Proc. Indian Acad. Sci. (Chem. Sci.), Vol. 106, No. 3, June 1994, pp. 729–734.

Stereochemical structure and biochemical activity of heme proteins

SAMARESH MITRA
Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India

Abstract. A correlation between the stereochemical geometry around the metal ion in heme enzymes and their catalytic properties is attempted. It is shown that spin–lattice relaxation time measurement of bulk water in the enzyme solution gives accurate information as to the presence or absence of water molecule at the sixth coordination site of the heme iron. The rate of formation of compound I, an intermediate in the catalytic cycle of the enzymes, is shown to be directly related to the nature of the sixth coordination site of the heme.

Keywords. Stereochemical geometry; heme enzymes; spin–lattice; catalytic cycle.

1. Introduction

The stereochemistry around the metal ion plays an important role in determining the biochemical activity of heme proteins (Perutz *et al* 1987; Perutz 1990). The heme iron in these proteins is usually coordinated to four pyrrole nitrogens of basal porphyrin ring and an axial nitrogen of proximal histidine. The sixth coordination site may either be vacant or occupied by a ligand such as water. The occupation (or otherwise) of the sixth coordination site in several heme proteins is crucial in determining their biochemical activity. Consider, for example, the catalytic cycle of peroxidase heme enzymes which catalyse the oxidation of inorganic and organic substrates by hydrogen peroxide (figure 1). The catalytic cycle involves two intermediates, compound I and compound II, which have been well characterised (Saunders 1973; Dunford and Stillman 1976). It has been postulated that binding of H_2O_2 to the sixth coordination site of the heme iron is essential for the formation of compound I (Dunford and Stillman 1976). If the sixth coordination site of the heme iron is occupied by a strong-field ligand such as cyanide, H_2O_2 cannot bind to it and the enzyme ceases to be active. Thus, a relationship seems to exist between the coordination geometry around the heme iron and the biochemical function of such heme proteins. This article reviews some of the studies carried out in our laboratory on peroxidase heme enzymes and myoglobin in this direction.

2. Methods and materials

Studies reported refer to two peroxidase heme enzymes, lactoperoxidase (LPO) and horseradish peroxidase (HRP), and to myoglobin. Methods for the isolation and purification of LPO and HRP are described in detail elsewhere (Modi *et al* 1989, 1990a, 1991a). Manganese reconstituted horseradish peroxidase (MnHRP) was

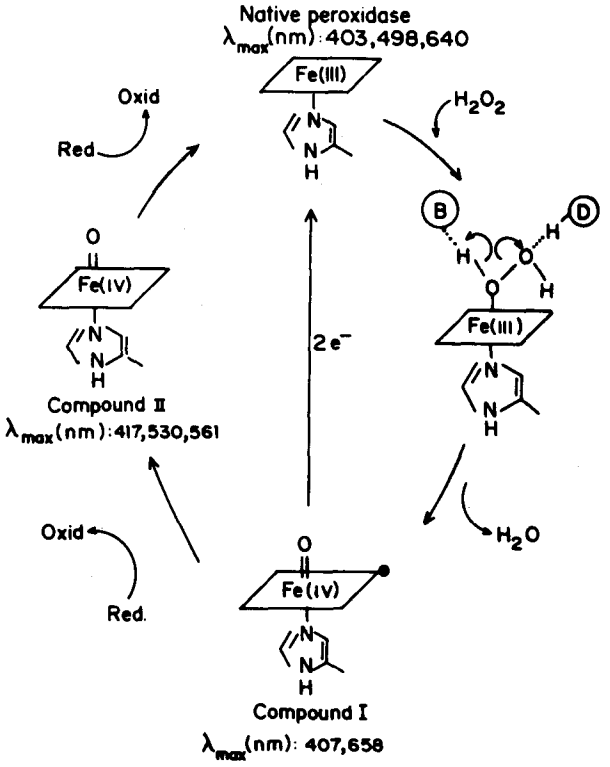

Figure 1. Catalytic cycle of peroxidase heme enzymes.

obtained by the method described earlier (Modi *et al* 1990; Saxena *et al* 1990). Chemical modification of sperm whale myoglobin was carried out as described by Modi *et al* (1991b). All NMR measurements were carried out on AM 500 MHz Bruker FT NMR spectrometer. Kinetic measurements were done using Hi Tech Stopped Flow equipment.

