OPTICAL, NMR AND EPR PROPERTIES OF HORSERADISH PEROXIDASE AND ITS DONOR COMPLEXES

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1. Introduction

Complexes of horseradish peroxidase (HRP) and hydrogen donors have only recently been demonstrated spectrophotometrically [1,2]. The donor increased the dissociation of the HRP–cyanide complex and altered the optical spectrum of HRP–H_2O_2 compound II much more than that of free HRP, but no formation of an active ternary HRP–H_2O_2–donor complex was assumed [1]. EPR spectra of HRP without peroxide or donor ('free' enzyme) [2–6] have been variously interpreted as corresponding to high spin [2–4] or thermally mixed high and low spin species [6]. A transition from a multi-species EPR spectrum for the free enzyme to a pure high spin spectrum has been observed upon the addition of benzhydroxamic acid [2], but no EPR or NMR studies of HRP–donor complexes have been published.

The present paper gives some optical, NMR, and EPR properties of HRP and its donor complex.** A recent analysis of the bacterial cytochrome c' EPR spectrum is applied to the EPR spectrum of HRP [7,8].

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2. Materials and methods

Three batches of HRP C2 [9] (III b, pI 8.8, R_Z 3.4) gave essentially the same results. All experiments but for a pH-study were performed in 100 mM sodium phosphate at pH 6.0, and only donors essentially uncharged at this pH were used.

Difference optical spectra (HRP–donor vs HRP) were registered at 25°C by means of standard Cary 15 or Acta III instruments. EPR spectra were done with a Varian E-4 spectrometer equipped with an Air Products LTD-3-110 cryostat, and NMR spectra with a Varian HR-220 instrument with a Fourier transform accessory. Relaxation time (T_1) measurements were accomplished as previously described [10].

3. Results and discussion

The optical changes caused by the donors were stable, showed isosbestic points, and were not altered by the presence of catalase (1% of HRP). Pronounced changes occurred at 411 nm where HRP and HRP–H_2O_2 compound II are isosbestic. The optical changes, yielding $\Delta\epsilon$ less than 5% in the Soret region, were therefore attributed exclusively to the formation of HRP–donor complexes. Scanning of the Soret region with Scatchard plots of ΔA between two

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wavelengths showed HRP to form 1:1 complexes with hydroquinone, catechol, resorcinol, guaiacol, aniline, *o*-toluidine and 3-amino-1,2,4-triazole with K_{diss} 3.2, 7.9, 4.0, 6.6, 26, 22 and 100 mM respectively. This gives conditions for equal binding proportions at 25°C, but the effects of temperature changes are unknown.

Aminotriazol and resorcinol were chosen for NMR spectroscopy because aminotriazole possesses a single observable proton and resorcinol a symmetrical structure. The effect of HRP on T_1 of the NMR observable protons at 25°C amounted to bound relaxation rates of 240 sec^{-1} for aminotriazole and 340, 200, and 210 sec^{-1} for the H (2), H (4, 6) and H (5) protons of resorcinol. For aminotriazol, T_2 was approximately equal to T_1, demonstrating that the observed relaxation effects were caused by paramagnetic interactions. Since the correlation time for diamagnetic interactions is expected to be the rotation time of the enzyme–substrate complex, diamagnetic effects of substrate binding should cause $T_1 >> T_2$. The observed equality of T_1 and T_2 hence leads to the conclusion of a short ($< 10^{-9}$ sec) correlation time, which is expected for the electron spin relaxation time of the heme iron. Using 10^{-11} sec for the correlation time gives estimated proton-iron distances of 6.1 Å for aminotriazole and 5.8, 6.2 and 6.3 Å for the H (2), H (4, 6) and H (5) distances.

In the EPR spectrum of free HRP at 4.2–30°K, several paramagnetic signals are superimposed (fig.1). Extensive EPR studies at various values of microwave power, temperature, and pH [11] revealed four distinct signals, denoted as a_1, b_1, b_2 and b_3 in fig.1b. The corresponding apparent g values are listed in table 1. The signals appeared in all HRP preparations, although in somewhat varying proportions.

Fig.2 shows EPR spectra of a sample of HRP, free and after the addition of increasing amounts of hydroquinone. At low concentrations (0.4 mM), this donor caused the nearly complete conversion from a composite spectrum to a single species spectrum, consisting largely of signal b_3. At higher concentrations ($\approx$ 10 mM) hydroquinone caused a further spectral conversion, from signal b_3 back to a spectrum composed of signals a_1, b_1, b_2 and b_3. Similar two-phase transitions to signal b_3 and back to a composite spectrum were seen as the concentrations of the other donors were raised [11], and they occurred

