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Equilibria between Horseradish Peroxidase and Aromatic Donors

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Equilibria between horseradish peroxidase and aromatic hydrogen donors have been analyzed spectrophotometrically and potentiometrically. The donors alter the peroxidase spectrum slightly but reproducibly with changes of two types. Donors of the two groups compete for the same binding site with no systematic difference in affinity for the enzyme. Donors with one aromatic ring are fairly loosely ligated, K_d 3 – 25 mM, but enlargement, or extension of the π-electron system, increases the affinity. A negative change in entropy and a large negative change in enthalpy upon binding indicates a specific donor-enzyme interaction, and the retention of the peroxidase by phenyl- but not by octyl-Sepharose® points at the involvement of aromatic amino acid(s) in the ligation of an aromatic donor. Substitution of the hematin vinyl groups by ethyl or acetyl groups does not affect K_d of the peroxidase-donor complex. Reduction of the iron atom to Fe(II), or its removal, influences K_d only modestly. The fluorescence of the protoporphyrin · apoprotein HRP C2 associate is not quenched by donors from either group. These observations are in accord with NMR and other data from the literature and point at a ligation of the donor only to the protein moiety. Our results do not support the assumption of an Fe(III) H_2O . . . donor hydrogen bond. The energy balance in the four-membered system free and donor-bound peroxidase Fe(III)/(II) has been analyzed. The model donors used in the present study modulate the redox properties only slightly. Plant peroxidases *in situ* may be donor-bound to a large extent.

Complexes between a peroxidase and the substances to be oxidized, the hydrogen or electron donor, were once considered to be optically inoperable, and the donor was stated not to be bound to the iron.[1] Later on indoleacetic acid was found to alter the spectrum of a plant peroxidase with maintenance of the essential features,[2] and such optical effects have been used in analyses of peroxidase-donor equilibria.[3–6] The donor and the sixth ligand to the iron atom exert a mutually negative effect on the binding without directly competing for the same site.[3,4] Equilibrium studies have thus led to the assumption of a separate donor binding site somewhat remote from the iron atom, and NMR spectroscopy has given distances of ≥ 3.2 to 10.6 Å between iron and various donor protons.[5,7–10]

The present paper further analyzes the equilibria between HRP and some aromatic donors and the energy balance between free and donor bound enzyme in its Fe(III) and Fe(II) forms. It also attempts to encircle the character of the donor binding site by examining equilibria with modified peroxidases. During this work it was noticed that some common donors caused opposite effects on the spectrum of HRP.

MATERIAL AND METHODS

HRP A2* (p*I* 3.9) and C2 (p*I* 8.8), and HRP C2 with 2,4-substituted hematins were prepared as described[11,12] and found to be homogeneous in gel electrophoresis and analytical isofocussing (Ampholine PAG-plates® pH 3.5 – 9.5, LKB, Stockholm). Apoprotein HRP C2 and protoporphyrin associated in the ratio 1:1 when allowed to react for 48 h in 120 mM sodium borate pH 9.1, excess porphyrin being removed on a Sephadex®-G 25 column in 100 mM sodium phosphate pH 6.0.[13] Superoxide dismutase (Cu-Zn) was a gift from Dr. S. Marklund, Umeå. Catalase was purchased from Worthington Biochemicals, New Jersey and freed from thymol on Sephadex®-G 25. Donor substances were of highest available grade and purified by suitable procedures until colorless. Styrene was distilled *in vacuo* and immediately diluted with ethanol. Buffers were filtered through Sartorius 0.2 μ membrane filters.

* HRP, horseradish peroxidase, donor: H_2O_2 oxidoreductase (E.C.1.11.1.7).

An Acta III Beckman spectrophotometer was used for difference spectrophotometry with HRP in both cuvettes. Absorbances were corrected for the dilution (< 1 %). Ethanol, as solvent for the donors, in these concentrations did not affect the spectrum of HRP or the dissociation constants. Fluorescence spectra were registered by means of a Perkin-Elmer model 1000 fluorescence spectrophotometer with 398 nm as exciting light. Experiments under argon were performed in a 1 cm cuvette fused from below to a water-jacketed vial, closed by a teflon plug carrying electrodes *etc.* Stirring was arranged by means of a magnetically operated gilded propeller. The cell was also equipped for the addition of solids when closed. After charging the cuvette was flushed for 2 h with humidified argon (< 2 ppm O_2) above the surface. This vessel was also used for potentiometry with optical determination of the ratio HRP Fe(III)/Fe(II).

