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"CHARACTERIZATION OF THE CHROMOPHORES IN HORSERADISH PEROXIDASE COMPOUNDS I AND II USING MAGNETIC CIRCULAR DICHROISM"

M.J. Stillman, B.R. Hollebone and J.S. Stillman

Dept. of Chemistry, University of Western Ontario, London, Canada and
Dept. of Chemistry, University of Alberta, Edmonton, Canada

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SUMMARY: Magnetic circular dichroism (mcd) spectra have been recorded for HRP I and II. Analysis suggests that the chromophores in both intermediate compounds are electronically similar yielding a $\pi \rightarrow \pi^*$ spectrum of the same band energies.

INTRODUCTION

Characterization of the electronic state of the iron protoporphyrin IX prosthetic group in native horseradish peroxidase (HRP) and its intermediate compounds HRP I and HRP II, has proved elusive despite considerable attention (1-8). Absorption spectra of these three species are well known and each can be readily identified by the bands observed in the 300-1000nm region (10-33000 cm^{-1}). However, there has been no clear assignment proposed for the origins of all the transitions observed. The additional parameters extracted from the mcd spectrum (the polarization properties of each transition, and the sign and magnitude of the angular momentum changes) measured on the same solutions used in the absorption experiment lead more readily to the characterization of the states involved (9, 10, 11). Mcd spectra of several heme proteins have appeared recently resulting in advances in both the interpretation of the absorption spectra and in understanding the relation between individual bands and the electronic state of the chromophore (8, 12, 13, 14).

We recently reported a photochemical reaction of HRP I in which a species is formed with a spectrum similar to that of HRP II. While the nature of this new species was not determined, these results emphasize the need for characterizing the electronic states of HRP I and HRP II.

Mössbauer studies (3) on I and II have not detected any differences in the two iron chromophores, although there must be significant electronic changes between the two species that give rise to the differing absorption spectra. Since mcd intensity is observed under all absorption bands and is sensitive to both the electronic and magnetic properties of each state involved in a transition (10) the mcd spectrum can provide the link between the absorption spectrum and such magnetically sensitive techniques as Mössbauer, EPR and magnetic susceptibility.

MATERIALS AND METHOD

HRP (grade 1) was purchased from Boehringer Mannheim GmbH as a salt suspension. Each sample was prepared for use by extensive dialysis against triply distilled water. The purity number (OD_{403}/OD_{280}) was in excess of 3.2. Preparation of compounds I and II was as previously described (15). Mcd spectra were recorded on a wide range, computer-controlled spectrometer built in this laboratory and will be described elsewhere (17). In agreement with past conventions (10,18), the sign of the mcd was taken as negative in the 510nm region of a solution of $CoSO_4$ in water. An Oxford Instrument Co. Ltd. magnet provided a 5 tesla field. Spectra at 280K were recorded repeatedly over 6 hours to follow the decay of each species; each spectrum took 8 minutes to record. Changes in spectral intensity within the first 10 minutes in both the mcd and absorption were less than 5% at this temperature.

RESULTS

The mcd spectrum of HRP I recorded at about 280K is shown in Figure 1. For comparison we have used absorption spectra recorded at 10K for both HRP I and HRP II. There is little wavelength shift in either absorption spectrum on going to 10K from room temperature, but considerable sharpening in each band is observed (2,15). The increased resolution aids assignment but is not essential, and all our conclusions can be derived using the more diffuse room temperature spectra.

The mcd in the region 12-22000 cm^{-1} comprises a series of overlapping bands with no distinct line shapes discernible. Of note is the absence of a clear Faraday A term under the Q_{00} or α band region of the porphyrin spectrum.

[†] Metal porphyrins have a $\pi \rightarrow \pi^*$ transition (the Q or α and β bands) in the 15-20000 cm^{-1} region that is characterized in the mcd by a derivative line shape known as a Faraday A term, in which the positive lobe lies to high energy of the cross-over point (10, 19, 20, 21).

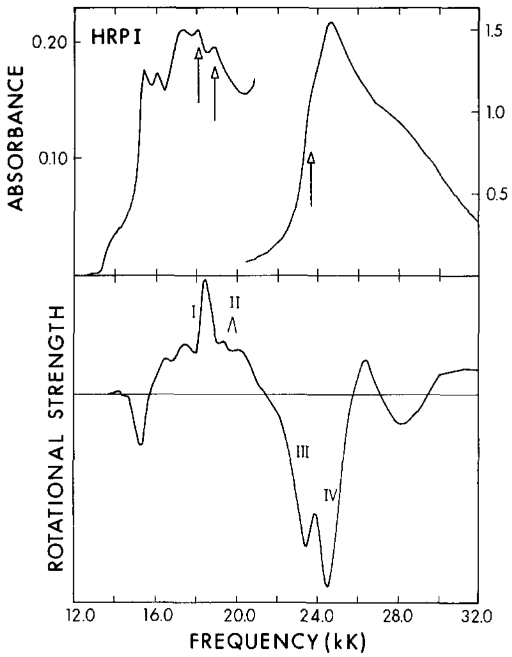

Figure 1. Mcd of HRP I recorded at 280K (bottom) and absorption recorded at 10K (top). The concentration of the mcd solution was 3.25 x 10^{-5}M.

