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Mössbauer studies of the ferryl, ferrous and ferric states of dehaloperoxidase from *A. ornata*

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ABSTRACT

Dehaloperoxidase (DHP) is a multi-functional catalytic globin from the marine worm *A. ornata*, whose physiological functions include oxygen transport and oxidation of toxic substrates present in its habitat. In the Fe(III) state, DHPA has an isomer shift of 0.42 mm/s, characteristic for high-spin heme proteins. Changes in pH have subtle effects on the electronic structure of DHP in the Fe(III) state detectable in the high-field spectra, which show a pH-dependent mixture of species with different zero-field splittings between 5 and 18 cm^{-1}. The short-lived intermediate obtained by direct reaction of the Fe(III) enzyme with H_2O_2 has an isomer shift of 0.10 mm/s, indicative of an Fe(IV)-oxo state and of an S = 1 electronic ground state confirmed by variable field studies. The O_2-bound state of DHP has an isomer shift of 0.28 mm/s and a high-field spectrum characteristic for diamagnetic heme complexes, similarly to other haemoglobins.

Overall, the isomer shift and quadrupole splitting of DHP in the four states studied are expectedly similar to both peroxidases and to myoglobin. The differences in electronic structure between DHP and other heme proteins and enzyme are observed in the high-field Mössbauer spectra of the ferric state, which show pH-dependent zero-field splittings suggesting a heme site in which the ligand field strength at the iron ion is tuned by pH. This tunability is correlated with variable electron-donating properties of the iron, which can perform multiple functions.

1. Introduction

Haemoglobins have been intensely studied since their discovery in the 1800's [1] and have provided an ideal subject for seminal magnetic measurements marking the birth of bioinorganic chemistry as shown by Bren et al. [2,3]. Soon after Rudolf L. Mössbauer discovered the recoil-free nuclear gamma ray absorption in 1958 [4,5], Gonser et al. used it to study the chemical structure of haemoglobin in preparations of human red blood cells [6]. Lang and Marshall studied purified ^{57}Fe-enriched rat haemoglobin, observing the distinct hyperfine interactions of reduced Fe(II) haemoglobin, oxy-haemoglobin, as well as complexes with CN^- and NO [7]. Since then, Mössbauer (MB) spectroscopy has been an essential tool for comparing electronic features of heme proteins and model complexes, including cytochromes, peroxidases, P450 monooxygenases, and globins [8–12].

As the first example of a multifunctional catalytic globin, the coelomic haemoglobin from the marine worm *Amphitrite ornata*, originally termed dehaloperoxidase (DHP, Fig. 1) due to its ability to catalyze the H_2O_2-dependent oxidative dehalogenation of 2,4,6-trihalophenols, has shown an unusual range of mechanistic functions: beyond its primary role as an oxygen transport protein, DHP has been shown to possess four additional activities necessary for the survival of *A. ornata* (peroxidase, peroxygenase, oxidase and oxygenase), all of which employ a common Fe(IV)-oxo intermediate. As such, and in contrast to

Abbreviations: CcP, cytochrome *c* peroxidase; DEQ, quadrupole splitting, ΔE_Q; DHP, dehaloperoxidase; Hb, haemoglobin; HRP, horseradish peroxidase; MB, Mössbauer; Mb, Myoglobin; ZFS, zero-field splitting, D; 5c, 6c, Five-coordinated, six-coordinated; δ, Isomer shift (mm/s); ΔE_Q, Quadrupole splitting (mm/s); $A_{xx, yy, zz}$, Principal components of the magnetic hyperfine tensor **A**; V_{zz}, Largest principal component of the electric field gradient (EFG) tensor; η, Rhombicity of the EFG tensor.

