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Circular dichroism studies of acid⇌alkaline transition in hemeproteins

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Abstract. Acid⇌alkaline transition in horseradish peroxidase and lactoperoxidase has been investigated by use of circular dichroism and nuclear magnetic resonance spectroscopy. The conformational changes associated with this transition in these hemeproteins have been monitored and pK of the transition determined.

Keywords. Circular dichroism; acid–alkaline transition in hemeproteins; NMR spectroscopy.

1. Introduction

The acid⇌alkaline transition in hemeproteins such as metmyoglobin (metMb) and methemoglobin (metHb) involves proton dissociation of bound water molecules (Antonini and Brunori 1970; Wuthrich 1970; Iizuka and Morishima 1975; McGrath and LaMar 1978; Modi *et al* 1990a). The ferric ion in these hemeproteins is axially coordinated to the 'proximal' histidine and a water molecule (Takano 1977). The acid⇌alkaline transition in these hemeproteins is therefore associated with the conversion of the high-spin 'aquo' form to the low-spin 'hydroxo' one and is characterised by sharp pH-dependent changes in their electronic and spectroscopic properties. In metMb and metHb, this transition has been extensively investigated and its pK is accurately known.

Such acid⇌alkaline transition has also been observed in other hemeproteins such as horseradish peroxidase (HRP) and lactoperoxidase (LPO) (Epstein and Schejter 1972; McGrath and LaMar 1978; Morishima *et al* 1977; Teraoka and Kitagawa 1981; Yamazaki *et al* 1982; de Ropp *et al* 1984; Shiro and Morishima 1986). It is however known from recent proton-NMR relaxation time measurements (Modi *et al* 1990a) that the water molecule is not coordinated to the heme iron in HRP and LPO, and that the sixth coordination site of the iron in these proteins is vacant. The acid⇌alkaline transition in HRP and LPO cannot therefore be associated with the protolytic reaction of the bound water molecule. The pH dependent proton NMR (Morishima *et al* 1977) and pH jump (Epstein and Schejter 1972) studies on HRP have indicated that the transition occurs at pH = 10·8 and is associated with the ligation of an ionised amino acid residue at the sixth coordination site of heme iron to form low-spin ferric heme HRP. If such ligation of a distal amino acid residue is indeed to occur,

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it is expected to bring about a concommitant conformational change in the protein backbone, which can be investigated by circular dichroism (Woody 1985; Johnson 1988). In the present paper circular dichroism (CD) spectroscopy has been used to study the acid⇌alkaline transition in HRP, LPO and metMb.

2. Materials and methods

LPO was isolated from unskimmed unpasteurised cow's milk as reported earlier (Modi *et al* 1989a, b, 1990b, 1991). metMb and HRP were obtained from Sigma Chemicals. The proteins were purified by standard chromotographic procedures and their purity checked spectrophotometrically. The pH of the protein solutions was measured directly in the cuvette or sample tube by an Aldrich extra-long combination electrode.

Circular dichroism studies were carried out on a J-600 spectropolarimeter. Quartz cells of 10 mm path length were used. All titrations were carried out at room temperature (*ca* 23°C). CD is expressed in terms of ellipticity (θ) given as

$$\theta = 2{\cdot}303(A_L - A_R)/4$$

where A_L and A_R are the absorbances of the left and right circularly polarised light. Optical spectral studies were carried out on a Shimadzu 2100 spectrophotometer fitted with TCC-260 temperature controller.

Proton NMR measurements were carried out on a Bruker AM 500 MHz FT-NMR spectrometer. The samples were lyophilised directly inside the NMR tube with excess of D_2O. The ^1H chemical shifts are referred to the ^1H signal of trace HDO as secondary reference at 4·75 ppm.

3. Results and discussion

The CD spectra of HRP in the range 200–250 nm were recorded in the pH range 7–12 (figure 1A). The band near 220 nm which is due to the n–π^* transition of the peptide backbone is known to be sensitive to conformational changes in the polypeptide chain (Hennessey and Johnson 1981). The CD at 220 nm does not show any significant change upto pH $\simeq$ 7. However, as the pH increases further, it shows changes which may be associated with the conformational change of the protein. The plot of θ vs pH for HRP shows a typical pH titration curve for an acid–alkaline transition with pK = 10·8 (figure 2). Similar experiments with LPO show that the CD remains almost unchanged upto pH $\simeq$ 10·7, above which it rapidly increases. The pK of this transition for LPO is deduced to be 12·0. These changes in CD with pH is evidently due to conformational changes in the polypeptide backbone of the protein which may be associated with possible movement of the amino acid residue (believed to be distal histidine here) towards the heme iron. The value of pK of this transition deduced above from the CD are listed in table 1, together with those obtained from NMR studies.

