0^\infty \varepsilon_A(\lambda)F_D(\lambda)\lambda^{-4}d\lambda$$

ε_A is the absorbtivity of the acceptor at wavelength λ. $F_D(\lambda)$ is the normalized fluorescence spectrum of the donor:

$$f_D(\lambda)d\lambda = \frac{F(\lambda)d\lambda}{\int_0^\infty F(\lambda)d(\lambda)}$$

where $F(\lambda)$ represents emission intensities per wavelength.

The peptide-bound single Trp was chemically modified as described.[19,20] Solid Ar'Br in 30-fold excess was added to the protein in 10 mM sodium phosphate, pH 6.0, with or without 8 M urea, but always containing 5 % (v/v) methanol. After 20 min in darkness the excess of Ar'Br, now as Ar'OH, and urea were removed on a Sephadex-G15 column (26×250 mm), pre-equilibrated with the above buffer. Then the protein was chromatographed on a short DEAE-Sephadex A-50 column in the same buffer to remove possibly remaining Ar'OH not bound to Trp; if this step is omitted Ar'OH, like other aromatics, will form a 1:1 reversible complex with holo-HRP and simulate binding to Trp. The modification of Trp was optically confirmed by bringing the apoprotein, if needed after re-splitting of the holoenzyme, to pH 10.0 (100 mM sodium carbonate) and to pH 4.5 (100 mM sodium acetate). Ar'trp protolyzes above pK_a 6.9 to give a pronounced light absorption with maximum at 410 nm.[20]

Results

Energy transfer. The Trp emission spectrum (Fig. 1) overlaps the absorption spectra of both NHA and porphyrin. Using the theory for dipolar interaction it is therefore possible to calculate the Trp-porphyrin and Trp-NHA distances. Trp fluorescence yield in Mp-HRP and PP-HRP is about 80 % of the yield for Trp in apo-HRP.

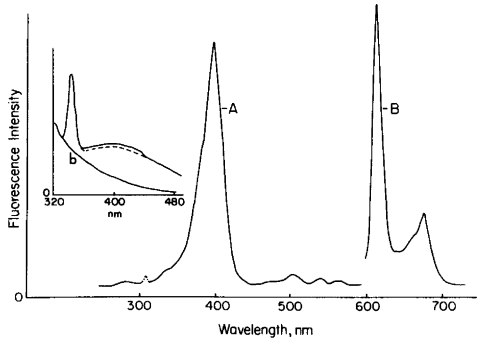

Fig. 1. *Fluorescence spectra of MP-HRP C.* The protein concentration was adjusted such that the absorption at the excitation wavelength was <0.1. A: Excitation spectrum using 620 nm as emission wavelength. B: Emission spectrum using 395 nm as excitation wavelength. ε_{300} = 9 mM^{-1}×9 mM^{-1}. —— apoHrp; ---- MP-HRP; b: background scattering from cuvette alone. The sharp maximum at ~344 nm results from Raman scattering. Slit width: 3 nm. 50 mM sodium phosphate, pH 6.5. Ambient temperature.

Table 1. The distances between tryptophan, heme, and NHA in horseradish peroxidase C.

Efficiency of energy transfer (%)		R_0/nm	R/nm
Tryptophan-heme	20	2.4	>2.2
Tryptophan-substrate	–	1.6	>2.0

Sensitized excitation at 290 nm of MP-HRP and PP-HRP confirms that some energy transfer from Trp to porphyrin occurs: the ratio of intensity at 300 nm to height of Soret band is only about 20 % of that found in hemoglobin as measured by the action spectrum of CO binding[21] or fluorescence excitation.[22] The values for R_0 and the experimentally determined distances are given in Table 1.

NHA quenched the Trp fluorescence by ≦5 %. With R_0 = 1.6 nm this gives an estimated Trp-NHA distance of >2 nm.

An attempt was made to use the intrinsic fluorescence of NHA to estimate the NHA-porphyrin distance. However, the fluorescence of NHA becomes quenched and undetectable when NHA is bound to HRP, porphyrin-HRP or apo-HRP. It is therefore not possible to calculate this distance on the basis of the Förster equations.

We were unable to reproduce the emission spectrum of HRP upon excitation at 390 nm previously reported.[23] Only the Raman scattering from water at 450 nm appeared.