Under the Soret band at ca. 25000 cm^{-1} we observe in the mcd an envelope that we provisionally interpret as the sum of an overlapping positive A term centred on about 23600 cm^{-1} and a negative B term centred on 24600 cm^{-1}. These two transitions are followed by broader and less well defined bands associated with the shoulders in the 26-30000 cm^{-1} region of the absorption spectrum. Through the increased resolution in the absorption spectrum at 10K the A and B terms may be identified as arising from the low energy shoulder and the band maximum. The multi-transition character of the HRP I Soret band has long remained puzzling because only one $\pi\rightarrow\pi^*$ transition is predicted in this region. Recently, however, Weiss has discussed the possibility of a 2nd band being present in the chlorophyll Soret region (22) and Nozawa et al., Vickery et al. and Springall et al. (8, 13, 23) have all reported complex overlapping of bands in this region for high spin ferric

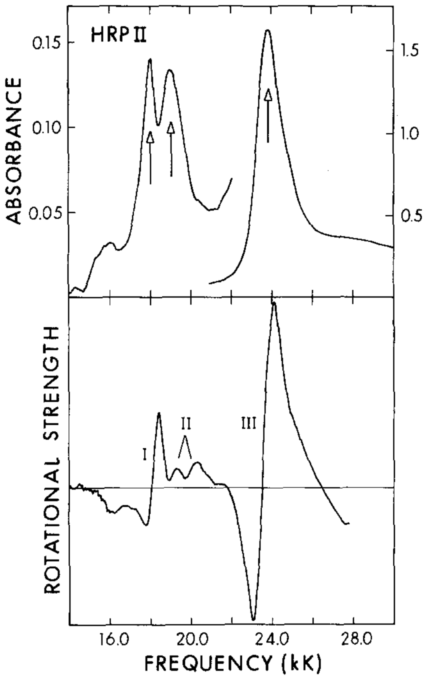

Figure 2. Mcd of HRP II recorded at 280K (bottom) and absorption recorded at 10K (top). The concentration of the mcd solution was 2.57 x 10^{-5}M.

myoglobin (Mb) and HRP. Stillman et al. (18) have similarly observed two degenerate transitions in the Soret region for the solution mcd of zinc phthalocyanine and its derivatives, and some phthalocyanine thin films (17).

The mcd spectrum measured for HRP II at 280K is shown in Figure 2. In this case the absorption spectrum is reminiscent of low spin derivatives of Fe(II)Mb and HRP with well resolved α and β bands and a single, sharp Soret band. Two symmetric, positive A terms identify the Q_{00} and B $\pi\rightarrow\pi^*$ transitions of the porphyrin ring, these are centred on 18100 and 23550 cm^{-1} and labelled I and III, respectively. The lack of detail in the mcd under the Q_{01} band appears characteristic of the $\pi\rightarrow\pi^*$ spectrum of protoporphyrin IX when bound to a protein as compared with the resolved bands observed for the free porphyrin (for example ref. 8, 9, 13, 23).

DISCUSSION

The considerable differences in the absorption spectra of HRP I and HRP II could be interpreted to represent transitions to very different π excited states in the two compounds (2, 6). A significant feature of the mcd spectra that is not apparent in the absorption spectra is the remarkable similarity between the HRP I and HRP II mcd bands labelled I, II and III in Figure 1 and 2. Close inspection reveals that these two sets of spectral line shapes occur at the same frequency and are associated with absorption bands of the same band widths and band centres, marked with arrows in Figures 1 and 2. Thus all the major bands observed for HRP II in both mcd and absorption spectra are present in the HRP I spectra. In addition there are several other transitions, including the prominent Soret band (labelled IV) associated with HRP I alone.

It is surprising to find such similar, major, spectral features for the protoporphyrin IX chromophore in the two chemically inequivalent molecules of HRP I and II. The spectra of other heme proteins encompass a wide variation in band frequencies and intensities for relatively minor chemical changes. The electronic configurations of the porphyrin and iron in HRP I and HRP II have been variously assigned, including, most recently, for HRP I, $[\mathrm{Fe(IV)HRP}]^{3+}$, and for HRP II, $[\mathrm{Fe(IV)HRP}]^{2+}$ (16).

Based on our mcd spectra, assignments of the spectra of both HRP I and HRP II are, in part: Q_{00} (α) at 18250 cm^{-1} (labelled I), Q_{01} (β) at 18920 cm^{-1} (labelled II), and B (α) at 23700 cm^{-1} (labelled III). In HRP II these are the only prominent bands. The other features observed in the HRP I spectrum might arise from charge transfer, if the second oxidising equivalent lies close to the ring or metal; or, in the Soret region, arise from an additional $\pi \rightarrow \pi^*$ transition.

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