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Heme CD as a probe for monitoring local structural changes in heme-proteins: Alkaline transition in hemeproteins

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Abstract. Structural change due to acid–alkaline transition in hemeproteins were monitored by circular dichroism measurements in the Soret region. It was observed that in cytochrome c and horseradish peroxidase, alkaline transition results in a large change in the heme CD due to significant conformational change in the heme cavity region. In metmyoglobin a simple protolytic mechanism associated with alkaline transition involves very small conformational changes.

Keywords. Hemeproteins; circular dichroism; alkaline transition; cytochrome c; horseradish peroxidase.

1. Introduction

Circular dichroism (CD) has been extensively used for conformational studies of proteins, nucleotides and other macromolecules in solution (Beychok 1968; Hsu and Woody 1971). The CD spectrum of proteins primarily arises from dipole–dipole interactions between the transition moments of different aromatic amino acid residues (Myer and Pande 1978). Restricted rotation of the amide bonds and other structural effects also contribute to the CD of proteins. The optical activity spectrum has been used to determine the bulk structural properties in various hemeproteins such as myoglobins, hemoglobins etc. (Myer and Pande 1978). Presence of the heme chromophore in hemeproteins gives rise to several new CD bands due to the heme electronic transitions. Although, the heme group being highly symmetric is expected to be devoid of optical activity (Moscowitz 1967), theoretical calculations have shown (Hsu and Woody 1971) that significant optical activity of the heme transitions arises from the interactions of heme with amino acid residues in the protein cavity. Dipole–dipole coupling interactions between porphyrin and the surrounding aromatic amino acid side chains of the protein have been shown (Hsu and Woody 1971) to have predominant effect on the CD of hemeproteins. Aromatic residues as far as 12 Å away from the heme group seem to contribute to the heme CD activity (Myer and Pande 1978). Heme-band CD spectra of various hemeproteins have earlier been reported in different oxidation states and with different axial ligands. These studies show that the CD spectrum in the heme absorption region is highly sensitive to conformation of the heme group inside the protein cavity.

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Hemeproteins have been found to show diverse effects on increase in *p*H of the solution (Antonini and Brunori 1971). The acid–alkaline transition in metmyoglobin (metMb) and methemoglobin (metHb) is associated with the conversion of heme-bound water molecule to the corresponding hydroxo form (Antonini and Brunori 1971) and results in a large change in the spectral properties of the heme group of the protein. Other hemeproteins such as, cytochrome c (Cyt c), horseradish peroxidase (HRP) and lactoperoxidase (LPO) also show *p*H-dependent changes (Morishima *et al* 1977; de Roop *et al* 1984; Shiro and Morishima 1986; Pettigrew and Moore 1990; Baker and Mauk 1992; Modi *et al* 1993), but unlike in metMb, the alkaline transition in these cases cannot be associated with a simple aqua-hydroxo transition (Dunford 1991), since these proteins do not contain axial water molecule coordinated to the heme ferric centre. *p*H-dependent proton-NMR studies on HRP (Morishima *et al* 1977) have indicated that the transition occurs with a $pK \sim 10{\cdot}8$ and is associated with the ligation of an ionized amino acid residue at the sixth coordination site of the heme iron of HRP. A similar type of transition has been proposed to occur at relatively higher *p*H ($pK \sim 12$) for LPO (Shiro and Morishima 1986). In the alkaline form of ferricytochrome c, it is generally believed that the thioether of Met80 in the native conformation is replaced by a nitrogenous ligand which possibly is the deprotonated ε-amino group of a surface lysine residue (Dickerson *et al* 1971).

Circular dichroism studies on the *p*H dependence of various hemeproteins would help to compare their structural aspects. In the present work, circular dichroism studies of metmyoglobin, cytochrome c and horseradish peroxidase have been reported at different *p*H levels. A comparison of the *p*H-dependent CD results obtained in the UV and Soret regions has been presented in order to demonstrate the sensitivity of the CD spectra in the heme-Soret region towards subtle structural changes in the heme environment in these hemeproteins.

2. Materials and methods

Horse heart metmyoglobin (metMb), horseradish peroxidase (HRP) and horse heart cytochrome c (Cyt c) were obtained from Sigma chemicals. Cyt c and HRP (type VIA) were used without further purification. metMb solutions were purified by passing through Sephadex column. *p*H variations of the different hemeprotein solutions were carried out in 100 mM *tris* buffer.