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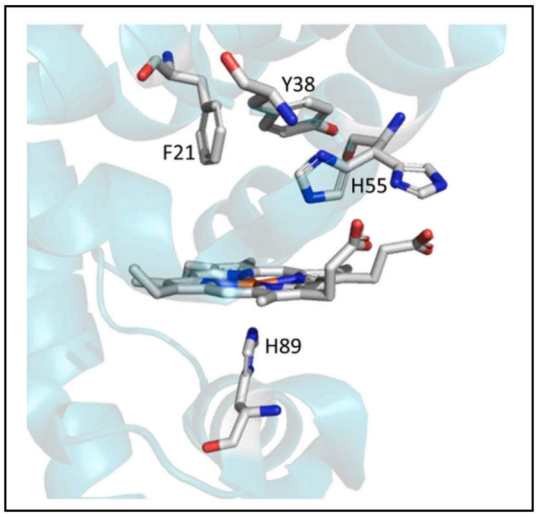

Fig. 1. The heme site and its protein environment in dehaloperoxidase from *A. ornata*. (PDB ID 1EW6, ref. (17)).

monofunctional hemoproteins, DHP has several different substrate-specific outcomes that can result from passage through a Compound I-type intermediate. The most exciting aspect of DHP has been the observation that different substrates can be oxidized by different mechanisms, and even trigger changes in protein structure needed to facilitate those mechanisms. Given that it is capable of the five major activities associated with heme proteins within a single active site, DHP is a prime example of a multifunctional protein that can advance our understanding of the structure-function correlation beyond what has been built from decades of study of monofunctional proteins. Thus, DHP is a unique enzyme platform for deeply interrogating questions relating the structure of the protein-substrate complex to the tuning of an enzyme's activity (complementing the perspective gained from the study of monofunctional proteins). As the multi-functional character of DHP challenges the traditional structure-function correlation in heme proteins, it is of interest to probe the electronic structure of this enzyme with Mössbauer spectroscopy in search of fundamental electronic structural features that may contribute to its reactivity.

Despite DHP being categorized as a globin according to the Structural Classification of Proteins (SCOP) database [13], it has little sequence homology to other known haemoglobins. As expected for a globin, the X-ray crystal structures of DHP isoenzyme A [14] and B [15] show that it has a globular structure and a histidine proximal ligand. However, DHP bears little resemblance to the fold of cyt *c* peroxidase (CcP), the prototype for the heme peroxidase family [16], and lacks the cysteinate proximal ligand in cyt P450s (monooxygenase) and chloroperoxidase (dechlorination) [14,17]. Thus, DHP is an example of a multifunctional protein in which the presence of either its peroxidase or peroxygenase activities cannot be readily explained based upon structural homology to other systems.

A second structural feature that sets DHP apart from other studied globins (e.g. myoglobin, haemoglobin), or even many heme peroxidases, is that it possesses a distal small molecule binding site that can accommodate a variety of different substrates (and inhibitors) [14,17–19]. Using stopped-flow UV–visible and rapid freeze-quench EPR spectroscopic methods, we have previously shown evidence that ferric DHP reacts with H_2O_2 to yield either Compound I or Compound ES, the latter being iron(IV)-oxo heme center with an amino acid radical [20]. We further showed that these species were the reactive intermediates responsible for oxidizing 2,4,6-tricholorophenol (TCP) to 2,4-dichlorquinone (DCQ) in the peroxidase mechanism, and for O-atom insertion into indole, nitrophenol, pyrrole and cresol in the peroxygenase mechanism [21]. The presence of multiple binding sites across the entire face of the distal heme cavity is consistent with—although not the only determinant of the multifunctional quality of DHP, a feature that is unprecedented for a haemoglobin [22]. An FTIR study of DHP-CO by flash photolysis concluded that binding of the substrate in the distal pocket may not be required for DHP function [23]. Moreover, a study correlating pH-dependence of EPR spectra with the rate of ES complex formation indicated that the low-pH, five-coordinated Fe(III) form is less active than the high-pH, six-coordinated form of DHP, proposed to have H_2O as the sixth ligand [24]. The data presented here suggest that the intrinsic electronic features of the heme and the extended hydrogen-bonding network affecting the donating abilities of the distal histidine have a contribution to the reactivity of the heme site.

Another property that stands out of for DHP is its large positive Fe^{3+}/Fe^{2+} reduction potential. For heme proteins, the Fe^{3+}/Fe^{2+}reduction potential was shown to be a good reporter for the coordination environment of the heme, being sensitive to single amino acid mutations of residues in the second coordination sphere of the iron [25,26] Table 1 presents the redox potentials for a selection of heme proteins. When added to the set of heme proteins in this table DHP is highly distinctive. Hypothetically, the exceptional characteristics listed above, including the differences in the sequence and redox potential of DHP could be correlated with differences in the electronic structure of the iron site, as reported by the Mössbauer parameters. In this study, we are presenting the MB characterization of the ferric, oxyferrous, ferrous and Fe(IV)-oxo states of DHP with the goal of uncovering features of the electronic structure of the heme iron giving rise to these distinctly different properties.

