Comparative analysis of catalases: spectral evidence against heme-bound water for the solution enzymes

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Abstract A recent X-ray structural analysis of *M. luteus* catalase indicates heme-bound H_2O *trans* to the proximal tyrosinate ligand, a finding in contrast to previous X-ray data reporting a 5-coordinate heme for bovine liver catalase. The presence of heme-bound H_2O, requiring displacement prior to substrate-binding, is likely to be catalytically significant for catalases. We have used magnetic circular dichroism (MCD) spectroscopy, a highly accurate method for assignment of heme spin- and coordination-states, to study native, solution forms of bovine liver, *M. luteus*, and *A. niger* catalases. All three enzymes display similar spectral features with the weak (~5 $\Delta\epsilon_M$ [moles·cm·Tesla]$^{-1}$) intensity typical of a 5-coordinate high-spin ferric heme. No evidence for H_2O-ligation, inducing a 6-coordinate heme, occurred upon variation of pH or buffer composition. Therefore, we suggest that the catalytically significant structure of catalases has an unoccupied heme binding site *trans* to the proximal tyrosinate heme ligand.

Key words: Catalase; Distal heme pocket; Magnetic circular dichroism (MCD) spectroscopy; 5-Coordinate vs. 6-coordinate heme systems; Axial H_2O-ligation

1. Introduction

Heme-containing catalase enzymes (EC 1.11.1.6 H_2O_2: H_2O_2 oxidoreductase) are high molecular weight, tetrameric proteins having identical iron protoporphyrin IX prosthetic groups and axial tyrosinate ligation [1–5]. Unlike enzymes such as the P450 mono-oxygenases for which there is considerable diversity in substrate identity, catalases display little or no functional diversity and thus have no (obvious) need for structural variability in their active-site environment(s). The X-ray structure of bovine liver (BL) catalase ([1] and refs. therein), revealed the presence of novel tyrosinate (O^-) ligation to the 5-coordinate ferric heme of the native enzyme. Tyrosinate is not known to be a ligand for any other native heme protein, although it has been proposed for the green chlorin (iron dihydroporphyrin) catalases from *Neurospora crassa* and *E. coli* [6,7].

It was recently reported for *Micrococcus luteus*** (ML) catalase [3], that the native ferric heme had H_2O as its 6th ligand. Furthermore, the heme-bound H_2O molecule was said to be hydrogen-bonded to a second H_2O, that was itself hydrogen-bonded to the distal histidine residue [3]. As shown in Fig. 1, there is thus a considerable difference between the proposed active-site structures for the BL and ML catalases. The catalase reaction:

$$H_2O_2 \rightarrow 1/2\ O_2 + H_2O$$

is one of the fastest enzyme reactions known, with a turnover number of 100,000 per second per active-center [1,5]. This reaction involves H_2O_2 ligation at the heme iron, as the 6th ligand, and a mechanism involving ferryl intermediates (for a review, see [5]). Thus, occupancy of the 6th ligand site, even by an easily displaced ligand such as H_2O, could be expected to have a significant impact on catalysis.

To resolve the issue of axial-H_2O ligation and address the important 'structure/function' correlation for catalases, we have examined the magnetic circular dichroism (MCD) spectra of BL, ML, and *Aspergillus niger* (AN) catalases. These enzymes thus represent mammalian, bacterial, and fungal species, respectively. Of the three catalases, only the latter (AN) has not been crystallized; however, the X-ray structure has been reported for the fungal catalase from *Penicillium vitale* [2].