Chemical modification. Intact HRP and its apoprotein were separately exposed to Ar'Br. The intact hemeprotein, at pH 4.5 and 10, gave no optical indication of a modified Trp, nor did its protein moiety after splitting. Thus Trp in intact HRP is inaccessible to Ar'Br. The presence of urea had no effect. Interestingly, the optical and catalytical properties of the enzyme appeared unchanged when urea was removed. The apoprotein, on the other hand, did react with Ar'Br, as evidenced by the spectra at pH 4.5 and 10 of the chromatographed apoprotein. This modification of the Trp residue did not prevent the subsequent recombination of apoprotein and heme. Once reconstituted, the holoenzyme showed a normal optical spectrum at 10.0 and 4.5 as found by difference spectrometry against HRP at the same pH. A fully dissociated (pH 10) Trp Ar'OH residue in the proportion 1:1 would have increased the Soret band absorption by about 7 % with some shift in position. After resplitting of the enzyme, the apoprotein spectrum again revealed the presence of a modified Trp.

The kinetic and substrate binding properties of the modified holoenzyme were not significantly altered (Table 2).

All experiments were made in duplicate, from the isolation of HRP to the final analyses. Mesoporphyrin is somewhat more stable than protoporphyrin and was used in the second series, with consistent results.

Discussion

The fluorescence of tryptophan is shown to be little quenched by protoporphyrin or mesoporphyrin or by addition of the substrate, NHA. This can be taken to mean that porphyrin (and heme) as well as NHA are ≧2 nm removed from

Table 2. Kinetic and substrate binding data of native and tryptophan-modified horseradish peroxidase.

Peroxidase	K_d/mM[a] donor		k_1/M^{-1}s^{-1} [b]	k_3/M^{-1}s^{-1} [b]
	Hydroquinone	Resorcinol	H_2O_2	Ascorbate
Native HRP	3.2	4.0	1.2×10^7	3.0×10^2
Trp-modified HRP	2.8	4.5	1.0×10^7	2.9×10^2

[a] K_d of the equilibrium HRP + donor ⇄ HRP·donor in 100 mM sodium phosphate, pH 6.0, 25 °C.

[b] k_1 and k_3 are rate constants (at pH 6.0) in the usual reaction mechanism for peroxidases:[1]

Fe(III)PO + ROOH $\xrightarrow{k_1}$ PO·compound I + ROH

Compound I + donor $\xrightarrow{k_2}$ compound II + products

Compound II + donor $\xrightarrow{k_3}$ Fe(III)PO + products

Trp. However, there are several caveats to the energy transfer experiments. The orientation factor $K^2 = \frac{2}{3}$ carries some uncertainty. With one chromophore transition fixed and the other at random, K^2 varies between $\frac{1}{3}$ and $\frac{4}{3}$ and R by ± 0.2 nm.[24] Another uncertainty in the calculation lies in the fact that the theory was made for point sources: this is certainly not the case for donor and acceptor used where the molecular sizes are large compared with the distances between them. A third uncertainty stems from the estimation of the value 1.4 for the refractive index of the medium between donor and acceptor. For halogen-free hydrocarbons $\eta = 1.3 - 1.5$.[25] The refractive index increment $d\eta dc'$ of hemoglobin (as model for HRP) $\cong 0.2$ ml/g[26] in combination with a partial specific volume of 0.75 ml/g and $\eta = 1.33$ for water gives $\eta = 1.6$, a maximum value. However, with $\eta = 1.3$ or 1.6 R will differ only by about 15 % since R_0 varies with η^{4-6}.

Additionally, an experimental problem arises from the low quantum yield in the Trp-porphyrin transfer, 0.02, which is equal to a previously reported value for HRP.[27] The same value was reported for two proteins with one Trp residue whereas six one-Trp proteins gave $\varnothing = 0.10$–0.44.[28] The low quantum yield means that under the actual conditions Raman scattering from the solvent is significant and more precise measure of energy transfer requires fluorescent lifetime measurements. The reason for the low quantum yield of Trp in the apoprotein is not known. It is known, however, that water will quench the quantum yield of indole.[29] On the other hand, we observed no pH-induced spectral effect on MP HRP, what may depend upon the lack of a proton-accepting base, e.g. water.

In the free apo-HRPC the single Trp residue reacts with Ar'Br to give a 1:1 product but without consequences for the catalytic properties of the reconstituted enzyme. This is in line with the result from fluorimetry that Trp is situated >2.2 nm from the heme/porphyrin. The distance from the donor (NHA) to Trp also appears to be >2 nm, since NHA did not quench the Trp fluorescence. Various NMR studies give the donor-heme distance as 0.5–0.8 nm (8 and references therein). A heme crevice peptide from affinity labelled HRP lacked Trp.[30] It would appear, therefore, that Trp is not involved directly in the catalytic site of HRP; none-the-less it does not exclude Trp from the electron transfer process in view of recent findings that electron transfer in proteins can occur over long distances.[31] The absence of a pH-dependent spectral effect on Trp-modified HRP suggests that Trp-117 dwells in a hydrophobic environment devoid of proton-accepting bases.

In summary, several lines of evidence give the single Trp in HRP a position quite remote from the catalytically active area. The situation is thus different from that in CCP.

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