Mössbauer spectroscopy probes directly the environment of the Fe nucleus and can detect both paramagnetic and diamagnetic iron. Parameters obtainable in zero-or small applied fields, namely isomer shifts and quadrupole splittings are good indicators for oxidation state and coordination number and geometry, spin state and sometimes ligand type. [30] Taken together they serve well in the identification heme proteins among other types of iron biological cofactors, i.e., non-heme iron. Among heme proteins in a particular oxidation state, e.g., ferric, MB isomer shifts and quadrupole splittings lie within a relatively narrow range, as would be expected for iron sites coordinated by the same protoporphyrin IX macrocycle. For example, isomer shifts for ferric horseradish peroxidase (HRP), cytochrome *c* peroxidase (Ccp), myoglobin, and cytochrome P450, (Table 1) are between 0.40 and 0.44 mm/s. However, it is clear from Table 1 that these parameters do not correlate well with the diversity of functions of these heme proteins and that DHP is expected to be similar with other hemes.

Table 1
Mössbauer (MB) parameters and reduction potentials for selected Fe(III) heme proteins and heme enzymes.

	(1) Isomer shift, δ (mm/s)[a]	(2) Quadrupole splitting ΔE_Q (mm/s)[a]	(3) Zero-field splitting, D (cm^{-1})[a]	(4) $Fe^{3+/2+}$ Reduction potential (mV)[b]	Ref. a for [1,2,3] b for [4]
DHP Fe^{III} (pH 5; pH 7)	*0.42*	*1.29*	*12, 14*	*+204*	a) This study; b) [27]
Horseradish Peroxidase (HRP)	0.40	1.60	14	−278	a) [10]; b) [25]
Cytochrome c Peroxidase (CcP)	0.40	1.2(1)	15	−183	a) [28]; b) [25]
Cytochrome P450	0.44	0.79	3.8	−303	a) [11];b) [29]
Myoglobin	0.42	1.24	10	+58.8	a) [12]; b) [26]

Diversity may however be reported by more sensitive parameters, related to the electronic structure of the heme complex, such as zero-field splittings (ZFS) and magnetic hyperfine interactions, which are determined by fitting the MB spectra in high applied magnetic fields. Interpretation of these parameters through the lens of ligand field theory gives insight into the electronic structure of hemes. For instance, MB spectroscopy has been effective in characterizing the resting states (Fe^{3+}), states in which oxygen is bound (Fe—O_2) as well as of high-valent Fe^{IV}-oxo intermediates, such as Compounds I-III and X in HRP and chloroperoxidase [10,31]. Thus, in this paper, we have examined the electronic structure of DHP with variable-field MB spectroscopy, searching for fundamental electronic structure features that might correlate with its distinct properties discussed above.

One electronic parameter determinable by MB, which was observed to be sensitive to the secondary coordination sphere in heme proteins is the zero-field splitting (ZFS). The effect of the second coordination sphere on the ZFS and on the spin state of a heme protein can be rationalized by considering the origin of ZFS in ligand field theory. High-spin hemes, such as met-haemoglobin, cytochrome *c* peroxidase (CcP), horseradish peroxidase (HRP) and DHP, have a ^{6}A ground electronic state [9,32,33]. Spin-orbit coupling can mix orbital excited states into the ground state, giving rise to large zero-field splittings and temperature-dependent quadrupole splittings, which are observed for these proteins [34]. Ligand field strength and therefore the coordinated ligands and their associated hydrogen bonding partners can influence the separation in energy between the ground state and the next orbital excited state. In turn, tweaks in the energy of the orbital excited states may affect the ZFS measured by spectroscopy. This mechanism has been invoked by Goodin and McRee [25] in a study of CcP in which they showed that a mutation of an Asp residue hydrogen-bonded with the proximal histidine can alter the zero-field splitting and also cause an increase in the redox potential of the heme iron and affect the anisotropy of the EPR signal of a nearby Trp residue. While the influence on the redox potential of secondary sphere mutations of residues on the proximal side of the heme has been demonstrated for DHP [27] the ZFS of any form of DHP has not been determined until this study.

