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The Temperature Dependence of the MCD Spectrum of Horseradish Peroxidase Compound I

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SUMMARY: The magnetic circular dichroism spectrum of the compound I species of horseradish peroxidase, which contains an iron (IV) porphyrin π-cation radical complex, has been measured between 273 K and 4.2 K. The spectrum is temperature independent between 273 K and 30 K. However, very strong temperature dependence is observed below 30 K. These data do not appear to fit the temperature dependence expected for the presence of a simple MCD C term, or combination of C terms, but suggest that an increase in the coupling between the S=1 iron (IV), and the S=1/2 porphyrin π-cation radical occurs forming a degenerate ground state. This increase in coupling below 30 K may be the result of a phase change in the protein which in turn affects the electronic structure of the heme group.

The horseradish peroxidase (HRP) compound I intermediate which is formed by the reaction of hydrogen peroxide with the native ferric enzyme:

$$\text{HRP} + H_2O_2 \longrightarrow \text{HRP compound I} + H_2O$$

has been studied by a variety of spectroscopic techniques (1-7). As a result of these investigations, and in particular, the magnetic circular dichroism (MCD) studies of the compound I species (6), and a series of porphyrin π-cation radical complexes (8) that have been proposed as models of the compound I species, the electronic configuration of compound I has been assigned as an Fe(IV) porphyrin π-cation radical (6). In addition, in this heme complex, one of the oxygen atoms from the hydrogen peroxide reactant is retained in a manner which appears to be similar to that observed for the compound I species of chloroperoxidase (2,9).

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While the MCD studies of HRP compound I at 273 K (6) together with ENDOR spectroscopy at liquid helium temperatures (1,2) provide strong evidence for the characterization of HRP compound I as a π-cation radical species, there remains the problem that the optical data were recorded at 273 K while the resonance data is from a ground state chromophore at 4.2 K. In the correlation of the results from these two techniques it is necessary to assume that the electronic structure of the compound I heme species at low temperatures is the same as at room temperature. However, structural changes at the heme site can occur in heme proteins when the temperature is lowered to 4 K; this has been demonstrated by the thermal spin changes observed at low temperatures (10).

MCD spectroscopy is sensitive to both the ground and excited state electronic configurations, and has been used to study the electronic structure of HRP compound I at 273 K (6). In this paper, we report the first MCD spectra recorded for this highly oxidized heme enzyme between 4 K and 100 K.

EXPERIMENTAL

Boehringer Mannheim lyophilized horseradish peroxidase (grade I) was dissolved in a 1:1 v/v solution of three times distilled water and glycerol (Fisher Chemicals Inc.). The purity index of this protein in three times distilled water measured as the ratio of A_{403}/A_{280}=3.3. The horseradish peroxidase solution was pretreated with hydrogen peroxide (1.2:1, hydrogen peroxide to protein), and allowed to stand at room temperature from twelve to twenty four hours before use. A second aliquot of hydrogen peroxide in a 1.2:1 ratio was added to the solution to form the horseradish peroxidase compound I species at 273 K. The compound I solution was then rapidly transferred to an aluminum cell, and plunged into liquid nitrogen to glass the solution. For the absorption spectra the glassed sample was transferred into an Oxford Instruments CF204 exchange gas optical cryostat which had been precooled to 100 K. The temperature of the sample was monitored using the Oxford Instruments CLTS temperature sensor mounted on the cryostat. The absorption spectra were obtained by placing the sample and cryostat in a CARY 17 spectrophotometer. The MCD spectra were obtained by placing a second sample of compound I in an Oxford Instruments SM 4 superconducting magnet which was mounted in a CD spectrophotometer built in this laboratory. The temperature of this sample was monitored using a Lake Shore Cryotronics Inc. carbon glass sensor attached to the sample cell.

All spectra shown in this paper were automatically digitized as they were recorded. Each spectrum has had the appropriate baselines subtracted and the intensities have been normalized and replotted using a computer. The absorption spectra are presented with scales of absorbance (A) and the MCD spectra are presented as $\Delta A_M/T^{-1}$. Depolarization of the circularly polarized light by the compound I sample in the CD spectrometer was estimated by placing an aqueous solution of dextro-[trisethylenediamine cobalt (III)] trihydrate before and after the sample. The sample depolarization was estimated at less than 10% over the spectral range studied, although, no adjustments to the ΔA_M values have been attempted.

RESULTS AND DISCUSSION

The absorption spectrum of horseradish peroxidase compound I measured at 85 K, shown in Fig. 1, is very similar to the 273 K and liquid helium temperature absorption spectra which have been reported previously (6,7). The broad series of overlapping bands in the visible and Soret transition regions of the 85 K spectrum are slightly better resolved than in the room temperature spectrum. This enhanced resolution is partly due to band narrowing, and partly due to an increase in intensity that occurs upon reducing the temperature from 273 K. The band narrowing observed in this spectrum is similar to the band narrowing measured in the spectra of ferric porphyrin complexes between 273 K and 4.2 K. There is no further significant change in the spectrum throughout the 300 to 750 nm region when the temperature is reduced from 85 K to 4.2 K.

