Nuclear Magnetic Resonance Evidence for the Absence of Iron Coordinated Water in Horseradish Peroxidase.

Amos Lanir and Abel Schejter

Department of Biochemistry, The George S. Wise Center for Life Sciences, Tel-Aviv University, Tel-Aviv, Israel.

Received November 21,1974

Summary: The pH dependence of longitudinal relaxation rates of water protons in horseradish peroxidase solutions indicates that water is not coordinated to the ferric iron of the enzyme.

The nature of the iron ligands in catalases and peroxidases is a problem of long standing in heme protein chemistry (1,2). The ferric iron of these enzymes is in the high spin state; by analogy to ferric-hemoglobin and myoglobin, it has been customarily assumed, though never conclusively proven, that one of the iron ligands in these heme proteins is a water molecule (2,3,4,5).

In order to obtain more direct evidence for the presence or absence of water in the first coordination sphere of the ferric peroxidase iron, a study was conducted on the pH dependence of the nuclear magnetic longitudinal relaxation rates (T_1^{-1}) of water protons in horseradish peroxidase (HRP) solutions. This method was previously used for the elucidation of structural details near the high spin ferric iron of various heme proteins (6,7,8). Since ferric horseradish peroxidase is in the high spin state in neutral solutions, it was expected that the existence of fast exchange of water molecules between the bulk of the solution and

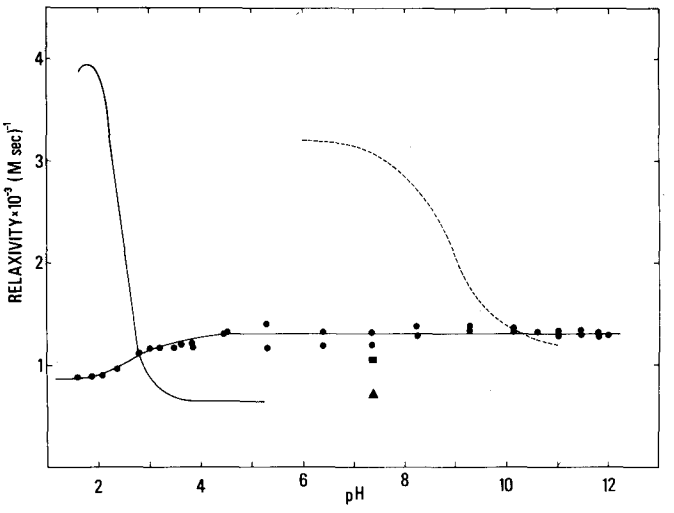

Fig. 1 Relaxivity as a function of pH for several hemoproteins. (-●-●-) horseradish peroxidase. (——) cytochrome c [taken from Lanir and Aviram (13)]. (---) methmyoglobin [taken from Fabry et al (12)]. ■ HRP-cyanide. ▲ Reduced HRP (with dithionite).

the first coordination sphere of the iron should result in a large enhancement of the proton relaxation rates of the water.

Methods

Values of T_1^{-1} at 100 MHz were measured using a spin echo attachment to a high resolution nmr spectrometer, by the 180°-90° null method (9). Horseradish peroxidase was a commercial preparation. The pH was adjusted with HCl or NaOH.

Results

The results at different pH's expressed at molar relaxivities are shown in Figure 1, where they are compared to results of similar experiments performed on ferric horse heart cytochrome c (8) and ferric myoglobin (7).

Two characteristics of the results are relevant to the problem of iron-water coordination: the absolute values of the molar relaxivities, and their pH dependence. In the first place, it can be seen that the value of the molar relaxivity of neutral ferric peroxidase is distinctly lower than neutral metmyoglobin and acidic ferricytochrome c, which have one and two water molecules bound to the high spin iron respectively. T_1^{-1} value in solutions of HRP is very close to that for the low-spin cyanide-HRP complex. Since the longitudinal electronic relaxation time of the low spin ferric ion is very short ($T_{1e} = 2 \times 10^{-12}$ sec)[11], T_1^{-1} value in solutions of cyanide-HRP may be considered as being very close to the diamagnetic contribution of ferric HRP to the relaxation. This value is somewhat higher than the molar relaxivity in the low spin, closed crevice cytochrome c in neutral solutions, which is reasonable considering the relative size of the two macromolecules. These results indicate that water is not coordinated to the iron in neutral peroxidase. Furthermore, ferric HRP undergoes a high spin transition with a pK of 10.8 (3,4,5). If a water molecule were coordinated to the iron at pH's below this pK, a decrease in the molar relaxivity should be observed as the protein is brought into the alkaline side of the transition. The observed fact is that the molar relaxivity is practically constant along the whole pH range investigated, that is, from pH 3 to pH 12. A small decrease in the acidic range, with an inflection point at about pH 2.5, is due to the well known changes occuring in HRP at acid pH that lead finally to the splitting of the heme group (10).