CD experiments were also carried out on HRP and LPO in presence of cyanide ion. Cyanide is known to bind (Behere *et al* 1985) strongly to the ferric ion of the enzymes at the sixth coordination site, making this site unavailable for the coordination by the distal amino acid residue. It is therefore anticipated that, in the

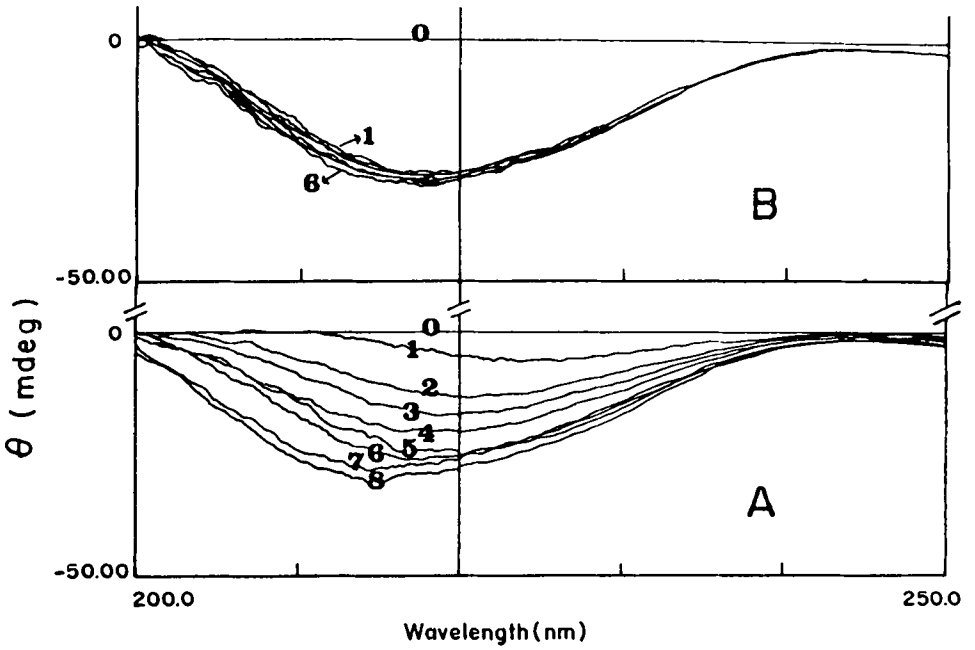

Figure 1. The variation of CD spectra of native HRP (A), and HRPCN (B). For (A), pH = 12·0 (1), 11·0 (2), 10·8 (3), 10·5 (4), 10·0 (5), 9·6 (6), 8·9 (7) and 7·0 (8). For (B) pH varies from 11·8 to 7·0 between 1 and 6.

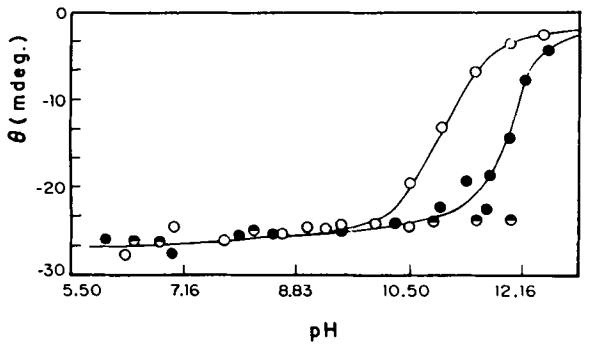

Figure 2. Variation in CD with pH for native HRP (○), LPO (●) and HRPCN (◓).

presence of cyanide, no conformational change should occur. The CD experiments carried out on HRP and LPO in presence of cyanide ion did not show any change over the entire pH range of measurements (figure 1B).

It has been shown that iodide and thiocyanate bind to HRP and the binding site is located away from the heme ferric centre and is near the heme 1- and 8-methyl protons involving propionic acid residue (Sakurada *et al* 1987; Modi *et al* 1989a, b). The pK of the acid⇌alkaline transition in HRP is not therefore expected to be affected by the presence of iodide and thiocyanate. The pH dependent CD experiments carried out on HRP in presence of iodide and thiocyanate show results identical to figure 1A with pK same as in native HRP (see table 1).