Magnetic circular dichroism (MCD) spectroscopy is known to be particularly sensitive to heme electronic structure, and particularly to both the spin-state (high-spin vs. low-spin) and the coordination-number (5- vs. 6-coordinate) [8–15]. As shown in Fig. 2, MCD intensities for transitions in the Soret region vary considerably upon changing the spin-state and coordination number of the ferric heme ([8], Andersson, L.A., in prep.) For example, the observed MCD intensity for a (fully) low-spin 6-coordinate heme protein is on the order of ± 110 $\Delta\epsilon_M$(moles·cm·Tesla)$^{-1}$, a factor making these species readily identifiable. In contrast, the MCD intensities for a 5-coordinate ferric heme (high-spin) are on the order of ± 5 $\Delta\epsilon_M$(moles·cm·Tesla)$^{-1}$, whereas those for a 6-coordinate ferric heme (also high-spin) have intensities of approximately ± 15–20 $\Delta\epsilon_M$ (moles·cm·Tesla)$^{-1}$ (Fig. 2). These data, and the spin- and coordination-state assignments, are from our studies of engineered recombinant myoglobins ([8], Andersson, L.A., in prep.), where changing the distal His residue (E7, His64) to Val, Leu, or Ile resulted in generation of 5-coordinate heme groups lacking the axial H_2O-ligation typical of wild-type myoglobin.

In this work, we report the MCD spectra for BL, ML, and AN catalases. The samples were examined at pH 7, and in both low and high pH buffers. In addition, the ML catalase was examined spectrally in a high-salt buffer analogous to that used

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Abbreviations: MCD, magnetic circular dichroism; BL, bovine liver; ML, *Micrococcus luteus*; AN, *Aspergillus niger*; KP^i, potassium phosphate; CD, circular dichroism; R_z, purity index A_{Soret}/A_{280}.

**N.B. *Micrococcus luteus* and *Micrococcus lysodeikticus* are the same organism!

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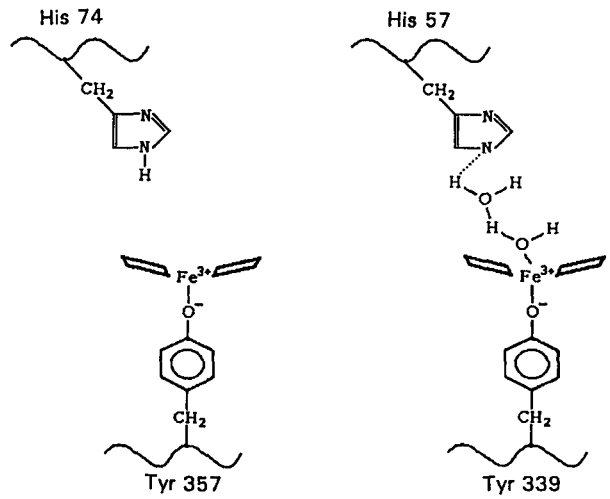

Fig. 1. Cartoon of the active-site structures proposed for native ferric catalases. Left, 5-coordinate Bovine Liver catalase [1]; right, 6-coordinate *Micrococcus luteus* catalase, with the proposed H_2O network [3].

for X-ray analysis (50 mM Na Acetate plus 0.6 M ammonium sulfate, at pH 5.20) [3]. In all cases, the MCD data are most consistent with a high-spin 5-coordinate heme moiety for each of the catalases. Altering the pH or buffer did not, in any case, have any major (or minor) effect on the spectra of the solution catalase samples, such as might be expected upon heme ligation of H_2O. Thus, we suggest that the catalytically significant form of native catalases is that in which the prosthetic heme moiety is 5-coordinate, without heme-bound H_2O.

2. Materials and methods

Bovine liver catalase was purchased from Boehringer Mannheim [R_z = 0.95]; *A. niger* catalase was purchased from Cal Biochem [R_z = 0.43]. *M. luteus* catalase was isolated and purified by Prof. J. Terner (Department of Chemistry, Virginia Commonwealth University) as described previously [4]. The catalases were examined by electronic absorption spectroscopy, in either 5 mm or 1 cm quartz suprasil cuvettes, using Varian-Cary 219, Hitachi U-2000, or Hitachi U-3200 spectrophotometers. Sample concentrations were determined using an ε_{Soret} = 100,000 $(M \cdot cm)^{-1}$ per heme [4]. MCD spectra were obtained on a state-of-the-art Jasco J-720 spectropolarimeter, fitted with a 1.5 Tesla (15 kiloGauss) electromagnet in the sample compartment, with the magnetic field direction parallel to the direction of light propagation. Following collection of the MCD/CD data, the samples were re-examined using electronic absorption spectroscopy. In no case was a change greater than ~1% observed.