We conclude, therefore, that ferric HRP has no water coordinated to its iron and has, in all probability, a closed crevice structure.

Discussion

Heme proteins can be classified according to the nature of their heme iron ligands, into two main groups, having "closed crevice" and

"open crevice" structures (1). In the first group, the iron atom is coordinated by two protein residues; in the second group, one iron ligand is a protein residue and the other a molecule of water. Cytochrome c is a typical instance of a closed crevice structure, while hemoglobin and myoglobin are examples of open crevice structures.

The hypothesis that catalase and peroxidase have open crevice structures, with an iron bound water molecule, creates difficulties in the interpretation of the pH dependence of the binding of catalase and peroxidase by ionic ligands. The affinity for ligands such as fluoride and azide, and for cyanide above the pK of hydrocyanic acid, decrease with increasing pH, indicating that a group in the protein becomes available for protonation upon formation of the complex. Alternative explanations such as binding by the acids HF, HN_3, HCN (11) or a structure in which a hydroxyl ion binds the iron instead of a water molecule (12), were criticized by George and Lyster, who proposed that peroxidases and catalases are closed crevice enzymes, a tyrosine phenolate ion being perhaps the sixth iron ligand (1, 13). A similar structure was also suggested by Phelps et al (14), in order to explain the difference in the reversible proton dissociation equilibria between peroxidase and apoperoxidase. More recently it was shown (15) that the kinetics of the pK=10.8 heme linked ionization of HRP does not support the commonly held view that this reaction is due, analogously to the pK 8 ionizations of methemoglobin and metmyoglobin, to deprotonation of an iron bound water molecule; instead, the reaction can be better interpreted as an ionization followed by a conformation change of the protein.

The nmr measurements reported in this communication indicate clearly that water is not bound to the iron of ferric HRP. Although the possibility of penta-coordination cannot be disregarded, our conclusion is that ferric HRP has a closed crevice structure in neutral solutions.

This result has an interesting implication. It is hard to conceive

how the low concentration of hydrogen peroxide required to activate peroxidase can suffice for iron binding, while the solvent concentration cannot. Thus one is lead to the suggestion that the initial event in the enzymic reactions of peroxidase is not the direct binding of hydrogen peroxide, but an oxidation starting at some other site in the heme protein molecule; a possibility that has been already contemplated by other authors (16).

References

1. George, P., and Lyster, R.L.J., Proc. Nat. Acad. Sci. (1958) 44, 1013-1029.
2. Ricard, J., Mazza, G. and Williams, R.J.P., Eur. J. Biochem. (1972) 28, 566 578.
3. Keilin, D. and Hartree, E.F., Biochem. J. (1950) 49, 88-104.
4. Harbury, H.A., J. Biol. Chem. (1957) 225, 1009-1024.
5. Ellis, W.D. and Dunford, M.B., Arch. Biochem. Biophys. (1969) 133, 313-317.
6. Wuthrich, K., in: "Structure and Bonding" (1970) 8, 53-121 (Springer Verlag, New York).
7. Fabry, T.L., Kim, J., Koenig, S.H. and Schillinger, W.E., in: "Probes of Structure and Function of Macromolecules and Membranes" (Edit. by Chance B., Yonetani, T. and Mildvan, A.S.)(1971) Vol. II 311-315.
8. Lanir, A. and Aviram, I., Arch. Biochem. Biophys. (in press).
9. Ginsburg, A., Lipman, A. and Navon, G., J. Sci. Inst. (1970) 3, 699-701.
10. Maehly, A.C., Biochim. Biophys. Acta (1952) 8, 1-17.
11. Chance, B., J. Biol. Chem. (1952) 194, 483-496.
12. Theorell, H. and Paul, K.G., Ark. Kemie. Mineral. Geol. (1944) 18A, No. 12
13. George, P., Lyster, R.L.J. and Beetlestone, J., J. Biol. Chem. (1961) 236, 3246-3251.
14. Phelps, C., Forlani, L. and Antonini, E., Biochem. J. (1971) 124, 605-614.
15. Epstein, N. and Schejter, A., FEBS Lett. (1972) 25, 46-48.
16. Dolphin, D., Forman, A., Borg, D.C., Fajer, J. and Felton, R.H., Proc. Nat. Acad. Sci. (1971) 68, 614-618.