Table 1. pK values for acid–alkaline transition for HRP and LPO in absence and presence of CN^-, I^- and resorcinol.

Heme protein	pK from NMR	pK from CD[c]
Mb	8·8[a]	—
HRP	10·8[b]	10·8
HRP–CN^-	—	—
HRP–I^-	10·6[c]	10·7
HRP–SCN^-	10·7[c]	10·8
HRP–Resorcinol	11·7[c]	11·9
LPO	12·2[d]	12·0
LPO–CN^-	—	—
LPO–I^-	> 12·8[c]	> 12·8
LPO–SCN^-	> 12·8[c]	> 12·8
LPO–Resorcinol	> 12·8[c]	> 12·8

(a) Schejter *et al* (1976); (b) Morishima *et al* (1977), de Ropp *et al* (1984); (c) present work; (d) Shiro and Morishima (1986)

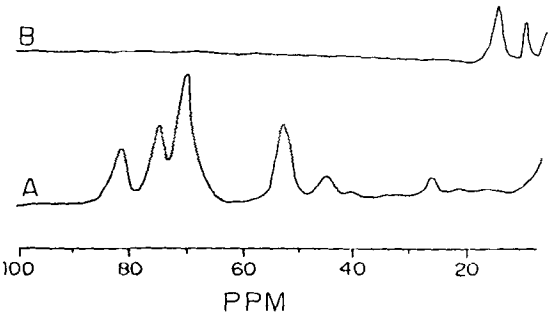

Figure 3. Hyperfine-shifted heme methyl proton NMR resonances in the 100–20 ppm range: HRP at pH = 6·8 (A) and 11·4 (B).

Aromatic donors like resorcinol bind to HRP through hydrophobic forces and hydrogen bonding (Burns *et al* 1975; Schejter *et al* 1976; Sakurada *et al* 1986; Hosoya *et al* 1989). The resorcinol is attached to a hydrophobic region in the protein interior and phenol oxygen is hydrogen bonded to the pyrrolic nitrogen of the distal histidine. If distal histidine is involved in the pH dependent conformational change, then addition of resorcinol to HRP will hinder the movement of the side chain residue, and until the above hydrogen bond is broken, the distal histidine would not bind to ferric ion and its movement will not take place. The CD measurements on HRP in presence of resorcinol show that pK of this transition is indeed affected (see table 1) suggesting possible involvement of distal histidine in the acid$\rightleftharpoons$alkaline transition in HRP.

Thiocyanate, iodide and resorcinol have been shown to bind to LPO at the site of distal histidine (Sakurada *et al* 1987; Modi *et al* 1989a, c, 1990). The pH dependent CD experiments carried out on LPO in presence of these substrates showed that the pK of the acid$\rightleftharpoons$alkaline transition was affected and increased to > 12·8 from its original value of 12·1. The exact value of pK could not be accurately determined here in the experiments since measurements at pH > 12·8 became difficult because of

the denaturation of the enzyme. Nevertheless, the experiments indicated large change in the pK of the acid⇌alkaline transition in LPO in presence of these substrates, which clearly suggests involvement of distal histidine in this transition.

The pK of the acid⇌alkaline transition in HRP and LPO in presence of iodide, thiocyanate and resorcinol was also determined by pH dependent studies of paramagnetic NMR shift of heme methyl protons; the values are included in table 1 for comparison.

The unusually high pKa of the acid⇌alkaline transition in LPO may also be related to the ionisation of amino acid residue in the distal pocket of the heme crevice. The exact nature of the distal amino acid residue which may be involved in LPO is not known. ESR of LPO at elevated pH is characteristic of low-spin species (Lukat *et al* 1987). It has also been found that the hyperfine-shifted heme proton resonances of LPO change from the characteristic high-spin spectrum at pH = 7·0 to a low-spin one at pH = 12·2 (Modi *et al* 1990a). These results suggest that a strong field ligand such as histidyl imidazole may occupy the vacant sixth co-ordination site of LPO, as in HRP. The CD experiments on LPO in presence of iodide and thiocyanate show that binding of distal histidyl imidazole to the heme iron might be involved in the acid⇌alkaline transition in this enzyme as well. This observation would be in agreement with the low-spin behaviour of LPO at higher pH, as seen in the NMR and ESR studies.

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