Circular dichroism (CD) studies were performed on J-600 (JASCO) spectropolarimeter and optical spectra were recorded using a Shimadzu UV-2100 spectrophotometer. All experiments were carried out at 25 ± 1°C.

3. Results and discussion

Figure 1 shows the CD spectra of Cyt c, metMb and HRP in the range of 300–500 nm at different *p*H. The CD spectrum of cytochrome c below *p*H 9 (figure 1a) has a negative peak at 416 nm and a positive peak at 403 nm which agree with the earlier report (Myer and Pande 1978). The integrated area under a CD band i.e., the rotational strength ($\mathbf{R}_k$) can be shown to be that given by the dot product between the electric transition dipole moment vector ($\boldsymbol{\mu}_e$) and the magnetic dipole moment ($\boldsymbol{\mu}_m$) of the chromophore (Schellman 1969):

$$\mathbf{R}_k = \boldsymbol{\mu}_e \cdot \boldsymbol{\mu}_m. \tag{1}$$

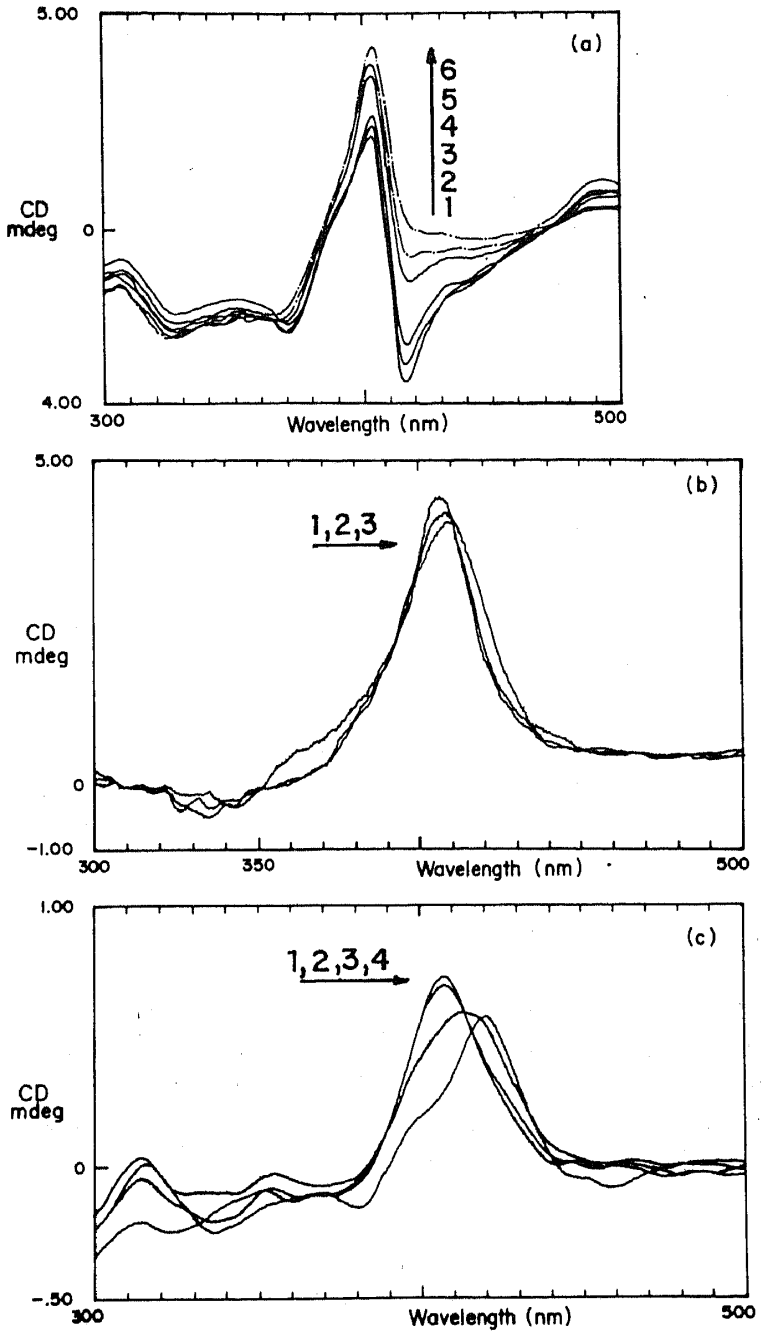

Figure 1. Circular dichroism spectra in 300–500 region at different *p*H for metmyoglobin, cytochrome c and horseradish peroxidase. The *p*H variations were performed by addition of dilute alkali (NaOH) to the hemeproteins solutions in 100 mM Tris buffer. The protein concentration were 91 μM for cyt c, 41·4 μM for metMb and 12·5 μM for HRP. The CD experiments were carried out in cuvettes of 1 mm path-length. (**a**) Cyt c: *p*H = 7·4(1), 8·3(2), 8·6(3), 9·3(4), 9·6(5), and 10·4(6) (**b**) metMb: *p*H = 7·4(1), 8·6(2), 9·3(3). (**c**) HRP: *p*H = 7·4(1), 10·4(2), 11·2(3), and 11·7(4).

The value of the electric transition dipole moment (μ_e) depends on the electron density distribution as well as the symmetry properties of the ground and excited electronic states of the chromophore. The magnetic dipole moment (μ_m) also depends on the

symmetry as well as spin density of the chromophore. $\boldsymbol{\mu}_e$ and $\boldsymbol{\mu}_m$ would thus change if the electronic state, spin state or oxidation state of the chromophore changes. Furthermore, the value of $\mathbf{R}_k$ would depend on the angle between the $\boldsymbol{\mu}_e$ and $\boldsymbol{\mu}_m$ vectors. The CD of the heme transition is thus expected to depend on the spin and valence state of the metal ion. Moreover, the $\pi - \pi^*$ transition in aromatic amino acids may couple with that of heme (or porphyrin) inside the protein cavity resulting in a significant CD activity of the heme transition. Restricted rotation of the heme group inside the protein cavity