In this paper we are presenting the Mössbauer spectroscopic characterization of dehaloperoxidase in the ferric, Fe^{II}-O_2 ("oxyferrous") form, as well as the MB characterization of the Fe^{IV}-oxo intermediate formed upon reaction of Fe^{3+}-DHPA with hydrogen peroxide. By comparing the MB parameters of DHP to other heme proteins and heme enzymes, such as myoglobin and peroxidases, we aim to gain insight into the fundamental electronic structure of the iron center in DHPA which is at least in part responsible for the distinct behavior of this protein among other heme proteins. In the simplistic assumption that the origins of DHP's multifunctional character may be broadly categorized in two different classes, i.e., properties related to the protein structure and properties related to the intrinsic electronic structure of the iron, our study presents data falling in the latter category. Thus, the present investigation of the MB parameters of DHP and their correlation with the tunable coordination environment of the heme, adds another perspective to the discussion of the factors that are responsible for the function of DHP.

2. Experimental methods

Cell Growth and isolation of ^{57}Fe-enriched DHP. Ferric DHP A enriched with Fe-57 was prepared by growing *E. coli* containing the DHP expression plasmid in minimal non-iron media and adding iron at induction time. The protein was purified according to published procedures for wild-type DHP B [35,36]. Iron levels were quantitated by measuring the intensity of the Soret band and by atomic absorption (AA) spectroscopy using the standard addition method. Mössbauer samples were concentrated (Amicon Ultra centrifugal filter, 10 kDa MWCO) to approximately 1.1 to 1.3 mM at pH 5.0 and 7.0, placed in Delrin cups and frozen in liquid nitrogen. The ferric samples were used to obtain the Mössbauer spectra of the resting state DHP and for subsequent preparation of other enzyme states.

Oxyferrous ($^{57}Fe^{II}$-O_2) DHP was prepared by reacting ^{57}Fe-DHP in KH_2PO_4 buffer (pH 5.0) with excess ascorbic acid in the presence of atmospheric oxygen, followed by desalting (P-10 column), and concentrated as described above. The ferryl state was prepared by reacting ferric ^{57}Fe-DHP in KH_2PO_4 buffer (pH 7.0) with H_2O_2 (200 mM) for 12 s before freezing the reaction solution in liquid nitrogen.

Preparation of ^{57}Fe-DHP by substitution of the porphyrin with ^{57}Fe-Protoporphyrin IX. One of the pH 7 samples was obtained by an alternative procedure. A modified Teale-Butanone extraction method [37,38] was employed as described by McGuire as follows: DHP dissolved in 100 mM KP_i was adjusted to pH 2.3–2.5 by dropwise additions of 0.1 M HCl and immediately mixed with an equal volume of ice-cold 2-butanone [39]. The mixture was vigorously shaken for 30 s and allowed to stand at 0 °C for 1 min until the deeply colored upper layer of butanone phase (heme-containing) separated from the colorless lower layer of aqueous phase (apoenzyme). The butanone phase was siphoned off, the remaining aqueous phase was treated twice more with 2-butanone, and finally dialyzed against 10 mM KP_i (pH 7.0) in quadruplicate to remove any trace butanone. The dialyzed solution was centrifuged to remove insoluble material, and the resultant apoenzyme in 10 mM KPi (pH 7.0) was mixed with an excess of ^{57}Fe protoporphyrin IX (purchased from Frontier Scientific, 2–4 mg) in 400 μL 0.1 M NaOH and allowed to stand at 0 °C for 30–60 min. The mixture was then passed through a carboxymethyl (CM) cellulose column equilibrated with 10 mM KPi (pH 7.0). The ^{57}Fe-DHP complex was adsorbed on the column, whereas the excess unbound porphyrin directly eluted with 10 mM KPi (pH 7.0). Elution of ^{57}Fe-DHP was achieved with 40 mM KP_i (pH 7.0). Reconstituted DHP made by this method showed identical UV–visible spectral features and Michaelis-Menten kinetics (benchmarked with 2,4,6-trichlorophenol as the substrate) as recombinant DHP. Additionally, X-ray structural studies of DHP reconstituted with non-native metal protoporphyrin IX (i.e., Mn^{2+}) also showed that DHP maintains its identical fold after this procedure [39]. The sample was buffer exchanged into 100 mM KP_i and 10% glycerol (*v*/v) and concentrated to approximately 1 mM before freezing in a Mössbauer sample cup. MB spectra of a pH 7 sample obtained by this reconstitution method and a pH 7 sample obtained by purification as holo-protein from ^{57}Fe-enriched *E. coli* are shown in Fig. S-3. The small differences in the overlaid spectra are within the experimental noise and similar to variations between preparations using the purification of holo-protein from bacteria grown on ^{57}Fe-enriched media.