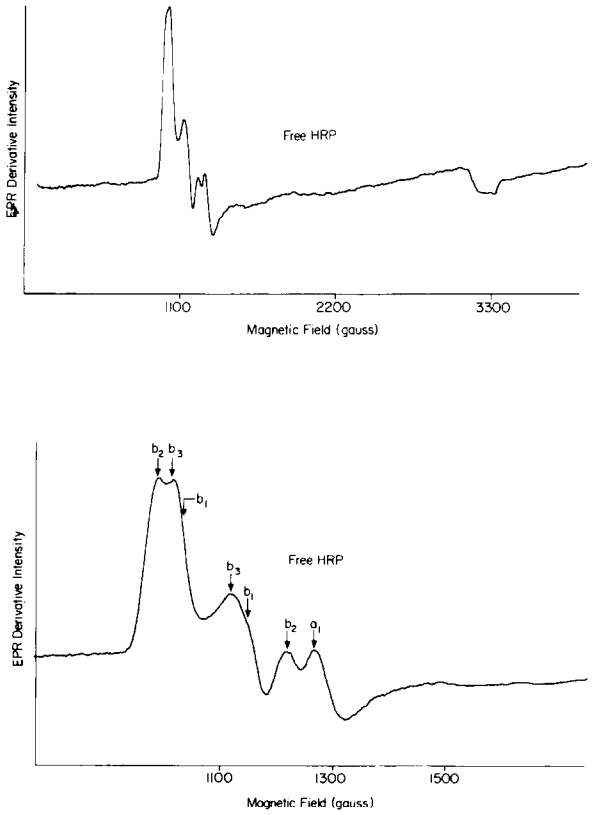


Fig.1. EPR spectrum of free 50 μM HRP at 4.2°K, 9.14 GHz and 5.0 mW power. (a) Field range 0–4000 gauss. (b) Field range 800–1800 gauss.

with all three samples of HRP, irrespective of the relative proportions of the signals in the EPR spectrum of the free enzyme.

The small isotropic signal at about 1525 gauss (g 4.28) is commonly seen in heme proteins and generally attributed to some iron impurity. It did not change upon the addition of donors to HRP.

Signals b_1, b_2 and b_3 have g values commonly observed for 'pure' high spin ferric heme complexes with slight rhombic distortions of the heme axial symmetry [12]. An EPR signal of type a_1 has been observed from the ferric form of the bacterial cytochrome c' [7,13] where, as now appears to be the case in HRP, the a_1 species occurs as a chemical mixture with high spin species [7]. The cytochrome c' a_1 type signals were characterized as due to quantum mechanical admixtures of intermediate and

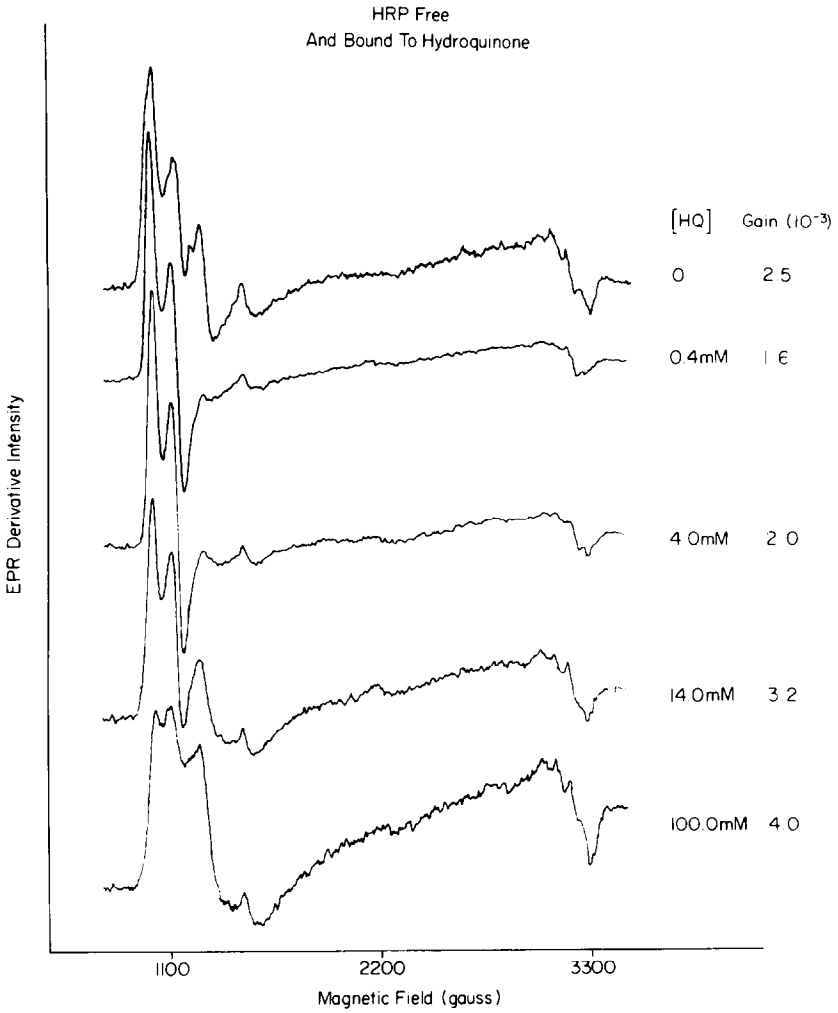
Table 1
EPR g values of free and donor-bound horseradish peroxidase

Spectral species	g values*	
	g_I	g_{II}
a_1	5.1	2.0
b_1	6.3, 5.6	2.0
b_2	6.6, 5.2	2.0
b_3	6.4, 5.7	2.0

* The apparent EPR g values were taken at the low field peak, zero-field crossing, and high field trough, respectively of spectra in the range 4.2–7°K.

high spin states, yielding a structural model with the heme iron sensitively poised between the out-of-plane high spin and the in-plane low spin positions and with axial ligands of about the same strength as in the high spin configuration [7,8]. We assign this model also to HRP.