and interactions of the polypeptide backbone with the heme may also give rise to non-zero rotational strength of the heme transition. Thus, any change in the protein configuration which changes the distance (or angle) between the heme and other aromatic amino acids would affect the CD of the heme transition of the protein. The prominent negative Cotton effect of cytochrome c in the heme region is considered to be due to heme–polypeptide backbone interactions (Myer and Pande 1978). The magnitude of the positive CD band has, on the other hand, been related to the difference in the axial ligand field strengths in cytochrome c (Myer and Pande 1978).

Increase in *p*H from 7·4 to 10·4 shows a systematic change in the heme region of the CD spectra of cytochrome c. The magnitude of the negative peak slowly decreases to zero with subsequent increase in the positive peak intensity (figure 1a). The CD in the intrinsic protein region (i.e. < 250 nm region) however, did not show any appreciable change with *p*H (data not shown). A plot of the ellipticity of the heme CD peak of cytochrome c at 403 nm as a function of *p*H has been shown in figure 2 (plot a). A large change (> 90% increase at 403 nm) in the ellipticity of the heme CD peak with increase in *p*H is observed, and a phenomenological *pK* of ~ 9·0 is obtained from the inflection point as seen from the plot in figure 2a. This *pK* matched closely with the *pK* of alkaline transition in cytochrome c reported earlier (Pettigrew and Moore 1990; Baker and Mauk 1992). Earlier (Baker and Mauk 1992) studies proposed that the alkaline transition in cytochrome c involves change in axial ligand of the heme at higher *p*H. The Fe-S- bond with the sixth axial ligand, Met80 possibly breaks at high *p*H resulting in a change in the configuration of the distal side of the heme which leads to binding of an amino acid with nitrogenous ligand (possibly a lysine residue with amino group deprotonated at high *p*H) in the sixth axial position of the heme (Baker and Mauk 1992). Thus the alkaline transition in cytochrome c is expected to involve change in the protein configuration in the vicinity of the heme group. It may be noted that there is no change in the spin or oxidation state of the iron in cytochrome c at higher *p*H.

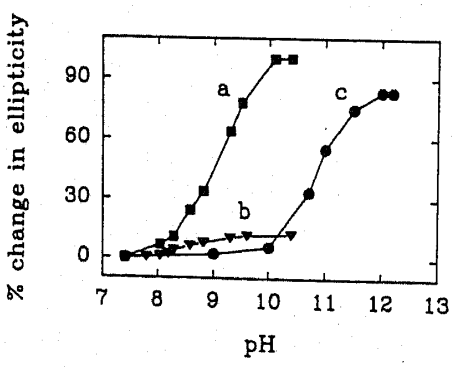

Figure 2. Percent changes in CD for (**a**) Cyt c at 403 nm, (**b**) Mb at 407 nm and (**c**) HRP at 407 nm, as functions of *p*H.