For NMR measurements the samples are lyophilised directly inside the NMR tubes with excess D_2O. The protein samples are treated with Cholex–100 (Bio Rad) to remove any traces of free metal ions, and samples are prepared in deionised double distilled water. T_1 measurements are made using $180°–\tau–90°$ pulse.

3. Results and discussion

3.1 *Structural and kinetic studies*

Accurate single crystal structural data available on metmyoglobin (Mb) show that the sixth coordination site of heme iron is occupied by a loosely bound water molecule which is stabilised in the coordination site through hydrogen bonding to distal histidine (Takano 1977). No such structural data are available for LPO and HRP. The question of the sixth coordination site being occupied (or not) by the water molecule in the peroxidases had therefore remained open. The question becomes further significant when the rate of formation of compound I in these systems is

Table 1. Water proton distance and rate constants of mylglobins and peroxidases heme enzymes.

	τ_c (s)	r (Å)	k ($mol^{-1}s^{-1}$)	Coordination at the sixth position
Mb	5×10^{-11}	2·7 (2·67)[a]	$1·0 \times 10^3$	Water coordinated
MnHRP	5×10^{-11}	2·6	$1·3 \times 10^4$	Water coordinated
HRP	$9·5 \times 10^{-11}$	3·6	$1·9 \times 10^7$	Vacant
LPO	$4·5 \times 10^{-10}$	4·5	$2·0 \times 10^7$	Vacant
Modified Mb	5×10^{-11}	3·8	$5·2 \times 10^7$	Vacant

[a]Single crystal X-ray data (Takano 1977)

compared (table 1). While the rate constant for HRP and LPO is almost identical, it is several orders of magnitude higher than that of Mb and MnHRP. This observation along with the uncertainty about the nature of the sixth coordination site in the peroxidases had prompted to determine the presence or absence of water at this coordination site in LPO, HRP and MnHRP.

3.2 *Determination of water in the coordination site (T_1 measurement)*

An elegant method to determine if water molecule is coordinated (or not) to the metal ion in these paramagnetic enzymes is to measure water proton relaxivity of the enzyme solution by NMR spectroscopy (Wuthrich 1970; Lanir and Schejter 1975). Since the ferric ion in these heme proteins at physiological *p*H is high-spin (Lukat *et al* 1987), it is expected that the existence of fast exchange of water molecules between the bulk of the solution and the first coordination sphere of the paramagnetic heme centre should result in large enhancement of proton relaxation rates of water (Dwek *et al* 1975; Modi *et al* 1989). The longitudinal (T_{1m}) and transverse (T_{2m}) paramagnetic relaxation times of bound substrate are given by the following equations (Solomon 1955; Bloembergen 1957):

$$\frac{1}{T_{1m}} = \frac{2\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right], \tag{1}$$

$$\frac{1}{T_{2m}} = \frac{\gamma_\gamma^2 g^2 S(S+1)\beta^2}{15r^6}\left[4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right], \tag{2}$$

where γ_I is the nuclear gyromagnetic ratio, g is the isotropic splitting factor, β is the Bohr magneton, S is the total spin of the ground electronic state of the paramagnetic ion, r is the distance of the resonating nucleus (here the hydrogen atom of the water molecule) from the paramagnetic metal ion; ω_I and ω_s are the nuclear and electronic Lamor precession frequencies respectively. Equations (1) and (2) include only those terms which arise out of dipole–dipole interaction between electron spin S and nuclear spin I, which is characterised by a correlation time τ_c that modulates this interaction. The distance between the water proton and metal ion centre can be obtained from

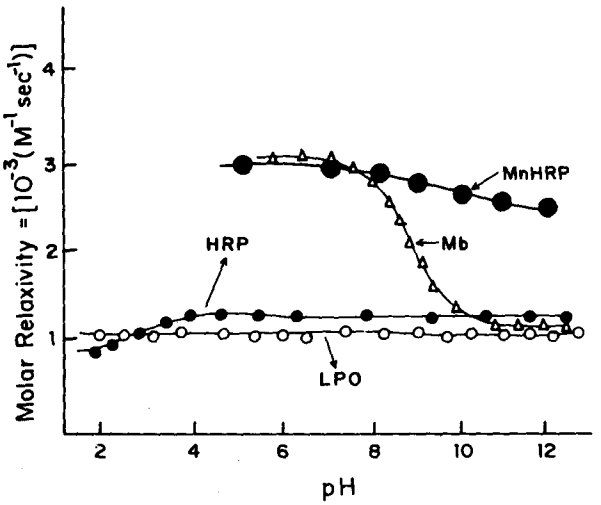

Figure 2. Molar relaxivity of HRP, LPO, Mb and MnHRP between *p*H 2–12.