Mössbauer Spectroscopy. Mössbauer (MB) spectra were recorded on a closed-cycle refrigerator spectrometer, model CCR4K (SeeCo, Edina, MN) equipped with 0.04 T (38 mT) and 0.07 T permanent magnets, maintaining temperatures between 4.5 K and 300 K. MB spectra in strong applied magnetic fields (1.0–8.0 T) were collected on liquid-helium cooled superconducting magnet instruments at Carnegie Mellon University (Dr. Alex Guo). The sample holders were Delrin 1.00 mL cups, frozen in liquid nitrogen. The isomer shifts are quoted at 5 K with respect to iron metal standard at 298 K. Mössbauer spectra were analyzed using the software WMOSS4 (Ion Prisecaru, www.wmoss.org) and SpinCount (Michael Hendrich, Ph.D., Carnegie Mellon University).

Spectral simulation and least-squares fits were produced using the quadrupole interaction Hamiltonian in Eq. (1) appended with the spin Hamiltonian for electronic spins (Eq. (2), where S = 5/2 for the high-spin ferric state) and S = 1 for Fe^{IV}=O, respectively.

$$\widehat{H}_Q = \frac{eQV_{zz}}{12}\left[\hat{I}_z^2 - \frac{15}{4} + \eta\left(\hat{I}_x^2 - \hat{I}_y^2\right)\right] \tag{1}$$

$$\widehat{H_{IS}} = D\left[S_z^2 - \frac{1}{3}S(S+1) + \frac{E}{D}\left(S_x^2 - S_y^2\right)\right] + \beta\hat{\mathbf{S}} \bullet \tilde{\mathbf{g}} \bullet \vec{\mathbf{B}} + \hat{\mathbf{S}} \bullet \tilde{\mathbf{A}} \bullet \hat{\mathbf{I}} + \widehat{H}_Q - g_n\beta_n\vec{\mathbf{B}} \bullet \hat{\mathbf{I}} \tag{2}$$

Symbols have the customary meaning [40,41].

3. Results and discussion

The Fe(III) state of ^{57}Fe-DHP. The samples of Fe(III)-DHPA enzyme in this study were prepared by two methods: the first was using a more traditional expression of DHP in minimal media supplemented with Fe-57 [42], while the second method was by reconstitution of apo-DHP with the ^{57}Fe-protoporphyrin IX cofactor. Both methods enabled the successful preparation of ^{57}Fe-DHP for study at pH 5 and 7, with the latter method providing a more economical use of the isotopically-labeled iron.

The spectra of DHP at pH 5, collected at a small applied magnetic fields of 70 mT at 6 K, show six-line patterns, indicating slow relaxation of the electronic spin on the Mössbauer timescale, and are characteristic for the S = 5/2 state of high-spin ferric hemes (Fig. 2 D, E). Simulations of the spectra in small applied magnetic fields allowed determination of the isomer shift and quadrupole splitting of 0.42(3) mm/s and $\Delta E_Q = 1.3$ (1) mm/s, respectively (Table 2), with an average magnetic hyperfine coupling ($A_{ave} = (A_{xx} + A_{yy} + A_{zz})/3$) of −19 T. For the determination of zero-field splittings we have collected spectra in high applied magnetic fields (see further).