Figure 1b shows CD spectra of the heme Soret transition of metmyoglobin at different *p*H. Similar to cytochrome c, the CD of metMb in the protein region (< 250 nm) does not change significantly with *p*H. However, the heme CD of metMb (figure 1b) shows a red shift in the CD peak with increase in *p*H from 7·4 to 9·3. A plot of ellipticity of the heme CD peak of metMb against *p*H shows a sigmoidal curve shown in figure 2b. The total change in ellipticity of the heme CD peak of metMb over the whole *p*H range was found to be much less than that observed in case of cytochrome c (figure 2a). The phenomenological *pK* obtained from figure 2b was ~ 8·8. This *pK* matched with that of aqua ⇌ hydroxo transition in metMb associated with deprotonation of the H_2O bound to the heme iron in aqua metMb reported earlier (Antonini and Brunori 1971). The aqua⇌ hydroxo transition in metMb has been shown to be associated with a high-spin ($S = 5/2$)⇌low-spin ($S = 1/2$) equilibrium (Antonini and Brunori 1971). The heme CD of metMb is expected to change with the spin state due to the alkaline transition of the protein. The small red shift of the heme CD spectra of metMb at high *p*H thus can be assigned to the aqua⇌ hydroxo transition in the protein which may not involve any significant structural change in the heme cavity. The nature of the CD band of the protein at low and at high *p*H(figure 1b) however, indicates that the configuration of the amino acid residues near the heme cavity may not change significantly with *p*H in metMb.

Figure 1c shows the CD spectra of HRP in the heme Soret transition region at different *p*H values from 7·4 to 11·7. The heme CD of HRP matched with earlier report (Strickland *et al* 1968) with a major positive band at 407 nm along with other weak bands in the region 300–390 nm (figure 1c). Increase in *p*H from ~ 7 to ~ 12 causes a significant red shift in the heme CD spectra of the enzyme. The CD spectra of the enzyme in the 200–250 nm range do not show any appreciable change with *p*H (figure 3). *p*H dependence of the ellipticity of the heme CD peak at 407 nm and at 422 nm (figure 2c) showed a large change (~ 80% increase at 422 nm) in ellipticity at high *p*H.

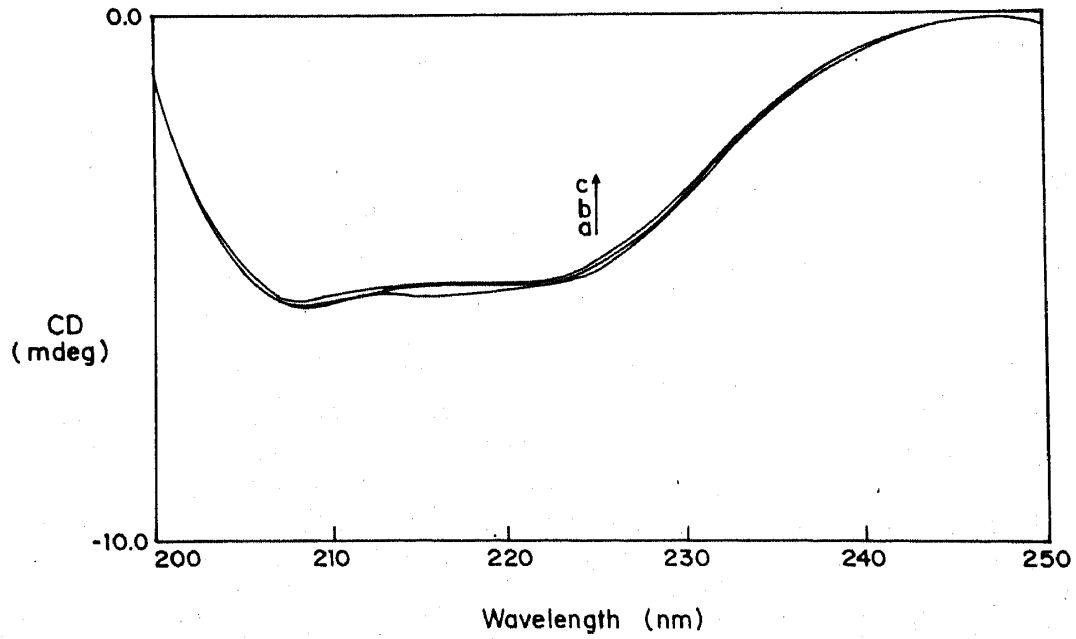

Figure 3. Circular dichroism spectra in 200–250 region at different values of *p*H for horseradish peroxidase (125 μM). The CD experiments were carried out in cuvettes of 1 mm pathlength.