(1) and (2) if τ_c is known. τ_c is generally obtained from the ratio of T_{2m}/T_{1m} (Dwek *et al* 1974; Modi *et al* 1989a, 1989b).

The molar relaxivity of the water protons of LPO, HRP, Mb and MnHRP has been reported over a *p*H range (Modi *et al* 1990a, 1990b). The results are summarised in figure 2. The figure shows that the molar relaxivity of LPO and HRP are almost similar but differ markedly from that of Mb which has a water molecule at its sixth coordination site. The distance of water protons from the metal ion can be calculated at $pH = 7$ with the help of (1) and (2). τ_c was obtained from the ratio T_{2m}/T_{1m}. The calculated distances are given in table 1.

The distances of water protons listed in table 1 clearly establish that water is not coordinated to heme iron in HRP and LPO, and that the sixth coordination site in these two enzymes is vacant. On the other hand, table 1 shows that water is coordinated to the metal ion in Mb (as is already known from X-ray data) and MnHRP. The observed *p*H dependence of molar relaxivity of figure 2 can now be easily understood. The low value of molar relaxivity and its *p*H independence in LPO and HRP is consistent with the observation that the sixth coordination site of heme iron in these enzymes is vacant. The 'anomalous' *p*H dependence of molar relaxivity in Mb is evidently associated with the aqua$\rightleftharpoons$hydroxo transition of coordinated water with $pk = 8{\cdot}8$ (Morishima *et al* 1977). In the acidic *p*H range aqua Mb is high-spin; in the alkaline *p*H range the coordinated water is deprotonated to hydroxo form to give hydroxo Mb which is known to be low-spin (Mitra 1982). The magnitudes of molar relaxivities at higher and lower *p*H extremities are thus consistent with their spin states.

The *p*H dependence of MnHRP needs further explanation. Table 1 shows that water is coordinated to the manganese ion in MnHRP. Its molar relaxivity is much higher than that of LPO and HRP, which is consistent with water being coordinated to it. However, the *p*H dependence of its molar relaxivity is quite different to Mb. The difference is due to the fact that aqua MnHRP and hydroxo MnHRP are both high spin (Behere *et al* 1981; Dugad *et al* 1984); hence the aqua$\rightleftharpoons$hydroxo transition

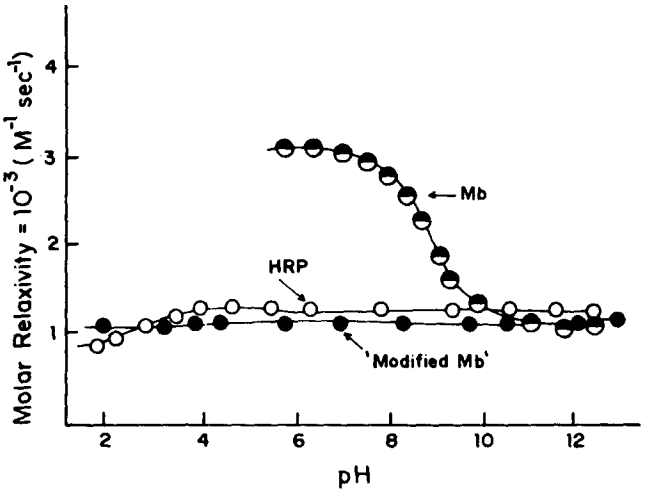

Figure 3. Molar relaxivity of modified Mb over *p*H 2–12. The HRP and Mb results are included here for comparison.

in MnHRP does not induce any spin state transition. The spin state of MnHRP remains the same over the entire *p*H range, thereby causing no change in the molar relaxivity.

3.3 *Chemical modification of Mb*

As mentioned in §3.1, water molecule coordinated to the iron in Mb is stabilised through hydrogen bonding to the distal histidine. If the distal histidine is chemically modified, this may affect the hydrogen–bonding and destabilise the coordinated water molecule. This strategy was achieved by cyanogen bromide which specifically modifies histidine (Modi *et al* 1990a; Shiro and Morishima 1984). Water proton molar relaxivity of modified Mb over the *p*H range is shown in figure 3. The data at *p*H = 7 was used to calculate the distance between the water proton and the heme iron, which clearly shows that the water molecule is no longer coordinated to the heme iron in the modified Mb (see table 1). The sixth coordination site of modified Mb is thus vacant, similar to that in LPO and HRP. The rate of formation of compound I for modified Mb was determined and found to be $5{\cdot}2 \times 10^7$ sec^{-1} mol^{-1} (table 1) (Modi *et al* 1991b).

