THE HAEM-ENVIRONMENT IN HORSERADISH PEROXIDASE AS SEEN BY PROTON MAGNETIC RELAXATION

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Received July 29, 1975

The water-proton fast exchange between the vicinity of the ferric haem-iron and bulk solvent in horseradish peroxidase solutions at neutral pH has been directly verified by measuring the proton magnetic relaxation times of the aliphatic protons from ethanediol in an otherwise deuterated solution. The implications of this finding are discussed with regard to the stereochemistry of the haem-surrounding.

The evaluation of the structural details of the immediate vicinity of the haem-iron is of considerable interest, since the haem-environment modulates the ligand-binding properties of the iron-ion enabling thus all the specifities among the haemoproteins. Solvent-proton magnetic relaxation technique (PMR) has already been applied in studies of various haemoglobins, but this communication is concerned with the haem-pocket in horseradish peroxidase (HRP) as seen by the temperature enhancement of the PMR rates in solutions at neutral pH. On the basis of similar experiments Lanir and Schejter (1) drew some conclusions on the nature of the sixth ligand to the iron-ion. Our results cast some new light on the stereochemistry of the haem-pocket in HRP.

Materials and Methods

Commercial liophylized HRP (Sigma, R.Z.=3.0) was dissolved in 0.1 M phosphate buffer in H_2O, pH 7.5 or in 0.1 M phosphate with deuterated ethanediol, $C_2H_4(OD)_2$, 35 v% in D_2O, pH measured 7.0. The details of the deuteration procedure have been described previously (2). The fluoride was added as the sodium salt (Merck). The carbonmonoxy-derivative was prepared by reduction with sodium dithionite (Merck) under the stream of carbonmonoxyde. The actual HRP concentrations were between 1 and 2 mM. The samples were qualitatively and quantitatively characterized by optical absorption spectra according to the data of Keilin and Hartree (3).

The longitudinal solvent-proton magnetic relaxation times, T_1, were measured by the π-t-$\pi/2$ pulse sequence at 24 MHz using a spectrometer built at "Jožef Stefan" Institute, Ljubljana, Yugoslavia. The data were further evaluated as described previously (2).

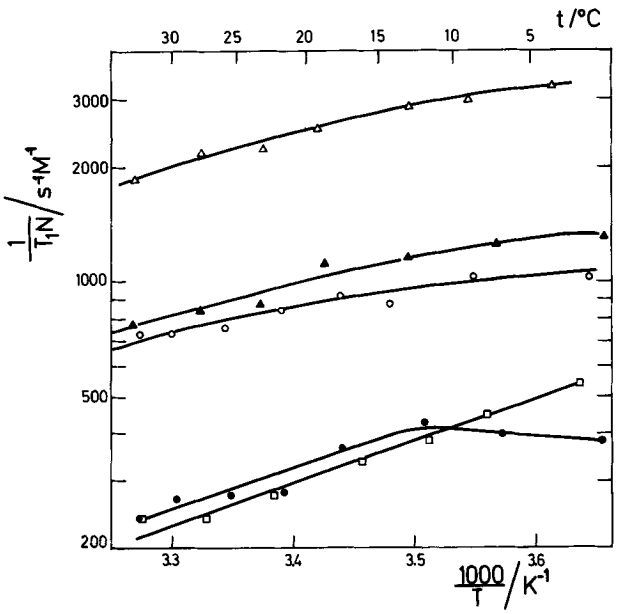

Fig. 1 The temperature enhancement of the molar longitudinal solvent-proton magnetic relaxation rates in horseradish peroxidase solutions at neutral pH. Circles: HRP; triangles: fluoride-HRP; squares: carbonmonoxy-HRP. Open symbols: ordinary aqueous solutions; filled symbols: deuterated solutions containing aliphatic protons of ethanediol.

Results and Discussion

The logarithms of the solvent-proton molar longitudinal magnetic relaxation rates in HRP solutions are plotted *v.* reciprocal absolute temperature in Fig. 1 with the linear temperature scale indicated as well. Open circles represent the PMR rates in the original HRP solution. These rates consist of the paramagnetic contribution of the high-spin iron-ion and the diamagnetic contribution due to the immobilization of water molecules within the hydration sheath of the macromolecule. The latter can be independently measured in a diamagnetic solution of carbonmonoxy-HRP (squares in Fig. 1). The pure paramagnetic term, obtained by subtracting the diamagnetic PMR-rates from the measured total relaxation rate, can yield structural data about the vicinity of the paramagnetic centre. This can be done, according to the well known Solomon's equation (4), if one knows which type of the relaxation mechanism governs the paramagnetically induced PMR rates. The value of the paramagnetic

rate of about 500 $s^{-1}M^{-1}$ throughout the temperature range measured is not sufficient *per se* to decide which of the two possible relaxation mechanisms (fast exchange *v.* outer sphere) dominates. We therefore applied our marker-method (2) by which only the relaxation of the aliphatic protons of certain lower alcohols is measured in an otherwise deuterated solution. Thus the molecules of ethanediol, inert towards the protein, would measure the same PMR rates as the water-protons in an ordinary aqueous solution, if they could equally approach to the iron ion in a fast exchange. If the measured PMR rates for the ethanediol marker are significantly lower than that of protons in an aqueous solution, it means that the interchange of the marker molecule between the haem-pocket and bulk solvent is hindered by the protein, i.e. that the haem-crevice is too narrow to accomodate the molecule of the marker. Alternatively, the rate of such an exchange may be too slow to affect the overall relaxation rates (2). In either case only the outer-sphere relaxation is measured. The PMR rates of the ethanediol marker by which the outer-sphere PMR is proved are plotted in Fig. 1 as the filled circles. This confirms definitely, in turn, that the fast-exchange mechanism dominates the observed relaxation rates in the ordinary aqueous HRP solution. We performed similar measurements with the fluoride-derivative of HRP (FHRP). The results are presented in Fig. 1 as triangles, open for the ordinary aqueous solution of FHRP and filled for the deuterated one containing the marker-molecule. The high molar relaxation rates measured in the aqueous solution are also due to the fast proton-exchange, as confirmed by the corresponding marker-experiment.