The reduction in temperature from 273 K to 4.2 K has a far more significant effect on the MCD spectrum of this species, Fig. 1. Like the corresponding absorption spectra, the 85 K MCD spectrum is very similar to the 273 K

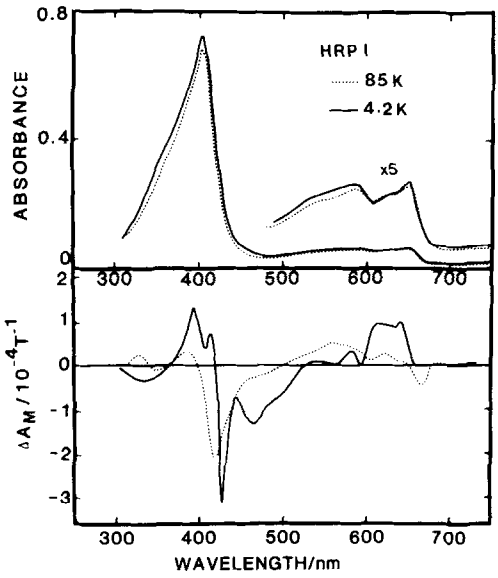

Fig. 1. The absorption (upper) and MCD (lower) spectra of HRP compound I at 4.2 K and 85 K. The spectra were obtained in a 1/1 v/v glycerol/water solution. The HRP concentration was 7.3×10^{-5} mol· L^{-1} and 4.4×10^{-5} mol· L^{-1} in the absorption and MCD experiments, respectively. The sample path length was 0.11 cm, and the magnetic field used was 4.58 T.

spectrum, including the increase in resolution and intensity which is the result of band narrowing that occurs with temperature reduction. As the temperature is reduced to 30 K the complexity of the visible region spectrum increases, and new bands at 420 nm and 460 nm begin to appear. Below 30 K, dramatic changes occur which result in the loss of intensity at 660 nm with a corresponding increase in the 640 nm band, and the subsequent dominance of the 300 nm to 500 nm region by the 420 nm and 460 nm bands. These spectral changes are fully reversible when the temperature is raised. The temperature dependent band at 420 nm appears to be derived from a small fraction of the photochemical product of HRP compound I(11,12) which is formed as an impurity during preparation of the sample, and as a consequence of the visible and UV light used to measure the MCD spectra.

The most striking feature of the spectrum below 30 K is the apparent relationship between the 640 nm and the 660 nm bands shown in Fig.2, and the simple increase in intensity with inverse temperature which is indicative of an MCD C term, that is observed in the 460 nm and 640 nm bands below 30 K. Coupling between the S=1 iron and the S=1/2 porphyrin to form a degenerate ground state as a set of three Kramers doublets which has been used to explain the observations of the temperature dependence of the EPR spectrum (5), can also be used to describe the appearance of the MCD C term (13). Above 30 K, however, the MCD spectrum is not significantly temperature dependent. This lack of temperature dependence above 30 K suggests that the iron and the porphyrin radical are not coupled together to the same extent as below 30 K. It is possible that a combination of two oppositely-signed C terms which coincidently fall in a single band envelope may give rise to the apparent lack of temperature dependence above 30 K. However, we feel that this is an unlikely explanation, since it is not clear why these C term bands should coincide so closely throughout the whole 300 nm to 750 nm region.

The observation of temperature dependence in the spectra of crystalline materials may be the result of simple Boltzmann population effects, or alternatively may be the result of phase or structural changes that can occur

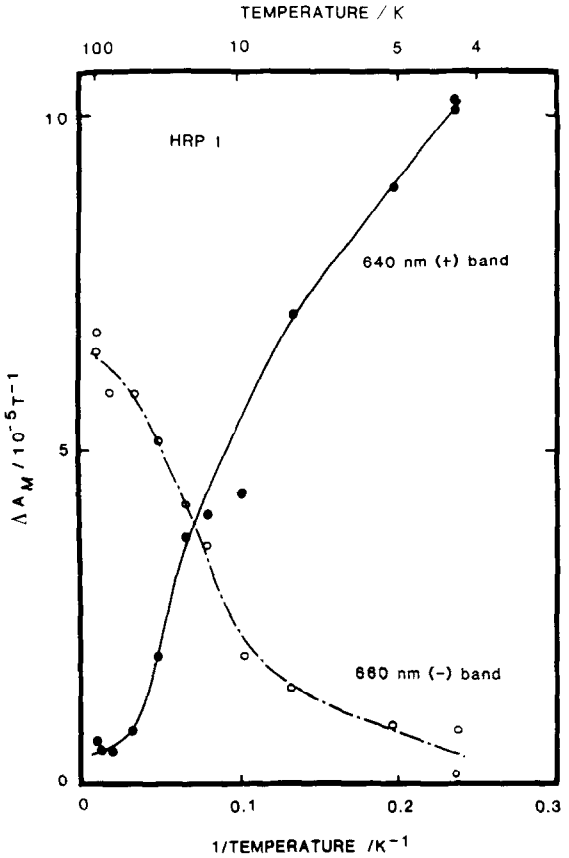

Fig. 2. The temperature dependence of the bands at 640 nm and 660 nm in the MCD spectrum of horseradish peroxidase compound I.

quite abruptly in the semi-crystalline environment of the heme pocket within the protein (10). It appears likely that below 30 K, a small temperature dependent phase or structural change has occured at the heme site which has resulted in the increase of the electronic coupling between the S=1 iron and the S=1/2 porphyrin π-cation radical, and from which a degenerate ground state has been formed.

The presence of this reversible phase change will complicate the estimation of the contribution made by the MCD C terms to the overall intensity in the spectrum at any given temperature. The complete analysis of the MCD C term contributions requires the knowledge of the magnetic field and temperature dependence of the spectrum below 4.2 K in order that the effect of thermal equilibrium can be separated from the normal C term contributions.

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