In zero-field, DHP samples (at both pH 5 and 7) exhibit broad spectra especially at low temperatures, with line-widths >0.4 mm/s. As observed previously for high-spin hemes, such as myoglobin, spectra sharpen in higher magnetic fields [28]. At temperatures of 100 K and higher we observed very broad doublets, due to fact that the electronic system enters fast relaxation on the MB timescale and that the magnetic features average out. (Fig. S-1C) This type of relaxation spectra were observed in the literature for the HRP-benzohydroxamic acid complex [10]. For the purpose of our study, we focused on the well-resolved spectra obtained at low temperature in variable magnetic fields.

Table 2
Mössbauer parameters for dehaloperoxidase from *A. ornata*.

	DHP Fe^{III} (pH 5)	Oxy-DHP	DHP Fe^{II}	DHP Fe^{IV}=O
S	5/2	0	0	1
δ (mm/s) (4.2 K)	0.42(3)	0.28(1)	0.83 (2)	0.10(2)
ΔE_Q *(mm/s) (4.2 K)*	1.3(1)	−2.26 (1)	1.50 (3)	1.53(2)
η	0	0.5	Not det.	0.6 (est.)
$A_{xx,yy,zz}/g_n\beta_n$ *(T)(% area)*	−18(1), −20(1), −18(1); 67%* −18(1), −20(1), −18(1); 19.5% −19(1), −20(1), −18(1); 13.5%	–	–	−19.5(1), −19.5(1), −1.0 (1)
$A_{iso} = (A_{xx} + A_{yy} + A_{zz})/3$ *D* (cm^{-1}), *E/D*	−19 T +12, 0.0 +10, 0.0 +8, 0.0	–	Not det.	+21, 0
Γ (mm/s)	0.38	0.30	0.30	0.35

* Spectral fitting indicated three distinct species with the relative areas indicated here. The numbers in parenthesis indicate the uncertainty in the least significant digit.

To better characterize the magnetic hyperfine interactions in DHP and determine the zero-field splittings, additional spectra were collected in variable large, applied fields between 3.0 and 8.0 T (Fig. 3). Typically, Mössbauer data are interpreted in terms of the spin Hamiltonian for an isolated spin sextet, S = 5/2, or ^{6}A orbital state. Thus, electronic parameters are reported in terms of spin Hamiltonian parameters, which allow a systematic comparison across different heme proteins. Using the spin Hamiltonian formalism (Eq. (2)), the whole set of spectra at 3.0, 5.0 and 8.0 T and 4.2 K was fitted simultaneously, in the assumption of slow fluctuation of the electronic spin on the MB time scale. Good spectral fits

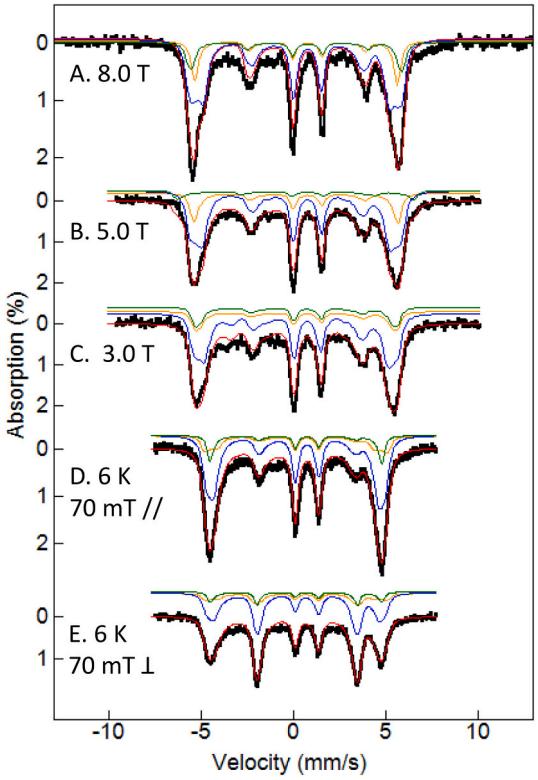

Fig. 2. Mössbauer spectra of a ^{57}Fe-enriched DHP sample at pH 5 in applied fields of (A) 8.0 T/ 4.2 K, (B) 5.0 T/4.2 K(C) 3.0 T/4.2 K; (D) at 6.0 K in 70 mT parallel field and (E) 6.0 K in 70 mT perpendicular field applied with respect to the incident γ-radiation. Spectral simulations (solid colored lines) were obtained with the parameters listed in Table 2.