3.4 *Stereochemical and kinetic data*

We are now in a position to rationalise the kinetic and stereochemical results in these heme proteins. Table 1 shows that LPO, HRP and modified Mb have similar coordination geometry with sixth coordination site of heme being vacant. The rates of formation of compound I in these enzymes are also found to be very similar. On the other hand water is coordinated to the metal ion in Mb and MnHRP, and rates of formation of compound I are several order of magnitude lower than those in LPO, HRP and modified Mb. As mentioned in the introduction, the binding of H_2O_2 to the metal centre is essential for the formation of compound I (Dunford and Stillman 1976). If the sixth coordination site of the metal ions is occupied by a water molecule,

as is the case in Mb and MnHRP, binding of H_2O_2 to the metal ions would be somewhat 'hindered', which will decrease the rate compared to that if the site were vacant, as in LPO, HRP and modified Mb. This is exactly what is experimentally observed here. The nature of the coordination geometry around the metal centre is therefore important in controlling the catalytic activity of the enzymes. The redox properties of the metal ion would also make some difference as is observed between Mb and MnHRP with similar coordination geometry but dissimilar metal ions at the centre.

4. Concluding remarks

Spin-lattice relaxation time measurements of bulk water in paramagnetic metal-enzymes promises to be useful in deciding if the water molecule is coordinated to the metal centre. The coordination geometry around the metal ion in these enzymes appears to modulate their catalytic activity, though other factors such as redox properties of the metal ion and heme cavity structure must be significant contributory factors in this process.

Acknowledgements

I thank Drs D V Behere, S Modi and A K Saxena who have contributed significantly to much of the work reported here.

References

Behere D V, Marathe V R and Mitra S 1981 *Chem. Phys. Lett.* **81** 57
Bloembergen N 1957 *J. Chem. Phys.* **27** 572
Dugad L B, Behere D V, Marathe V R and Mitra S 1984 *Chem. Phys. Lett.* **104** 353
Dunford H B and Stillman J S 1976 *Coord. Chem. Rev.* **19** 187
Dwek R A, Williams R J P and Xavier A V 1974 *Metal ions in biological systems* (ed.) H Sigel (Marcel Dekker, New York) **4** 62
Lanir A and Schejter A 1975 *Biochem. Biophys. Res. Commun.* **62** 199
Lukat G S, Rogers R and Goff H M 1987 *Biochemistry* **26** 6927
Mitra S 1982 In *Iron porphyrins II* (eds) A B P Lever and H B Gray (Reading, MA/New York: Addison-Wesley/Benjamin)
Modi S, Behere D V and Mitra S 1989a *Biochemistry* **28** 4689
Modi S, Behere D V and Mitra S 1989b *Biochem. Biophys. Acta* **996** 214
Modi S, Behere D V and Mitra S 1989c *J. Biol. Chem.* **264** 19677
Modi S, Behere D V and Mitra S 1990a *J. Inorg. Biochem.* **38** 17
Modi S, Behere D V and Mitra S 1990b *Biochem. Biophys. Acta* **1038** 164
Modi S, Behere D V, Mitra S and Bendal S D 1991a *Chem. Commun.* **830**
Modi S, Deodhar S, Behere D V and Mitra S 1991b *Biochemistry* **30** 118
Morishima I, Ogawa S, Inubushi T, Yonezawa T and Iizuka 1977 *Biochemistry* **16** 5109
Perutz M F 1990 *Mechanism of cooperativity and allosteric regulation in proteins* (Cambridge: University Press)
Perutz M F, Fermi G, Luisi B, Shaanam B and Liddington R C 1987 *Acc. Chem. Res.* **20** 309
Saunders B C 1973 In *Inorganic biochemistry* (ed.) G L Eichhorn (Amsterdam: Elsevier) p. 988
Saxena A K, Modi S, Behere D V and Mitra S 1990 *Biochem. Biophys. Acta* **1041** 83
Shiro Y and Morishima I 1986 *Biochemistry* **25** 1986
Solomon I 1955 *Phys. Rev.* **99** 559
Takano T 1977 *J. Mol. Biol.* **110** 537
Wuthrich K 1970 *Struct. Bonding* **8** 53