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The active site structure of *E. coli* HPII catalase

Evidence favoring coordination of a tyrosinate proximal ligand to the chlorin iron

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E. coli produces 2 catalases known as HPI and HPII. While the heme prosthetic group of the HPII catalase has been established to be a dihydroporphyrin or chlorin, the identity of the proximal ligand to the iron has not been addressed. The magnetic circular dichroism (MCD) spectrum of native ferric HPII catalase is very similar to those of a 5-coordinate phenolate-ligated ferric chlorin complex, a model for tyrosinate proximal ligation, as well as of chlorin-reconstituted ferric horseradish peroxidase, a model for 5-coordinate histidine ligation. However, further MCD comparisons of chlorin-reconstituted myoglobin with parallel ligand-bound adducts of the catalase clearly rule out histidine ligation in the latter, leaving tyrosinate as the best candidate for the proximal ligand.

Magnetic circular dichroism spectroscopy; Active site structure determination; Tyrosinate proximal ligand; EPR spectroscopy; Catalase; Dihydroporphyrin

1. INTRODUCTION

The 2 catalases found in *E. coli* are both heme enzymes but differ in that the more prevalent one, designated HPI, contains a protoheme prosthetic group, while the second catalase, HPII, has a dihydroporphyrin or chlorin [1]. Iron chlorins have been found as the prosthetic group in several heme proteins. In addition to HPII catalase, *E. coli* also contains a heme *d* terminal oxidase that has 3 heme groups, 2 protoheme and 1 chlorin [2]. The prosthetic groups of the green catalase from *N. crassa* [3,4] and of sulfmyoglobin [4–6] have been shown to be iron chlorins. Myeloperoxidase has been proposed to contain an iron chlorin prosthetic group [7,8] but recent evidence is more consistent with a formyl-porphyrin [9,10]. While the type of prosthetic group in HPII catalase has been established, the proximal ligand to the iron has not been identified to date and is the focus of this study.

Magnetic circular dichroism (MCD) spectroscopy [11] has been extensively applied to the study of protoheme-containing proteins and has developed into a powerful method for axial ligand identification [12–15]. Comparison of the MCD spectra of structurally defined iron protoheme complexes either in proteins or synthetic models in different oxidation and ligation states with parallel derivatives of a structurally undefined, protoheme protein can often lead to the establishment of the axial ligands of the latter in a straightforward manner [12–15]. Recently, we have extended this approach to the investigation of iron chlorin systems. We have adopted a 3-fold approach including experiments with chlorin model complexes [16], with chlorins reconstituted into well-studied proteins such as myoglobin [17] and with naturally occurring chlorin proteins such as the present study with HPII catalase. The results show that MCD spectroscopy can be used as a probe of iron chlorin coordination structure in the same way as has been done repeatedly with iron protoheme systems.

Abbreviations: MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; HRP, horseradish peroxidase; OEC, octaethylchlorin; MeC, methylchlorin.

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2. MATERIALS AND METHODS

HPII catalase was purified as previously described [1]. The lyophilized protein was resuspended in deionized water (Continental) and centrifuged to remove insoluble material. The protein solution was then dialyzed against 50 mM sodium phosphate buffer, pH 7.0, concentrated by ultrafiltration and stored at 77 K. Horse heart myoglobin (Sigma, Type III) was further purified as described by Dawson et al. [18]. Horseradish peroxidase (Sigma, Type VI) was used without further purification. The synthesis of iron methylchlorin (MeC, 2,2,4-trimethyldeuterochlorin, featuring a gem-dimethyl substituted periph-

eral carbon) has been previously described by Chang and Sotiriou [19]. Iron octaethylchlorin chloride, Fe(OEC)Cl, was prepared by a published procedure [20]. Iron chlorin-reconstituted myoglobin and horseradish peroxidase were prepared by standard methods [17]. All other chemicals (Aldrich or Sigma) were reagent grade and used without further purification.

Ferric protein-ligand complexes were prepared by addition of ligand stock solution to saturating conditions. The pyridine-CO derivative of the extracted HPII catalase prosthetic group was prepared from the pyridine hemochrome [9] by CO addition. The same complexes of MeC and OEC were generated anaerobically by dissolving Fe(MeC)Cl or Fe(OEC)Cl in neat pyridine followed by reduction with solid $Na_2S_2O_4$ and CO addition. The ferric OEC phenolate complex was prepared by the published method [21]. An extinction coefficient of 118 $mM^{-1}\cdot cm^{-1}$ at 405 nm for native ferric HPII catalase was calculated using the pyridine hemochrome assay [22]; a value of 83 $mM^{-1}\cdot cm^{-1}$ at 423 nm was used for the pyridine hemochrome of the enzyme based on the average value for MeC and OEC [16].