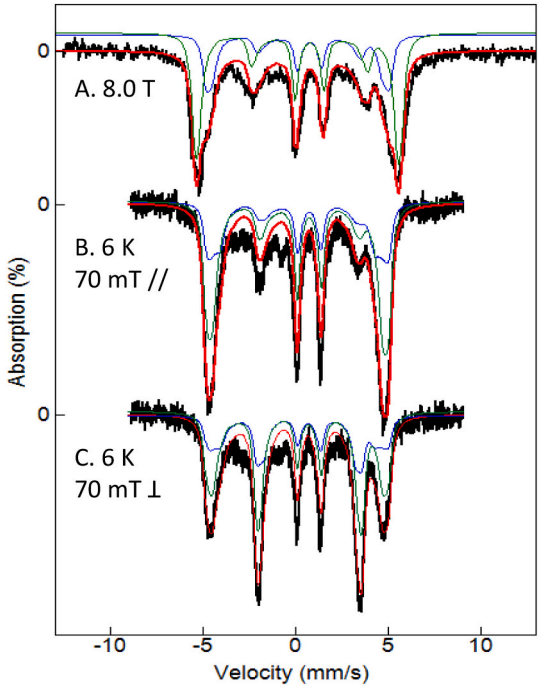

Fig. 3. 4.2 K-Mössbauer spectra of ^{57}Fe-DHP A prepared at pH 7, in applied fields of (A) 8.0 T, (B) 70 mT parallel and (C) 70 mT, perpendicular field applied with respect to the incident gamma radiation. The raw data are shown in black. The solid colored lines are spectral simulations with the parameters discussed in the text (Table 2).

for the data set at all magnetic fields required a superposition of three high-spin Fe^{III} species (S = 5/2), all having common values for the low-field parameters. The high-field spectra (between 1.0 and 8.0 T) show that all species present are iron(III) and have axial zero-field splittings, varying between 8 and 12 cm^{-1}. The majority species, accounting for 67% of the total MB absorption, has D = 12 cm^{-1} (blue line in Fig. 2A) and a slightly axial magnetic hyperfine coupling tensor with principal components $A_{xx,\ yy} = -18(1)$ T, $A_{zz} = -20(1)$ T.

Spectra of multiple samples of the enzyme at pH 7 were collected in magnetic fields of variable strengths and at variable temperature. In small fields of 300–700 G, the spectra of the pH 7 samples are sharp, with well resolved features, almost identical to the pH 5 data (Fig. 3 and also Fig. S-1). The main difference between the samples at the two pH-values is observed by comparing the MB spectra in large applied fields. Thus, at 8.0 Tesla, we observe a species with a larger D-value, namely 18 cm^{-1} (36%, blue line in Fig. 3A), present along with the majority species which has D = 10 cm^{-1}, which is reminiscent of the pH 5 sample. Thus, our least-square fitting of the spectra in high filed for the pH 7 enzyme shows a slightly different mixture of species (Fig. S-1, Table S-1) including a species with larger D. Larger D-values are expected for a stronger ligand field, as could be caused by changes in the bond distance of the axial histidine or conversion from a five- to six-coordinated heme [25].

The analysis of high-spin ferric heme MB spectra has been definitively established by decades of studies [9,40,41,43,44]. As shown in Table 1, the isomer shifts and quadrupole splittings of ferric DHPA are in the range for heme proteins, such as ferric myoglobin, horseradish peroxidase and the high-spin form of cytochrome P450. In our data no low-spin forms have been observed.

The zero-field splittings of heme proteins are usually large (5–15 cm^{-1}) and E/D values are small, typically smaller than 0.1.(Table 1) From this point of view DHP is no exception; the high-field spectra of DHP show multiple species with zero-field splittings varying between 8 and 18 cm^{-1}, with larger values observed for the pH 7 form. These values similar to those seen in cytochrome *c* peroxidase, but also myoglobin (Table 1). The MB spectral characteristics of heme enzymes, for example HRP and other peroxidases [10,45] are sensitive to pH variations and to the presence of substrates or inhibitors, which bind in the second coordination sphere.

