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Low-temperature magnetic circular dichroism investigation of the active site of chloroperoxidase

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Abstract Magnetic circular dichroism (MCD) spectra for near UV and visible spectral regions of chemically reduced chloroperoxidase from *Caldariomyces fumago* have been recorded at temperatures from near 293 to 2.15 K. The spectra of reduced chloroperoxidase at 4.2 K were compared with those of photolysis products of its carbon monoxide complexes. Obtained results give evidence for high rigidity of the active site in chloroperoxidase and strongly suggest that thiolate is a protein-derived ligand of the heme iron in the reduced enzyme. The unusual high-spin ferrohemoproteins temperature dependence of the Soret MCD closely resembles that of the substrate-bound cytochrome P-450$_{cam}$.

Key words: Chloroperoxidase; Magnetic circular dichroism; Low temperature; Photolysis

1. Introduction

Chloroperoxidase (CPO) is a unique enzyme which catalyzes peroxide-dependent halogenation of organic substrates. It also exhibits classic peroxidase and catalase activities. Extensive spectroscopic studies of CPO have supported the conclusion that thiolate is the protein-derived ligand coordinated to the heme iron of the enzyme [1,2]. The same has been demonstrated for the cytochrome P-450 [1,3,4], the only other thiolate-ligated heme protein known. These two enzymes, however, have rather distinct catalytic properties. Clearly, the differences in reactivity are due to the differences in protein environment of the heme in the two enzymes.

Magnetic circular dichroism (MCD) spectroscopy, especially at low-temperature, is very sensitive to subtle changes of both electronic and structural heme group parameters and may, therefore, be very useful in studying the role of the protein moiety in the formation of the active site structure in heme proteins. This was demonstrated in our earlier investigations of hemoproteins with a histidine-coordinated heme iron [5–7]. Recently, an active site of camphor-free and -bound cytochrome P-450$_{cam}$ was characterized by low-temperature MCD spectroscopy [8,9]. In this paper we report the first low-temperature MCD studies of the reduced CPO at two pH values, as well as the first studies of metastable photoproducts of its CO complexes.

2. Materials and methods

CPO from *Caldariomyces fumago* (Sigma; R.Z. 1.1 (absorbance at 403 nm to that at 280 nm)), was used without further purification. The enzyme was dissolved in 0.2 M citrate-phosphate buffer (pH 3.5 or 6.5). To obtain transparent glasses for the low-temperature measurements the enzyme solutions were mixed with glycerol (Sigma) at a volume ratio of 1:1.5, respectively. The oxidized enzyme was reduced under anaerobic conditions by adding a few crystals of sodium dithionite (Merck) at room temperature. The CO complex was prepared by incubating the reduced enzyme at an appropriate pH value in CO atmosphere for 30 min with occasional shaking. The MCD spectra were recorded at various temperatures down to 2.15 K in a magnetic field of 1.45 T using a magnetic dichrograph equipped with a cryostat. Application of the MCD method, experimental techniques and details of low-temperature measurements are described in [5]. The same cells with an optical pathlength of 1.0 or 0.5 mm were used for recording MCD and absorption (Shimadzu UV-160A spectrophotometer) spectra.

3. Results and discussion

3.1. Evidence for high rigidity of the active site and for thiolate ligation of the heme iron

Fig. 1 shows the Soret MCD spectra at 4.2 K of the reduced CPO at pH 6.5 and those of the reduced camphor-free and -bound cytochrome P-450$_{cam}$ (P-450$_{cam}$) obtained earlier [8,9]. The three spectra have similar shapes and characteristic features which are rather distinct from those of the histidine-ligated heme proteins [5–7]. Differences observed between the three spectra reflect peculiarities in geometry of the heme iron coordination sphere imposed by the protein moiety. Except for some differences in the peak intensities, the MCD spectra of the reduced CPO at pH 3.5 and those of the photoproducts at pH 3.5 and 6.5 (not shown) appeared to be very similar to the spectrum displayed in Fig. 1 for the reduced CPO at pH 6.5 (see inset in Fig. 1 where the main Soret peak intensities are drawn vs. their wavelengths).

Some evidence, discussed earlier [5,8], suggested that after photolysis of a ligand at cryogenic temperatures the heme stereochemistry (the geometry of the heme local environment) remains unrelaxed even in the photoproduct of a protein-free heme bound to 2-methylimidazole. Therefore, the difference between MCD spectra for the unligated heme in the reduced fully relaxed hemoprotein and in the unrelaxed metastable photoproduct may be considered as a measure for the restrictions imposed by the protein moiety at the proximal heme stereochemistry changes which occur upon ligand binding or release. Previously, cases of weak (myoglobin, hemoglobin), moderate (neutral HRP), and strong (alkaline HRP) protein constraints of heme local structure have been observed [5–7]. Results presented in the inset of Fig. 1 show that CPO refers to the latter

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Abbreviations: MCD, magnetic circular dichroism; RR, resonance Raman; NMR, nuclear magnetic resonance; CPO, chloroperoxidase; P-450$_{cam}$, bacterial cytochrome P-450$_{cam}$; HRP, horseradish peroxidase.