The spectropolarimeter was calibrated daily for intensity with ammonium *d*-10-camphor sulfonate (Jasco) and was calibrated bimonthly with neodymium glass for wavelength. The MCD/CD data for each sample were obtained using the following experimental conditions: 1 nm bandwidth; 25 scan accumulation; 200 nm/min scan rate, 0.5nm resolution; 13.9 kiloGauss (1.39 Tesla) magnetic field. Final MCD data were corrected for natural circular dichroism and for the buffer blanks using the Jasco software, and were then corrected for concentration, cuvette, and magnetic field. The data presented in Figs. 3 and 4 have been smoothed using the Jasco software. Samples were maintained at ~14°C during data collection, by use of a custom-made flow-through cell holder and dedicated water bath [16]. Final MCD data are presented in units of $\Delta\varepsilon_M$ $(moles \cdot cm \cdot Tesla)^{-1}$, where Θ_M $(deg \cdot cm^2 \cdot dmol^{-1} \cdot Tesla^{-1})$ = 3300 $\Delta\varepsilon_M$.

3. Results and discussion

3.1. Magnetic circular dichroism spectra

Fig. 3 presents MCD spectra of the native ferric catalases from Bovine liver, *M. luteus*, and *A. niger*, obtained at pH 7. The overall spectral patterns are closely analogous for the three catalases, although there are minor differences. The spectrum reported here for the BL catalase at 14°C is closely similar in both shape and pattern of intensities to that previously reported for native BL catalase at room temperature [17–19].

The MCD spectral pattern for the native catalases consists of several key features (described from high- to low-energy). These include: (i) a roughly W-shaped feature in the Soret region, having two negative bands at ~400 nm and ~429 nm. The latter feature is red-shifted by almost 10 nm for the AN catalase, to ~439 nm. (This shift is not matched by any concomitant change in the Soret region electronic absorption spectra between the AN vs. BL and ML catalases. The electronic absorption spectra for the three catalases are essentially identical [4].) The higher energy MCD trough, at ~403 nm, has the strongest intensity in the Soret region, of ~5 $\Delta\varepsilon_M$ $(moles \cdot cm \cdot Tesla)^{-1}$. (ii) The MCD spectra of the three catalases have a broadly positive region, between ~450 nm and ~535 nm, again with an overall intensity of ~5 $\Delta\varepsilon_M$ $(moles \cdot cm \cdot Tesla)^{-1}$. (iii) Above ~540 nm, the spectrum of AN catalase differs markedly from that of the BL and ML catalases. The AN catalase spectrum has a second W-shaped pattern, with the left trough at ~560 nm, and the right trough at ~577 nm; the small positive feature is at 563 nm. In contrast, the MCD spectra in this region for both the BL and ML catalases have only a broad negative feature at ~560 nm, with an unresolved shoulder at ~578 nm. (iv) Above ~590 nm,

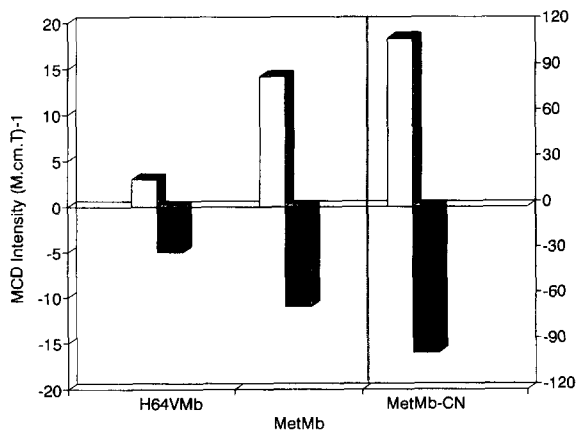

Fig. 2. Bar graph of the Soret region MCD spectral patterns typical for different spin- and coordination-states of ferric heme (iron protoporphyrin IX) systems. *Left*: high-spin, 5- coordinate heme, represented by the engineered recombinant human cardiac myoglobin, His64 → Val [8]; *Middle*: high-spin 6-coordinate heme, represented by wild-type human cardiac metMyoglobin [8]; *Right (note scale change)*: low-spin, 6-coordinate, represented by the human cardiac metMyoglobin-CN^--adduct (Andersson et al., in prep.).