For potentiometry were used a PHM 4 (Radiometer, Copenhagen) battery powered potentiometer, a Radiometer calomel electrode K 4112, and home-made bright platinum electrodes in soda glass. These were soaked in concentrated nitric acid for 10 min., rinsed in distilled water, and heated to glow in an ethanol flame. When so treated the electrodes differed ≤ 0.2 mV at 20 – 80 % reduction of HRP. The calomel electrode together with a saturated KCl bridge in 4 % (w/v) agar (Noble, Difco) was checked against a hydrogen gas electrode and found to agree within 1 mV with the given value of + 244.4 mV (*cf.* Table 6).

Measurements were performed at 25 °C unless otherwise stated with cuvette temperatures monitored by means of a thermocouple. pH 6.0, 100 mM sodium phosphate, was chosen for all experiments since studied donors are practically uncharged at this acidity.

The increment in light absorption upon saturation of HRP with donor, the dissociation constant of the HRP·donor complex, and the stoichiometry donor: HRP were obtained from Hanes or Scatchard plots. Rectilinear regression lines were given by least-square fittings, the correlation coefficients usually being > 0.995 (> 0.975 in the titration of HRP Fe(II) with donor). Equilibria between HRP and donor are expressed as dissociation constants, K_d = [HRP] [donor]/[HRP·donor]. Donor was usually added in large excess over HRP and hence [free donor] ~ [total donor].

Many donors generate peroxide in aerobic milieu. Some similarities between the difference spectra (HRP·donor – HRP) and (compound I or II – HRP) raised a suspicion that part of the spectral effects might be caused by peroxide formation. This possibility could be rejected:

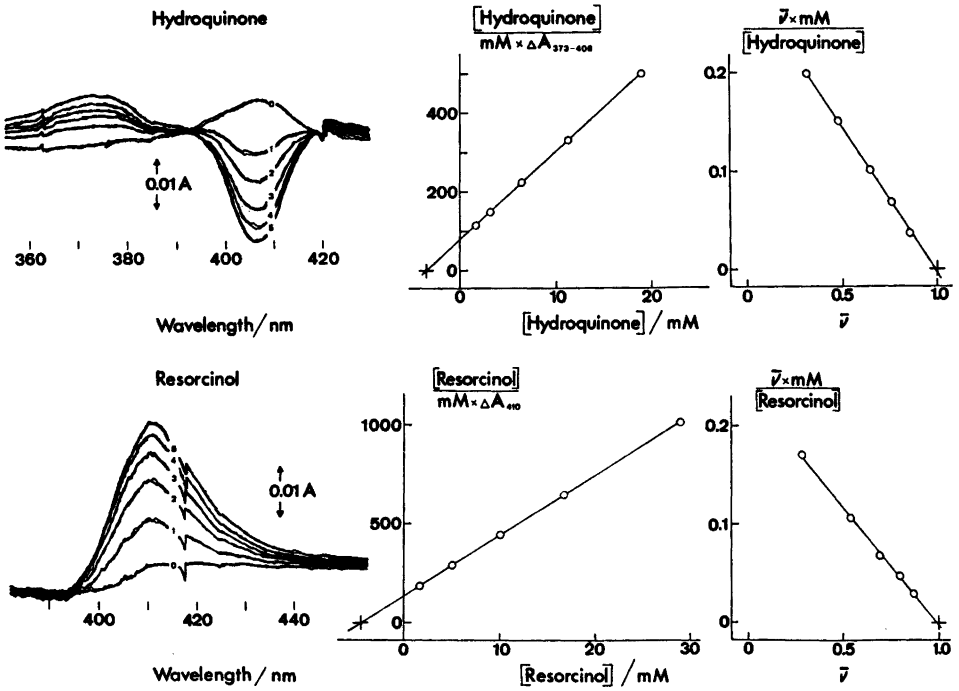

Fig. 1. Difference spectra in the Soret region between HRP C2·donor and free HRP C2 with Hanes' and Scatchard plots of ΔA and [donor].HRP ~ 10 μM.