The optical and EPR spectral changes induced by the donor exclude the possibility of a strong donor-iron bond, and hence favor the idea of donor binding at some other site. The latter mode of binding has previously been suggested from optical studies [1,2, 14]. The EPR changes at low donor concentrations are consistent with a conformational change at the



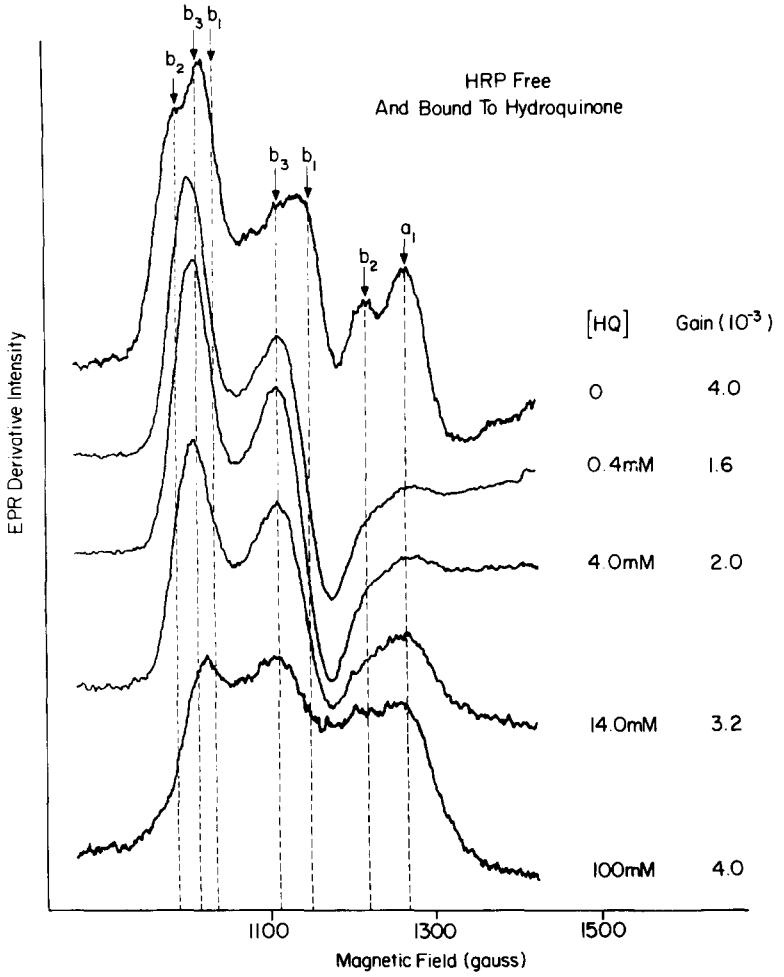


Fig.2. EPR spectra at 10°K of HRP, 50 μM, free and upon the addition of increasing amounts of hydroquinone. Conditions are otherwise the same as in fig.1. (a) Field range 750–3500 gauss. (b) Field range 900–1450 gauss.

iron site, imposing a greater degree of specificity and stabilizing the heme iron in a distinct high spin configuration. This model of enzyme–substrate binding is in accord with the 6 Å donor proton–iron distance estimated from NMR data. The 'reverse' effect of high donor concentrations on the EPR spectrum is probably less specific in nature. This assumption is corroborated by the fact that $\Delta A_{obs}/\Delta A_{max}$ in the Scatchard plots ranged from 0.15 to 0.90, leaving little room for changes in the Soret region at higher donor concentrations. The two-phase effects in these equilibrium studies have a kinetic correspondence in the occurrence of optimal donor concentrations [15].

Thus, the following model for the binding of donor to enzyme is arrived at: (i) in the absence of substrate, an enzyme molecule can assume a conformation corresponding to either of three high spin states or a quantum mechanically mixed intermediate and high spin state, yielding a composite EPR spectrum of the solution; (ii) at low donor concentrations, one donor molecule binds at a site about 6 Å away from the iron, thereby inducing a shift to a distinct high spin form (b_3); (iii) at high donor con-

centrations additional, probably unspecific, allosteric changes occur, again yielding a composite EPR spectrum. Implicit in this model is the assumption that the binding of the donor to HRP can strongly affect the planarity of the heme iron.

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