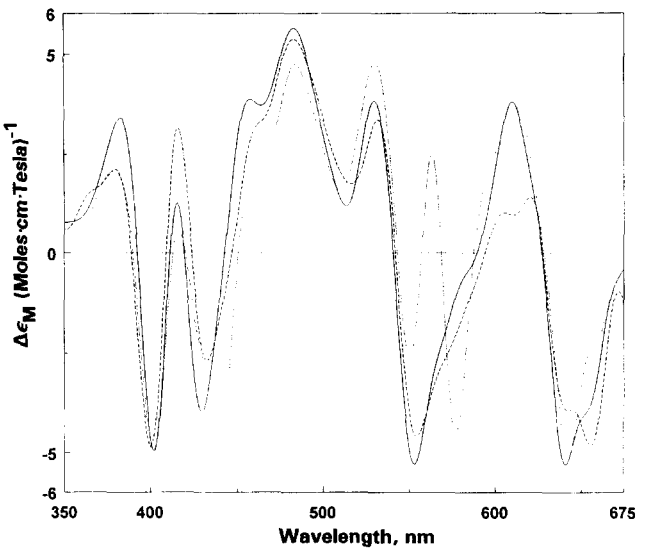

Fig. 3. MCD spectra of the catalases at pH 7, in 100 mM KP_i buffer: solid line, BL catalase (15.4 μM); dotted line, AN catalase (17.8 μM); dashed line, ML catalase (16.1 μM). Experimental conditions for the spectral analysis are described in section 2.

the spectra of the three catalases are again closely similar. There is a positive transition at ~610 nm and a negative feature at ~640 nm, which has a low energy shoulder above 650 nm. This shoulder is resolved for the ML catalase.

Thus, with minor exceptions, the overall spectral patterns (both shape and intensity) observed for both the AN and ML catalases are closely similar to that of the BL catalase. The prosthetic heme moieties of the bovine liver enzyme are known to be 5-coordinate high-spin ferric [1,4,5,17–19]. Furthermore, the general MCD spectra of the three catalases have no feature with an (absolute) intensity greater than ~5 $\Delta\varepsilon_M$ (moles·cm·Tesla)$^{-1}$. The intensity pattern is thus generally similar to that for the 5-coordinate engineered recombinant human myoglobins (Fig. 2) [8]. It is also on the order of that observed for 5-coordinate horseradish and peanut peroxidases [20], although the Soret MCD intensities of peroxidases are slightly more intense – a factor we attribute to the strongly hydrogen-bonded histidine/histidinate 5th ligand (Andersson, L.A., in prep.). The Soret MCD data for the three catalases are clearly considerably weaker in intensity than that typically observed for a 6-coordinate high-spin ferric heme, having water bound axially, of which metaquoMyoglobin is the best known example (e.g. Fig. 2, [8]).

3.2. Variation of experimental conditions

To determine whether the catalases would bind H_2O at the heme upon altering the buffer conditions, we also prepared *M. luteus* catalase in a buffer analogous to that used for the X-ray report [3]: pH 5.2 Na Acetate, 0.6 M ammonium sulfate. The MCD spectral data for this sample are shown in Fig. 4, (dotted line), vs. MCD data for *M. luteus* catalase in a salt-free buffer at pH 5 (Fig. 4, solid line). The addition of the salt did not have any significant effect on the MCD data for the *M. luteus* sample; in particular, the high-salt buffer did not induce any heme-ligation of H_2O, based on MCD intensities (compare Figs. 2 and 4).

The MCD data for the ML catalase are not completely identical between the pH 7 and the pH 5 samples (*dotted line*, Fig. 3 vs. solid line, Fig. 4). Some minor differences occur, e.g. for the band at ~429 nm, which is decreased in intensity, and for the bands above ~550 nm. However, none of these is on the order of what might be expected were H_2O to bind at the heme.