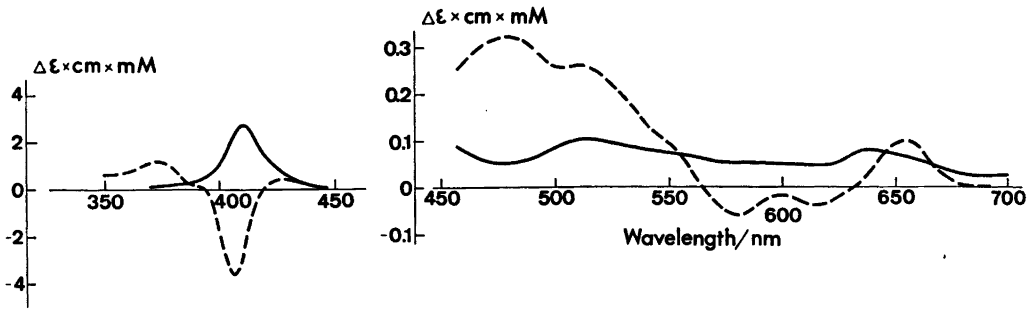

Fig. 2. Difference in ε_{mM} between HRP·donor and free HRP. Resorcinol, —; hydroquinone, - - -.

a. During titration with a donor the difference spectrum (HRP·donor-HRP) showed stable isosbestic points. HRP and compound II are isosbestic at 411 nm, close to the maximum absorbance change with resorcinol.

b. Hanes and Schatchard plots gave straight lines.

c. Calculations of the dissociation constants for an HRP·donor complex gave the same numerical value when based on measurements at 380 – 420, 490 – 510 and 620 – 660 nm.

d. Attempts to simulate observed changes in absorbance by assuming contributions of 1 – 4 % of compound I or II or both were unsuccessful.

e. The addition of a donor caused no oxygen uptake detectable by means of a Clark O_2-electrode (4004, Yellow Spring Instruments, Ohio).

f. The presence of superoxide dismutase, catalase, or both in amounts up to 10 mol per cent of HRP changed the absorbances only by their own light absorptions.

g. Identical results were obtained when the titrations of HRP with donor were made in open cuvettes and in closed cuvettes under argon with the addition of solid donor.

h. The presence of a donor should rapidly reduce any peroxide eventually formed.

RESULTS

Optical characterization of HRP C2·donor complexes. The addition of hydroquinone to HRP broadened the Soret band asymmetrically with an essentially hypsochromic shift of absorption and some reduction of maximum absorbance (Fig. 1). The 500 nm band in the HRP spectrum also migrated towards shorter wavelength whereas the 640 nm band shifted in the opposite direction, both bands with some increase in intensity. Some absorbance was lost in the 570 – 630 nm range (Fig. 2). Resorcinol, on the other hand, brought about a bathochromic shift of the Soret band (Fig. 1) and a general increase in absorbance in the visible range (Fig. 2). The 500 nm band became somewhat reinforced and red-shifted, and the 640 nm band was reinforced without shift in position. There was no decrease in absorbance within the range 380 – 700 nm when resorcinol was added to HRP. The spectral effects due to hydroquinone and resorcinol were thus essentially opposite in character.

The occurrence of two spectral types raised the question whether hydroquinone and resorcinol bind to the same site in HRP. The addition of increasing amounts of one donor (D_1) to a solution containing HRP and the other donor (D_2) yielded a hyperbolic increase in absorbance with the apparent dissociation constant equal to K_d of HRP·D_1. Fig. 3 shows that K_d of HRP·D_1 increased linearily with the concentration of D_2 and *vice versa* and also that the observed value of $\Delta\varepsilon$ approached the value calculated from independent determinations of K_d and $\Delta\varepsilon$ for HRP·D_1 and HRP·D_2. Thus D_1 and D_2 compete, and the competition is governed by simple equilibria between HRP, D_1 and D_2.

In an attempt to map what property of the donor molecule determines the spectral type a few more HRP-donor complexes were examined (Table 1). Spectra were consistently of the type induced by either hydroquinone or resorcinol and always of the same type in the Soret and visible regions. All complexes were of the 1:1 composition, usually with K_d of the same order of magnitude. However, flavone and α-naphthol