Electronic absorption and MCD spectra were recorded using Varian Cary 210 or 219 spectrophotometer and a Jasco J-500A spectropolarimeter equipped with a 1.5 T electromagnet. Data acquisition and handling were carried out as previously described [13]. EPR spectra were recorded at 21 K using a Bruker ESP-300 spectrometer.

3. RESULTS AND DISCUSSION

Fig. 1 shows a comparison of the MCD spectra of the ferrous pyridine-CO derivatives of octaethylchlorin (OEC) and methylchlorin (MeC) and of the naturally-occurring chlorin extracted from HPII catalase. The MCD spectra of model complexes have been red-shifted in all figures due to the lack of the 2 conjugated vinyl groups that are present on the HPII chlorin [1]. This procedure was first used by Vickery et al. [23] in comparing the MCD spectra of octa-alkylporphyrins with those of protoporphyrin IX. The fact that the 3 spectra displayed in Fig. 1 are nearly superimposable establishes that complexes of OEC and MeC can be used as models for the natural chlorin system. We have recently demonstrated the utility of using octa-alkylporphyrins as models for protoheme in MCD studies [18].

The MCD spectrum of the native ferric high-spin state of HPII catalase is displayed for the first time in Fig. 2A. It is compared to the spectrum of the ferric OEC phenolate complex which closely matches the

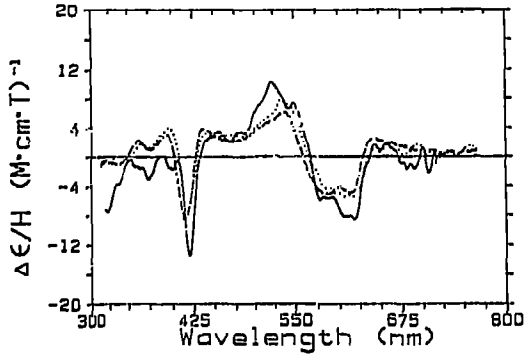

Fig. 1. MCD spectra of the ferrous pyridine/CO complexes of the extracted heme from HPII catalase (——), OEC (------), and MeC (·····). The OEC and MeC spectra have been red-shifted by 15 nm, see text.

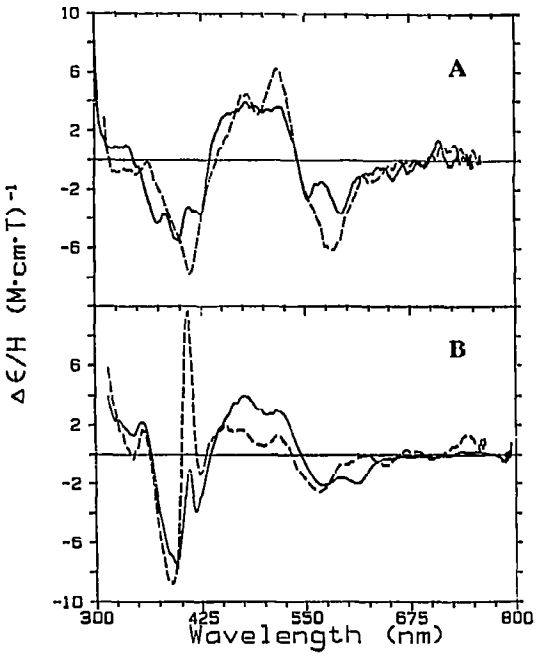

Fig. 2. (A) MCD spectra of native HPII catalase in 50 mM potassium phosphate buffer, pH 7.0 (——), and Fe(OEC)(phenolate) in methylene chloride (------). (B) MCD spectra of ferric MeC-HRP (——) and MeC-Mb in 100 mM potassium phosphate buffer, pH 6.0 (------). The OEC, MeC-Mb and MeC-HRP spectra have been red-shifted by 10 nm, see text.

spectrum first reported by Stolzenberg et al. [21] as a model for tyrosinate ligation. A very similar spectrum was also reported for the 5-coordinate acetate model ferric OEC complex while that of the analogous thiolate adduct was different, especially in the Soret region [21]. The spectra in Fig. 2A by themselves are consistent with an oxyanion as proximal ligand in HPII catalase, likely a tyrosinate anion as in bovine catalase [24]. However, the MCD spectrum of a 5-coordinate histidine-ligated ferric chlorin system (Fig. 2B), generated by reconstituting iron MeC into horseradish peroxidase (HRP) [17], also resembles that of native HPII catalase. The corresponding 6-coordinate aquo/histidine complex of MeC-reconstituted myoglobin [17], however, is clearly different (Fig. 2B). Taken together, these lines of evidence narrow down the likely possibilities for the coordination structure of the native ferric high-spin state of HPII catalase to either a 5-coordinate tyrosinate or histidine ligated complex.