MCD spectra for all three catalases were also obtained in both acidic and basic buffers (data shown only for pH 5 ML catalase). These MCD spectra are closely analogous to those of the solution samples at pH 7, particularly for the BL and AN catalases. In no case was there any indication of an increase in the MCD intensities, particularly in the Soret region, as might logically might be expected were there to be ligation of H_2O and formation of a 6-coordinate catalase complex. H_2O-binding *trans* to a tyrosinate-ligated heme has been shown to result in an increase in MCD intensities upon lowering the pH for the MCD spectra of engineered recombinant mutant-metmyoglobin, where the proximal histidine residue (His93) was replaced with tyrosine (Andersson, L.A., Willingham, T.R. and Morishima, I., in prep.).

4. Conclusions

As shown herein, the solution MCD spectra for the native ferric catalases from Bovine liver, *Aspergillus niger* and *Micrococcus luteus* are clearly analogous to one another. Furthermore, no change in intensity was observed for any of the catalases upon lowering (or raising) the pH, or upon duplicating the buffer used for generation of crystals of the *M. luteus* catalase. The overall pattern of MCD intensities is on the order of ~5 $\Delta\varepsilon_M$ (moles·cm·Tesla)$^{-1}$, a value roughly 2/3 lower than that observed for a typical 6-coordinate high-spin ferric heme protein having H_2O bound at the heme (e.g. Fig. 2).

If we make the assumption that ligation of H_2O at the heme moiety of catalase can be expected to induce an increase in the MCD spectral intensities, as is observed between 5- and

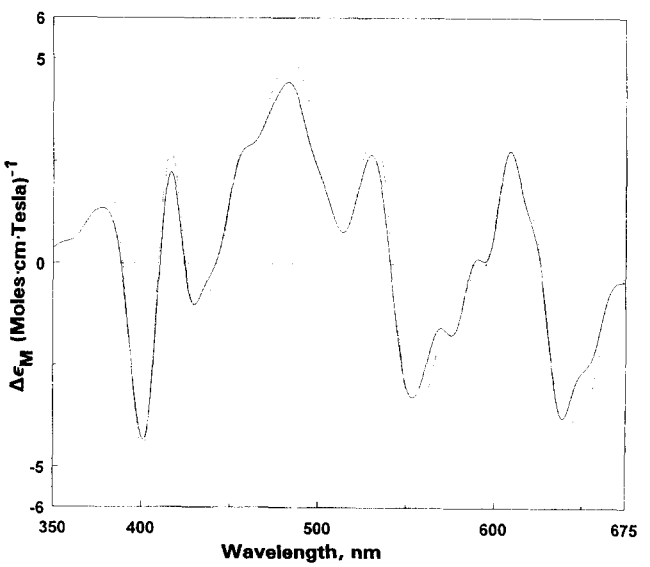

Fig. 4. Solution MCD spectra of *M. luteus* catalase, duplicating conditions used for X-ray analysis. Solid line, ML catalase (17.25 μM) in pH 5.0, 20 mM Na Acetate buffer; dotted line, ML catalase (17.25 μM) in pH 5.2, 50 mM sodium acetate buffer, plus 0.6 M ammonium sulfate. Experimental conditions for the spectral analysis are described in section 2.

6-coordinate metMyoglobin species (Fig. 2, [8]), then these data indicate that H_2O is not likely to be bound to the catalases in solution under the conditions evaluated. The MCD spectra reported herein are most consistent with the three native catalases having prosthetic heme groups that are 5-coordinate and high-spin in solution. This finding is also is agreement with our previous study of these three catalases by resonance Raman spectroscopy [4].

Thus, we conclude that the solution form of native catalases from mammalian, fungal, and bacterial species has 5-coordinate heme prosthetic groups lacking axially bound H_2O *trans* to the tyrosinate axial (5th) ligand. It is likely that this coordination state is of catalytic significance, given that an open 6th ligand site is more easily occupied during catalysis.

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