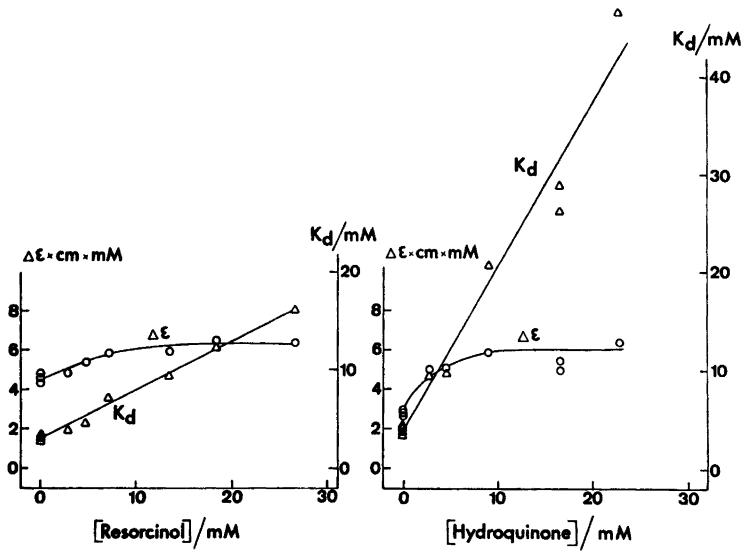

Fig. 3. Titration of HRP C2·donor 2 with donor 1. Both cuvettes contained initially HRP (~10 μM) and the concentrations of donor 2 given by the abscissa. The change in light absorption upon the addition of increasing amounts of donor 2 to the sample cuvette was registered (left $A_{373}-A_{406}$, right A_{410}).

$\Delta\varepsilon_{mM}$ gives the difference between sample and reference cuvettes at these wavelengths on the basis of total HRP present. K_d is the apparent dissociation constant of HRP·donor 1 at the given concentration of donor 2.

were more firmly, and aniline and its derivatives less firmly bound than simple phenols [5] when compared in suitable solvents. The ability of a substance to evoke spectral effects of either type does not guarantee a substrate function. Acetic acid (K_d~70 mM) and pyridine (K_d~1 M) in their uncharged forms elicited spectra of the hydroquinone type whereas some vinylpyridines (K_d 100 – 500 μM) produced the other type.[14]

The circular dichroism spectrum of HRP C2 was surprisingly little affected by the addition of a donor.[15] Resorcinol shifted the ellipticity in the range 380 – 680 nm rather uniformly towards more positive values, $\Delta[\theta] = 1.9 \times 10^3$ degrees cm^2 mol^{-1} at 605 nm. The change followed a hyperbolic curve giving $K_d = 4.5$ mM, close to the value 4.2 mM from optical absorbance determinations. Hydroquinone had very little, if any, effect on the CD spectrum.

Peroxidase-donor redox equilibria. The potentiometric determination of E_h and the simultaneous optical determination of the degree of oxidation offered no difficulties with native and artificial, hematin-substituted HRP. Table 2 shows that also the mixture of HRP and HRP·donor at a donor concentration close to K_d appeared as a single redox system when titrated. Potentiometric measurements on the system HRP Fe(III)·donor/HRP Fe(II)·donor require donor concentrations close to saturation for both forms. However, even 0.95 saturation of HRP Fe(III) means nearly 0.1 M donor and even higher for Fe(II), *i.e.* concentrations of an aromatic compound which might perturb heme-protein interrelations as found with myoglobin.[16] To minimize such adverse effects redox titrations were performed at several donor concentrations below 0.75 saturation of HRP Fe(III), plots of [donor]/ΔE_m *vs.* donor giving E_m at saturation (Table 3).

Donor binding site. A determination of the enthalpy and entropy of binding would give additional information about the nature of the HRP·donor association. The change in free energy upon binding was obtained from $\Delta G' = RT \ln K_d$ (Table 1) and the change in enthalpy from Arrhenius plots (Fig. 4). The slopes of

Table 1. Complexes between HRP C2 or protoporphyrin – apoperoxidase C2 and various donors. Number of titrations within parentheses. HRP C2 9 – 11 μM. $\Delta\varepsilon$ refers to the wavelength couple (373 to 383) nm – (403 to 406 nm) for the hydroquinone type spectra and to the given wavelength for the resorcinol type spectra. $\Delta\varepsilon$ gives the difference between HRP C2·donor and free HRP C2.