From the pure paramagnetic PMR rates due solely to the fast-exchange mechanism, knowing the actual correlation time, τ_c, the iron-to-proton inter-spin distance, r, can be calculated from the Solomon's equation (4). There is a lack of a fully elaborated theory for the PMR in solutions of macromolecules bearing paramagnetic centres with *anisotropic* g-tensors (5), but a reasonable *lower* limit for r may be obtained using the EPR data of Asakura *et al.* (6) for $\tau_s(\equiv\tau_c)$. It is known from the magnetic susceptibility measurements (7)

Table 1

Derivative	$(1/NT_1)^a$	g[b]	n[c]	τ_c[d] ($\times 10^{10}$s)	r, Å	τ_c[e] ($\times 10^{10}$s)	r, Å
H R P	625	2	1	0.9	3.2	1.0	3.3
			2		3.6		3.7
		6	1	0.9	4.7	1.0	4.8
			2		5.2		5.3
F H R P	1750	2	1	2.7	3.3	5.1	3.6
			2		3.7		4.1
		6	1	2.7	4.7	5.1	5.2
			2		5.3		5.9

a. paramagnetically induced relaxation rate at 10°C, normalized to 100% high-spin for HRP
b. The insertion of g=6 is not strictly correct, and is used only to obtain an estimate of a *possible* r when the protons exchange roughly parallel to the x-y plane of the haem.
c. number of fluctuating protons.
d. determined from the EPR line-width (6).
e. assumed on the basis of EPR/PMR ratio for methaemoglobin.

that at room temperature about 82% of the haem-irons in a HRP solution are high-spin. Therefore, our relaxation data must be normalized to 100% high-spin iron. The values of r calculated for different numbers of fluctuating protons, different correlation times and g-values (for directions of proton exchange parallel and perpendicular ot the haem-plane) are given in Table 1. The values of τ_c were calculated from the EPR line-widths taken at 10°C in HRP solutions (6) using the formula $T_{2,S}(\equiv\tau_S) = 2/\Delta\omega\sqrt{3}$; these values are the *shortest* possible τ_c's. The τ_c's in the right column in Table 1 are the values estimated on the basis of comparison of the correlation times derived from EPR line-widths (6) and from PMR frequency dispersion (8,9) both for aquo- and fluoro-*methaemoglobin*. The values of r in the left column are the *shortest possible* interspin distances and, therefore, two main conclusions emerge. (*i*) The

increase of the paramagnetically induced relaxation rates, when going from the original HRP solutions to FHRP, is due to the increase of the electron-spin relaxation time, and *not* to a smaller electron-nucleus spin-separation. (*ii*) From the PMR data it is not possible to draw conclusions about the nature of the sixth ligand to the iron-ion, contrary to the one derived in Ref. 5, since the exchanging protons are at least 3.2 Å apart from the iron-ion. (The protons of a coordinated water molecule should be separated from the haem-iron about 2.6 Å.) It has been proposed that in acid solutions of methaemoglobin and metmyoglobin the sixth-ligand water molecule, observed by X-ray studies, does not take place in the exchange mechanism, but other solvent-water molecule(s) in its vicinity (10,11). This has recently been further substantiated (2,9). There is no particular reason to expect another mechanism in the neutral HRP solutions, if the sixth ligand in HRP were also a water molecule.

Our experimental results are very similar to those of Lanir and Schejter (1) who ascribed the observed relaxation rates to the outer-sphere mechanism. One of their main arguments for this was the comparison with the pH-titration curve of metmyoglobin obtained by Fabry *et al.* (12). However, the latter data are four times too high (probably owing to an error in normalization, for comparison see Refs. 2 and 13). Lanir and Schejter (1) found a pH-independent relaxation even above the photometric pK (≈10.8) in HRP solution. This is rather puzzling, since the lowering of the total electronic spin should be reflected to a certain degree even in the outer-sphere relaxation, because it is induced by the similar dipolar interaction between the nuclear and electronic spins. When interpreting the PMR-titration data in haemoprotein solutions special attention has to be paid, because it has been found for aquomethaemoglobin that photometric and PMR pK values are very different (12) resulting from different ionizations. However, the pK values measured by the two methods (PMR and photometry) at *room* temperature in aquometmyoglobin solution are similar just by mere chance. Mildvan *et al.* (13) gave convincing evidence that the temperature dependencies of pK values observed here by the two techniques are completely different.

Our results show that the haem-pocket in HRP is too narrow to accomodate the ethanediol molecule. This together with the fast-exchange behaviour for H_2O makes it similar, in the PMR sense, to the haem-pocket in *Chironomus*-haemoglobin (3). The effect of the fluoride ion on the electronic relaxation of the iron-electrons, similar in magnitude to that observed for methaemoglobin when the ligand-water is substituted by fluoride (8,9), is consistent with a sedentary water molecule at the sixth ligand-site of the haem-iron in HRP.

We thank Dr. S. Maričić for stimulating and helpfull discussions. This work has been supported by the PL-480 grant 02-004-1.

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