The phenomenological *pK* obtained from the *p*H dependence of ellipticity of the heme CD peaks was ~ 10·8, which matched with earlier reports of the *pK* of the alkaline transition of HRP (Morishima *et al* 1977; de Roop *et al* 1984). Earlier NMR studies have shown that the alkaline transition of HRP is associated with a high-spin ($S = 5/2$)⇌low-spin ($S = 1/2$) transition (Morishima *et al* 1977; Modi *et al* 1993). Native HRP does not have any axial water bound to the heme (Modi *et al* 1993), thus the alkaline transition in this enzyme is expected to involve ligation of an amino acid residue in the distal side of the heme cavity. The alkaline transition of the enzyme has recently been shown to be associated with an enhancement of the intrinsic tryptophan fluorescence, suggesting a structural change in the distal region of the heme pocket (Das and Mazumdar 1995). The protein intrinsic CD spectra (< 250 nm) is insensitive to such local structural change (see figure 3) which is at variance with the earlier report of Modi *et al* (1993). *p*H dependence of the heme CD of HRP may arise both from the change in spin state of iron in heme (from high-spin to low-spin) and from the structural changes near the heme leading to changes in interactions of the heme with aromatic amino acid residues. It is worth noting that the position of the heme CD peak at high *p*H (figure 1c) is similar to that of the cyanide complex of HRP (Willick *et al* 1969). Both the alkaline form of HRP and the cyanide complex have low-spin heme (Fe^{3+}) prosthetic group. Thus, unlike in case of cytochrome c, the change in the heme CD of HRP on alkaline transition cannot be directly correlated with structural changes associated with the amino acid residues near the heme cavity. The observed change in heme CD at alkaline *p*H is possibly dominated by the change in the spin-state of the heme moiety and contribution of structural change in the heme cavity, if any, may be smaller than that of the former. The heme CD spectrum of the cyanide complex of HRP however, has been earlier (Willick *et al* 1969) interpreted as a reflection of conformational changes in the immediate vicinity of the heme group, which might be associated with movement of the metal ion with respect to the heme plane on change in ligation and spin state. Although the position of heme CD peak of the cyanide complex of HRP and that of the alkaline form showed close similarity with each other, the nature of the CD spectra in these two species are quite different. The alkaline form showed a positive shoulder near 400 nm and a negative hump at ~ 460 nm which are absent in the cyanide complex. These differences in the general feature of the heme CD spectra of the alkaline form of HRP possibly correspond mainly to the change due to structural change in the distal heme pocket associated with the alkaline transition.

A comparison of the relative changes in CD at the major heme band is shown in figure 2. In case of Cyt c and HRP, percentage changes in heme band are quite large (~ 90%) whereas much less but systematic changes were observed in metMb (~ 10%). Though a comparison between the percent CD changes in different hemeproteins cannot be directly correlated with conformational changes, still it may be noteworthy that metMb shows a very small change in heme-CD where only small conformational changes are expected to occur due to acid-alkaline transition, whereas in Cyt c and HRP, the changes are due to binding of distal residues to heme at the axial positions and conformational changes are obviously much larger in these two hemeproteins.

Conclusions

*p*H dependent CD of the heme region is sensitive to the local structural changes due to alkaline transition in hemeproteins and the *p*K for this transition can be accurately

determined. CD of the intrinsic protein region was found to be insensitive to these local structural changes, possibly because these changes do not seem to affect the helical content and other higher order structural properties of the whole protein. The large changes in the heme CD of cytochrome c and HRP due to alkaline transition can be directly correlated to the structural changes in the heme cavity region whereas only minor changes in CD of metMb are observed resulting from spin-state change of the metal ion in the heme prosthetic group. These studies demonstrate that heme CD spectra can be used to monitor subtle structural changes in hemeproteins.

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