Donor		Spectrum of complex Max.	Min.	Max.	$\Delta\varepsilon$ cm mM	K_d/mM	Donor/ HRP C2
HRP C2 in 100 mM sodium phosphate, pH 6.0							
Hydroquinone	(3)	373	406	423	4.6 ± 0.3	3.2 ± 0.3	0.98 ± 0.01
Phenol	(4)	375	403	420	2.7 ± 0.3	6.2 ± 1.3	1.00 ± 0.06
Catechol	(2)	383	404	420	3.4	7.9	0.97
Aniline	(2)	380	406	426	8.3	26.0	0.99
o-Toluidine	(2)	380	405	423	8.9	17.6	1.04
p-Toluidine	(2)	377	403	423	3.2	7.0	0.98
Benzene	(1)	379	405	424	9.0	25.6	0.99
Styrene	(1)	378	404	423	5.6	3.8	1.04
Nitrobenzene	(1)	~382	406	426	–	10.8	1.01
Resorcinol	(4)	410			2.8 ± 0.1	4.0 ± 0.5	1.01 ± 0.03
Guaiacol	(7)	408			3.7 ± 0.6	7.4 ± 1.5	1.00 ± 0.07
Coniferol	(2)	409			1.6	1.2	1.02
Mesidine	(2)	416			2.1	7.8	1.09
Aminotriazol	(2)	415			3.4	98.6	0.94
HRP C2 in 10 mM sodium phosphate with 30 percent (v/v) ethanol, pH 6.0							
α-Naphthol	(1)	373	406	flat	5.3	4.8	0.96
Flavone	(1)	376	405	flat	5.8	3.4	0.99
Hydroquinone	(1)	373	407	flat	6.1	23.4	0.99
Resorcinol	(1)	415			1.5	81.5	1.00
Protoporphyrin – apoperoxidase C2 in 100 mM sodium phosphate, pH 6.0							
Hydroquinone			402	419	5.2 (419 nm)	5.6	1.02
Resorcinol			396	415	3.9 (415 nm)	2.8	1.00

Table 2. Redox titration of HRP C2 Fe(III) with dithionite. Phenosafranine 1.5 – 2 μ M.

Per cent reduction 580 nm	640 nm	$E_{m,pH\ 6.0}$/mV 580 nm	640 nm
HRP C2 47.5 μM			
20.5	21.7	−207.4	−206.5
36.0	36.8	−207.8	−207.3
50.5	51.6	−207.8	−207.8
65.5	65.9	−208.2	−208.4
81.8	81.0	−207.7	−207.7
Mean ± SD		−207.7 ± 0.5	
HRP C2 72.3 μM with 7.3 mM guiacol			
21.0	21.2	−209.1	−209.4
34.8	35.1	−210.0	−209.7
52.3	52.7	−210.3	−210.3
72.8	73.4	−210.3	−209.4
84.0	84.8	−210.3	−209.7
Mean ± SD		−209.9 ± 0.4	

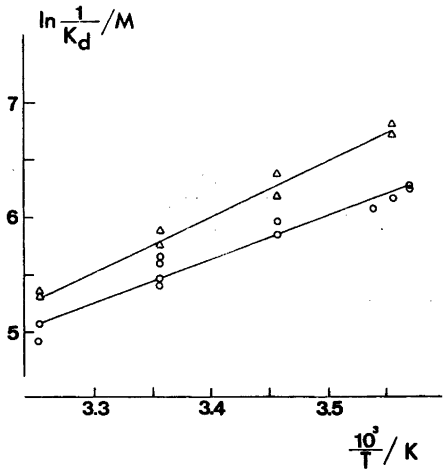

Fig. 4. Arrhenius plots of dissociation constants K_d and temperature for the equilibria between HRP and hydroquinone (△) or resorcinol (○).

Table 3. Redox titration of HRP C2 Fe(III) with dithionite in the presence of donors. HRP C2 50 – 70 μM, phenosafranine as mediator 1.5 – 2 μM.

Donor	mM	$E_{m,6.0}$/mV	$E_{m,6.0}$/mV at saturation with donor
None		−207.7	
Hydroquinone	1.13	−214.2	−243.7
	5.42	−226.1	
	10.40	−231.8	
Resorcinol	1.02	−209.7	−225.4
	4.45	−214.8	
	10.34	−217.8	
Phenol	7.97	−219.9	
Guaiacol	7.31	−209.9	

ln K_d^{-1} *versus* $1/T$ and hence ΔH for the binding of hydroquinone and resorcinol differed little but significantly ($p < 0.01$), assuming ΔC_p constant, as did ΔS, calculated from $\Delta G = \Delta H - T\Delta S$. Table 4 reveals two peculiarities of the HRP-donor complex formation as compared to some other reactions between protein and ligand: a negative entropy change and a large negative enthalpy term.

The ability of HRP to bind aromatic donors with little specificity initiated affinity chromatography of the enzyme on phenyl-Sepharose®. HRP C2 was adsorbed by the top layer of a column from 900 mM K_2HPO_4 and eluted when this solution was saturated with benzene or when a gradient 900 – 150 mM K_2HPO_4 was applied. HRP C2 was not retained by octyl-Sepharose® from 900 mM K_2HPO_4.