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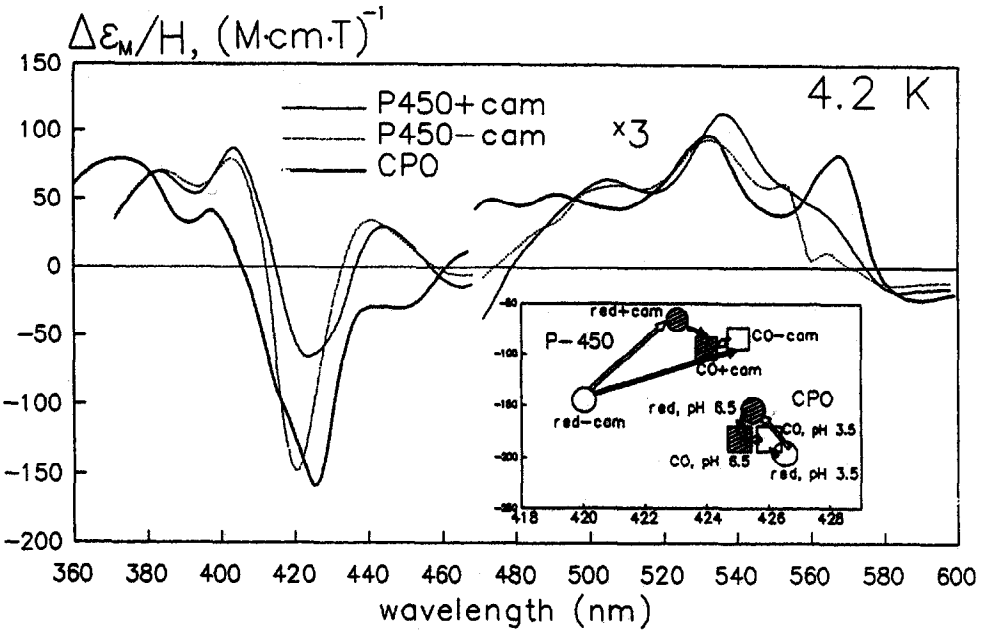

Fig. 1. Comparison of MCD spectra recorded at 4.2 K for reduced CPO in 0.1 M citrate-phosphate (pH 6.5) buffer-glycerol (1 : 1.5 v/v) solvent mixture with those for reduced camphor-free and -bound P-450$_{cam}$ obtained earlier [8]. CPO concentration, 0.13 mM; magnetic field, 1.45 T. Inset shows intensities of the main Soret MCD peak at 4.2 K vs. their wavelengths for the reduced forms (circles) of CPO at pH 3.5 (open) and 6.5 (hatched) and camphor-free (open) and camphor-bound (hatched) P-450$_{cam}$, and for the photoproducts of their carbon monoxide complexes (squares, open for CPO at pH 3.5 and camphor-free P-450$_{cam}$, hatched for CPO at pH 6.5 and camphor-bound P-450$_{cam}$).

case. Its proximal heme stereochemistry is locked by the protein moiety into a fixed geometry, independent of the ligand binding or pH changes. The active site rigidity in CPO is higher than in P-450$_{cam}$, since substrate or ligand binding to camphor-free P-450$_{cam}$ causes much larger changes in the MCD spectra (inset in Fig. 1).

Numerous investigations of CPO [10–14] clearly reveal an ionizable group with a pK value of about 4.7–5.5 for the ferrous enzyme and for its CO complex. Two distinct, acid and alkaline, forms of the CO-bound CPO were observed by ^{17}O NMR [12] and RR spectroscopy [13]. The same has been demonstrated for HRP isozymes A and C [12]. The data obtained by NMR and RR spectroscopy suggest hydrogen bonding of the CO ligand in the acidic form of CPO with a protonated distal amino acid residue while this bonding appeared to be much weaker than in the cases of HRP and cytochrome c peroxidases [12,13].

In addition, two low frequency modes in the RR spectrum of CO–CPO were observed to shift substantially upon the change of pH from 6.0 to 3.3. These modes have been tentatively assigned to $\delta(C_bC_\alpha C_\beta)$ bending of the vinyl groups and to the $\delta(C_bC_1C_2)$ bending vibration of the C_b- propionate bonds [13]. The changes are not linked directly to hydrogen bonding of the CO ligand but indicate some conformational change in CPO caused by the change of pH.

Our results are not in contradiction with the above RR and NMR data since we dealt with the unligated heme group, and in our experiments solely the changes at a proximal side of the heme were followed by MCD spectroscopy. The results imply that changes occurring at the distal side or at the periphery of the heme group in CPO are the local ones and do not spread to the proximal heme side through either a *trans*-effect upon ligand binding or a global conformational change upon ionization of amino acid(s).