In order to better define the active site structure of HPII catalase, we have compared the MCD spectra of several low-spin ferric ligand adducts of the enzyme with those of parallel complexes of chlorin-reconstituted myoglobin (Fig. 3) as models for a histidine-ligated chlorin system. The results using azide and cyanide are shown in Fig. 3. The MCD spectra of ligand complexes of chlorin-reconstituted HRP closely match those observed for analogous adducts of chlorin-recon-

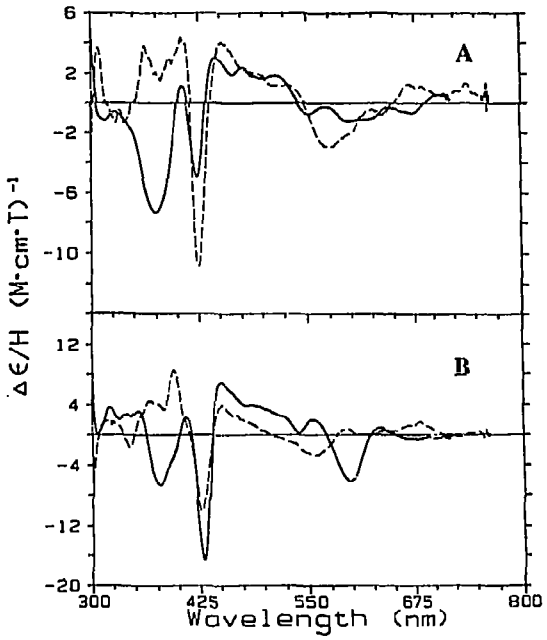

Fig. 3. MCD spectra of the (A) ferric azide and (B) cyanide derivatives of HPII catalase (KCN, 10 mM; NaN_3, 5 mM) (——) and MeC-Mb (KCN, 40 mM; NaN_3, 50 mM) (------). The buffers used were the same as those described in the legend to Fig. 2. The MeC-Mb spectra have been red-shifted by 15 nm, see text.

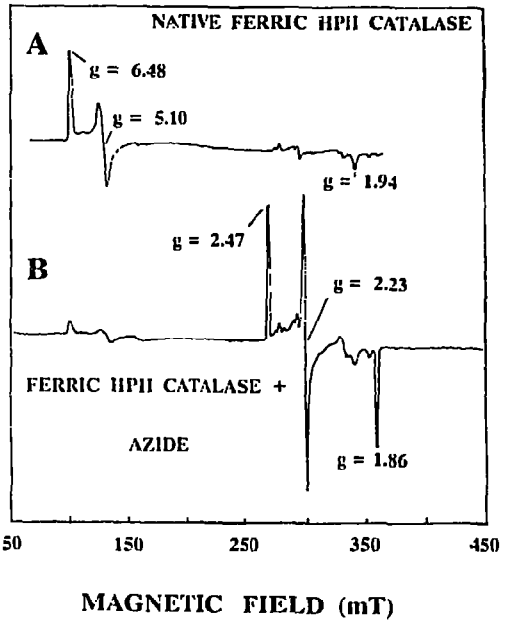

Fig. 4. EPR spectra of (A) native ferric HPII catalase and (B) the ferric-azide HPII catalase complex at 21 K. The EPR spectrometer was operated at modulation frequency of 100 kHz, a modulation amplitude of 10 G, a microwave power of 10 mW, a microwave frequency of 9.27 GHz and a time constant of 0.163 s.

stituted myoglobin [17]. Except for the presence of a sharp trough near 425 nm in all 4 spectra in Fig. 3, the spectra of the respective ligand adducts of the 2 proteins are sufficiently different to state with confidence that the proximal ligand in HPII catalase cannot be histidine. This leaves tyrosinate as the most likely identity of the proximal ligand in HPII catalase. This conclusion is consistent with the recent nucleotide sequence analysis of the gene which encodes HPII catalase and the predicted amino acid sequence in which Tyr^{415} in HPII catalase is proposed to be the counterpart of Tyr^{357}, the proximal ligand to the heme iron of bovine catalase [25]. The three-dimensional structure of HPII catalase is currently being determined by X-ray crystallography [26].

Additional support favoring proximal tyrosine ligation comes from the use of EPR spectroscopy. Addition of azide to the ferric enzyme converts its EPR spectrum from that typical of high-spin ferric heme iron (Fig. 4A) to that of a low-spin state (Fig. 4B). The g-values of the latter (g = 2.47, 2.23, 1.86) are virtually identical to those of several ferric OEC model complexes prepared by Stolzenberg et al. [21] with nitrogenous ligands *trans* to phenolate (g = 2.46–2.47, 2.25, 1.85–1.87) and differ from a complex with 2 nitrogenous ligands (g = 2.51, 2.37, 1.73).

In summary, we have used MCD and EPR spectroscopy to probe the nature of the proximal ligand in HPII catalase. While no single result definitively establishes the identity of the ligand, a range of data have been presented which favor tyrosine as the proximal ligand for the 5-coordinate chlorin iron in *E. coli* HPII catalase. Additional studies are underway to further examine this issue.

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