Donor complexes with other forms of HRP. HRP A2 and C2 differ in their reactions with peroxides and donors,[17] and their complexes with some donors were therefore compared (Table 5). On the whole C2 will bind an aromatic donor more firmly than A2 does, but available data reveal no simple correlation between binding and rate constants. When simultaneously chromatographed on phenyl-Sepharose® (0.5 M sodium sulfate in 10 mM sodium phosphate, pH 6.2, 25 °C) HRP A2 was eluted at 6.4 and HRP C2 at 11.2 void volumes, in agreement with expectations from the K_d-values.

K_d and spectra of donor complexes with some artificial peroxidases, 2,4-substituted in the hematin, showed no drastic response to the substitutions (Table 6). K_d seems to depend more upon the donor than upon the hematin.

Table 4. Thermodynamic parameters for the formation of ligand-protein complexes (selected ligands from Refs. 37 and 38).

Protein	Ligand	t/ °C	$\Delta G'$/ kJ mol^{-1}	$\Delta H'$/ kJ mol^{-1}	$\Delta S'$/ kJ mol^{-1} K^{-1}	Ref.
HRP C2 Fe(III)	Hydroquinone	25	−14	−40	−87	This
	Resorcinol	25	−14	−32	−64	paper
β-Lactoglobulin	Pentane(K_1)	25	−32	− 9	77	37
	Butane	25	−27	− 5	74	
Apomyoglobin	Dodecanoate	25	−37	−13	81	38
	Protoporphyrin	25	−48	−12	121	
Cy P450 Fe(III)	Camphor	21	−32	0	110	39

Table 5. Complexes between HRP A2 and some donors. Number of titrations within parentheses. HRP A2 9 – 11 μM.

Donor		Spectrum of complex Max.	Min.	Max.	$\Delta\varepsilon$ cm mM	K_d/mM	Donor/ HRP A2	K_dA2/K_dC2
Hydroquinone	(2)	373	405	430	7.1(373 – 405)	11.7	0.99	3.7
Guaiacol	(2)	374	405	420	2.8(374 – 405)	33.2	1.03	8.3
Resorcinol	(2)	~376	406	–	3.9(406)	12.0	0.98	3.0
Mesidine	(1)	418				20.0	1.07	2.6

Table 6. Effects of substitutions at the 2,4-positions of the hematin on the equilibria between HRP C2 and for donors, pK_a and E_m (*cf.* text). These values of E_m are 8 – 16 m V more negative than published values.[48]

2,4-Substituent	pK_a[12]	$E_{m,6.0}$mV	K_d/mM Hydroquinone	Phenol	Resorcinol	Guaiacol
Ethyl	11.8	−228	4.6	6.6	4.5	8.5
Vinyl (recomb.)	11.1	-207.8 ± 1.0 ($n=3$)	3.3	6.0	5.1	11.6
Acetyl	9.0	− 83	3.6	5.5	6.5	18.7

The spectral type of the HRP·donor complex as well as the magnitude of the wavelength shift persisted from one of these peroxidases to another.

Resorcinol and hydroquinone combined with protoporphyrin-apoprotein HRP C2 to give 1:1 complexes with K_d values only slightly larger than those found with HRP C2 (Table 1). The fourbanded, neutral etioporphyrin-type spectrum persisted when the donor was added, and no new bands appeared in the range 370 – 700 nm. With both donors the difference spectrum in the Soret region was of the hydroquinone type though shifted 10 nm towards shorter wavelength. The fluorescence of the protoporphyrin-apoprotein HRP C2 associate did not respond, in any way, to hydroquinone or resorcinol in concentrations equal to 0.5 – 3 K_d.

DISCUSSION

Plant tissues are rich in potential donors with two or more aromatic groups, or other structures that increase the affinity for HRP (Table 1). The benzene-styrene couple confirms that coniferol is a stronger ligand than guaiacol. For $K_d = 1$ mM the feasible donor concentration of 0.2 % of the plant tissue will suffice to complex 90 % HRP C2, assuming a molecular weight of 200 and uniform distributions. An ESR spectrum of fresh horseradish root gave signals in the $g \sim 5 - 6$ region with intensities corresponding to the contents of HRP found by extraction, and the relative intensities of the signals in this region approached those from pure HRP at near saturation with donor.[18] Plant peroxidases may be donor-ligated to a large extent in the living tissue, and knowledge of the affinities between a plant peroxidase and donors available *in situ* should give some guidance about the biological functions.