Chloroperoxidase, as well as cytochrome P-450, exhibit a

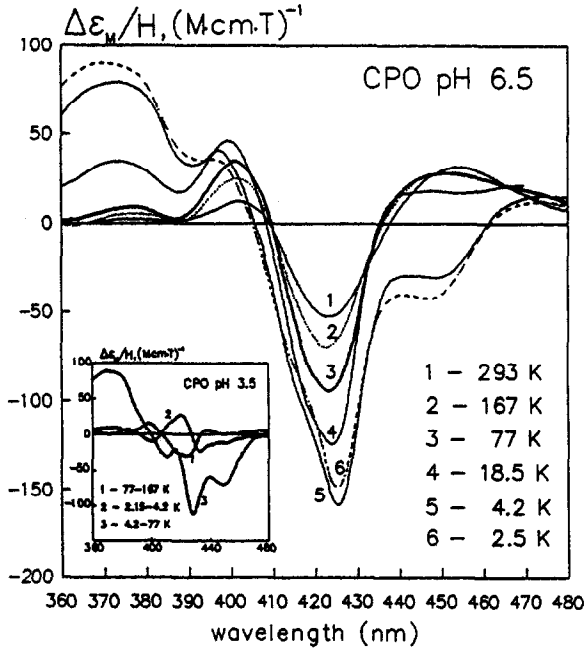

Fig. 2. MCD spectra recorded at different temperatures for the reduced CPO at pH 6.5 in citrate-phosphate buffer-glycerol (1 : 1.5 v/v) mixture. Concentration, 0.13 mM; magnetic field, 1.45 T. Inset displays the difference MCD spectra for the reduced CPO at pH 3.5 obtained for various temperature intervals.

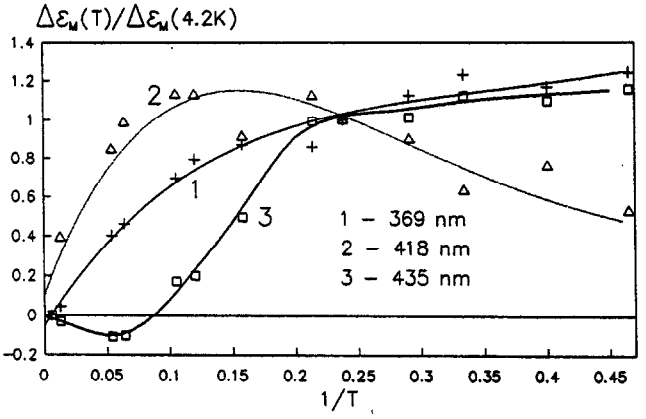

Fig. 3. Temperature profiles of MCD intensities represented as a ratio of intensity at a given temperature to that at 4.2 K obtained at different wavelengths for the reduced CPO at pH 6.5. To exclude temperature independent contribution in MCD the spectrum at 167 K was subtracted from those recorded at lower temperatures. Magnetic field, 1.45 T.

unique hyperporphyrin ('split Soret') absorbance and MCD spectra of their reduced CO complex, which strongly suggests ligation of a thiolate ligand *trans* to CO [15,16], since such unique spectra arise exclusively from [RS-Fe^{2+}heme-CO] adducts [16–18]. So, our results strongly suggest that thiolate is the 5th axial ligand in the reduced chloroperoxidase. This conclusion is in accordance with the results of numerous earlier physical studies of the reduced high-spin chloroperoxidase by room-temperature MCD, RR, NMR and Mossbauer spectroscopy (see [1] for review).

3.2. *The Soret MCD changes with temperature*

Fig. 2 shows MCD spectra at various temperatures for the reduced CPO at pH 6.5. Similar spectral changes have been obtained at pH 3.5. The room temperature spectra are in accordance with those obtained earlier by Dawson et al. [16]. By contrast to other high-spin ferrous hemoproteins studied (except for the cytochromes *P*-450), the variations of MCD intensity with temperature are small, and the shape of the Soret MCD changes with lowering the temperature below 18 K. The difference spectra drawn in the inset of Fig. 2 clearly illustrate that MCD changes may be even of opposite signs for various temperature intervals.

In Fig. 3 temperature dependences of the MCD intensity at 369, 418 and 435 nm are compared. For ease of comparison, the temperature dependences are normalized to the MCD intensity at 4.2 K. To avoid disturbance of the results by the effect of line narrowing with lowering the temperature, the spectrum at 167 K was subtracted from those at lower temperatures.

Temperature profiles shown in Fig. 3 resemble those obtained earlier for the substrate-bound $P\text{-}450_{cam}$ [8,9]. In the case of $P\text{-}450_{cam}$, a complex temperature behaviour of MCD spectra has been quantitatively described with an assumption that two reduced high-spin forms of the enzyme co-exist and differ by a temperature factor of their MCD spectra [8,9]. However, our attempts to simulate temperature-dependent MCD spectra of the reduced CPO by superposition of MCD spectra originating either from two different enzyme forms or two overlapping bands in the Soret region were unsuccessful. Further experimental investigations and theoretical analysis are needed to explain the unusual temperature behaviour of MCD spectra observed for hemoproteins with thiolate-ligated heme iron.

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