The hydroquinone induced spectral changes in the Soret region are of the type previously reported for several donors [3–6] whereas the changes due to resorcinol have been seen only with hydroxamic acids.[4] Neither shift is accompanied by expected alterations of the visible spectrum, although the hydroquinone induced effects on the 500 and 640 nm bands agree.[19,20] The two types of HRP·donor spectra are similar to the main types of the cytochrome P 450·substrate spectra, but much less pronounced. In the latter case the substrates profoundly modulate the spin state of the heme protein. During an ESR study of HRP differences due to hydroquinone and resorcinol were sought for with entirely negative result.[18] The competitive mode of binding, the identical effects on the ESR spectrum, and the near agreement in thermodynamic and redox properties of the complexes clearly bring it out that the two donors bind to the same site in HRP. Hence the spectral differences reflect some property, inherent in the donor molecule but so far not identified. Ethylbenzene, benzene, styrene, and nitrobenzene, although no substrates of HRP, all produced spectra of the hydroquinone type, and the electron distribution within the donor can thus hardly be decisive. Nitrogenous donors can give either type.

The donors used in the present model study associate only loosely with HRP and nearly as well with the Fe(II) as with the Fe(III) enzyme. The design of HRP may not allow for a Fe(III)/(II) modulation by the donor since this system does not operate in the peroxidase cycle with compounds I and II, but reactions

involving compound III may be affected by a strong, biological ligand.

HRP Fe(III) and Fe(II) associate with donors to form a system with four equilibria [21,22]

$$\begin{array}{ccc} \text{Fe(II)} & \xrightarrow{K_r/G_4} & \text{Fe(II)donor} \\ E_e/G_1 \Big| & & \Big| E_{ed}/G_2 \\ \text{Fe(III)} & \xrightarrow[K_o/G_3]{\text{donor}} & \text{Fe(III)}\cdot\text{donor} \end{array}$$

where E_e and E_{ed} represent the midpotentials (50 % oxidation) of the free enzyme and the enzyme-donor complex, and K_o and K_r the dissociation constants of the enzyme-donor complexes in the Fe(III) and Fe(II) forms. Table 7 summarizes $\Delta G'$ values for HRP C2.

Energy conservation requires that $\Delta G'_1 + \Delta G'_4 = \Delta G'_2 + \Delta G'_3$,[22] which seems to be the case within reasonable limits of error. This also confirms the method of extrapolating E_m to its value for donor-bound HRP Fe(III) and Fe(II). The uncertainty in the value of K_r may be the major source of the deviation from zero; $K_r = 13.4$ and 7.9 mM for hydroquinone and resorcinol would give $\Delta G' = 0$. According to our experience K_o and the redox equilibria can be determined with better reproducibility than K_r, which is conveniently obtained from

$$E_{ed} - E_e = \frac{RT}{nF} \ln \frac{K_o}{K_r}$$

Hydroxamic acids with a planar, aromatic nucleus, and aromatic donors compete for a site in HRP, conceived as a largely apolar region of the apoprotein. This binding was considered the main driving force in the enzyme-ligand association with some contribution from a hydrogen bonding between the hydroxamic acid and an iron-linked water molecule,[4] an outer sphere association. Essentially the same conclusion emerged from studies of the interaction between a donor and the sixth ligand.[3] The thermodynamic parameters distinguish HRP from the other three proteins in Table 4 and suggest a more specific arrangement than a hydrophobic bonding mediated by alkyl groups and with water redistribution as a dominating feature.[23] Two other modes of attachement seem possible: to an aromatic structure by $\pi - \pi$ interaction or to a cleft in HRP, narrow enough to exclude water even in its donor-free form. Without being definitely disproved the second alternative is made less likely by the very rapid initial HRP-donor association [3] and the ability of HRP to oxidize big molecules such as cytochrome c.[24] The first alternative is supported by two observations. (a) HRP C2 was retained by phenyl- but not by octyl-Sepharose.® An aliphatic, apolar binding area in HRP should have caused the opposite behaviour on the two columns. Interestingly, rabbit liver cytochrome P 450 is retained by octyl-Sepharose®.[25] (b) In the aqueous polymer two-phase system with polyethyleneglycol (PEG) and dextran aromatic substances show a distinct preference for the PEG phase, whereas proteins close to pI usually prefer the dextran phase.[26] HRP A2 and C2, unlike the other proteins studied simultaneously, stubbornly stuck to the PEG phase at various acidities and electrolyte compositions.[27] Taken together these observations strongly suggest an aromatic, exposed structure in HRP as donor binding. The free energy of the donor-HRP ligation amounts to 9 – 18 kJ mol^{-1} (Table 1). Even this value, low for an enzyme-substrate complex, is much too high for an aromatic interaction and auxilliary forces must operate. Another two possibilities will be discussed: a hydrogen bond between the donor and an iron-linked water molecule, and an interaction directly between the donor and the porphyrin.

The strength of the hydrogen bond would depend, at given steric relations, upon the nature of the donor and the acidity of the water molecule. The tighter binding of phenols than of aniline derivatives is in accord with O–H···O being a stronger bond than O–H···N. This points at an appreciable contribution from

Table 7. Free energy balance in the system HRP Fe III, Fe II, and donor.

	Hydroquinone	$\Delta G'$/ kJ mol^{-1}	Resorcinol	$\Delta G'$/ kJ mol^{-1}
K_r/mM	18.9	−9.8	9.2	−11.6
K_o/mM	3.2	−14.2	4.0	−13.7
E_{ed}/mV	−243.7	+23.5	−225.4	+21.7
E_e/mV	−207.7	+20.0	−207.7	+20.0
		0.9		0.4

this bond to the associating forces, but other observations fail to confirm a mechanism with a hydrogen bond to the water. The replacement of the hematin vinyl groups by ethyl or acetyl groups shifts pK_a for the reversible transition of HRP C2 between the brown, high-spin form and the red, low-spin form from 11.1 to 11.8 an 9.0, respectively.[12] The transition has been attributed to a dissociation $Fe(III)H_2O = Fe(III)OH^- + H^+$ (but *cf.* reviews [22,29]). However, the 600-fold difference in acidity between the extremes does not show up in the dissociation constant of the HRP·donor complex (Table 6). Further, protohematin HRP A2 and diacetyldeuterohematin HRP C2 with nearly equal pK_a (9.2 and 9.0) bind donors with different strengths, unlikely if a hydrogen bond had a major role (Tables 5 and 6). HRP C2 Fe(II) and the iron-free protoporphyrin· apoprotein HRP C2 associate bind donors nearly as firmly as HRP C2 Fe(III) does (Table 1). Finally, benzene, nitrobenzene, and styrene are ligated as tightly as the phenols. In summary, an $Fe(III)\cdot H_2O\cdots$donor hydrogen bond contributes but little, if at all, to the stability of the complex between HRP and a donor. The absence of such a bond would be in accord with indications from NMR and ESR spectroscopy,[30,31] which deny the existence of an iron-bound water molecule.

The perturbations of the spectra of HRP or its iron-free form by a donor resemble the spectral effects seen when aromatic compounds form charge transfer complexes with metalloporphyrins or free porphyrins.[32,33] Various combinations of charge accepting or donating ligands with several metalloporphyrins revealed no simple correlation between stability constants of the complexes and any parameter of the ligand but for one: large molecules tended to give more stable complexes.[34] Charge transfer complexes with aromates require, however, a narrow interplanar distance and reasonably good matching. Various applications of NMR have given the distances between Fe and ligand protons as ≥ 3.2 (ethanediol [7]), $5.8-6.3$ (aminotriazole, resorcinol [8]), $\geq 7.0-8.5$ (*p*-cresol [5]), $9.2-10.6$ (indolepropionic acid; H (4) [9]), and ≥ 10 (2,4-dichlorophenoxyacetic acid [10]) Å with the donors inclined so as to give observed protons about equal distances to the iron atom. In the hematin disc the distance between the iron atom and a pyrrolic 2-carbon atom is ~4.5 Å.[35] Neither this distance nor the inclination permits this kind of porphyrin-donor interaction. The conclusion is supported by the results from CD spectrophotometry. An asymmetric position of the donor close to the chromophore should have a considerable impact on the Cotton effect of the hematin. The observed slight perturbation is compatible with a donor position remote from the hematin. Alternatively, a donor position perpendicular but close to the hematin disc would give a modest CD effect (*cf.* histidine as fifth ligand in hemeproteins [36]), but this interpretation is rejected by the NMR results. Finally, charge transfer between a porphyrin and an aromatic ligand will quench the fluorescence,[33] but nothing in that direction occurred when a donor was added to the protoporphyrin·apoprotein C2 associate. A direct interaction involving charge transfer between the prosthetic group and a donor can be excluded. Hence the ligation of the donor is mediated only by the protein moiety, the strength of the bonding requiring favourable stericity to support the aromatic amino acid residue bond.

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