The pH dependence of the EPR spectra of ferric DHP has been previously reported from our laboratories [24] and by others. [46] Thus, changes in pH from pH 7 to 5 introduced enough perturbations in the hydrogen bonding network in the distal side of the heme to cause motions of the His 55 residue and decrease in function [24]. EPR spectra for DHPA showed mixtures of different species at pH values from 7 to 4, the higher pH form being high spin six-coordinated, with an H_2O molecule as the sixth ligand (pH 7, 6c) with a high rate of ES formation, while the low-pH form being termed high-spin five-coordinated (5c), having a low rate of compound ES formation. Empirical deconvolutions indicated two overlapping signals with *g*-values near 6 and 2, typical for high-spin heme proteins with near-axial symmetry (E/D < 0.01), which reinforces the E/D determinations from our MB data. Our data show multiple ZFS at both pH values, including a spectral component with a larger ZFS at pH 7, which is not present at pH 5. Changes in the coordination environment of the iron that increase the electron donating properties of a ligand can cause and increased ligand field, lowering excited states and thus increasing the mixing of excited states into the ground state, in turn increasing the ZFS. Thus, our observation of a larger ZFS suggests this type of changes, which include shortening of the Fe-N_{His} bond, due to shifting hydrogen bonds, or addition of a water ligand in the proximal position, or both. For other peroxidases (see Introduction) EPR studies have shown that ZFS and redox potentials are tunable through mutation of the residue involved in hydrogen bonding with the proximal histidine ligand [25]. The pH-dependence of the ZFS seen here correlates with the pH-dependence of the rate of ES formation in DHP observed previously and suggests that in this enzyme the electron-donating power of the iron ion is more sensitive to its second coordination sphere than in other peroxidases. This property of the heme iron has been proposed to be a determinant of the peroxygenase function [22]. Thus, the pH-dependence of ZFS can be explained by changes in the H-bonding of the proximal ligand *and* the distal ligation. Tuning of the ZFS corresponds to tuning of the ligand field strength, which in turn controls the iron ion's donating power, responsible in part for the multifunctionality of DHP.

The oxyferrous (heme-O_2 complex) and ferrous states of DHP. Aerobic incubation of DHP with ascorbate generated the oxy-haemoglobin analog of DHP in variable yields. When ascorbate and oxygen were present but not in a great excess, the incomplete reduction enabled observation of three species (Fig. S-2): i) the unreacted ferric form accounting for 23% of the total iron signal, ii) a new MB spectral component accounting for 40% of the total iron with $\delta = 0.83$ mm/s that is indicative of a high-spin Fe^{II}, which we assign as the ferrous state (i.e., deoxy); and iii) a second new MB spectral component accounting for 37% of the total iron, with an isomer shift of 0.21 mm/s (compared with 0.42 mm/s for ferric DHP) and a relatively large quadrupole splitting of 2.20 mm/s, which we assign as the oxyferrous form of DHP (i.e., Fe^{II}-O_2). To further validate this assignment, the reduction was performed in the presence of abundant O_2, yielding a 100% conversion of the ferric DHP to the of the oxy-DHP form, as demonstrated by the isomer shift of 0.28 mm/s and the quadrupole splitting of 2.26 mm/s (Fig. 4). To ascertain the identity of the oxygenated DHP, we collected spectra in high applied magnetic fields. The spectrum in Fig. 4B exhibits splittings that are attributable solely to the nuclear Zeeman interaction, which is conclusive evidence for a species with a diamagnetic (S = 0) electronic ground state [41], with parameters similar to those of oxy-myoglobin. The high-field spectrum enabled us to determine the sign of the quadrupole splitting, which in this case is negative, as also observed in the literature for the O_2-complexes of HRP, cytochrome P-450, and myoglobin [10]. For comparison the data for oxy-myoglobin and for the O_2-complex of HRP are given in Table S-2 [10,12].

The Fe^{IV}=O species of DHP. Samples of ferric DHP (pH 7) used for the above studies were treated with excess hydrogen peroxide at room temperature and rapidly frozen in liquid nitrogen within 12 s to generate the high valent Fe^{IV}=O (ferryl) oxidation state of DHP. The MB spectra at 4 K and 179 K (Figs. 5 and 6A) show that 78% of the ferric dehaloperoxidase spectrum was converted principally into a new doublet with

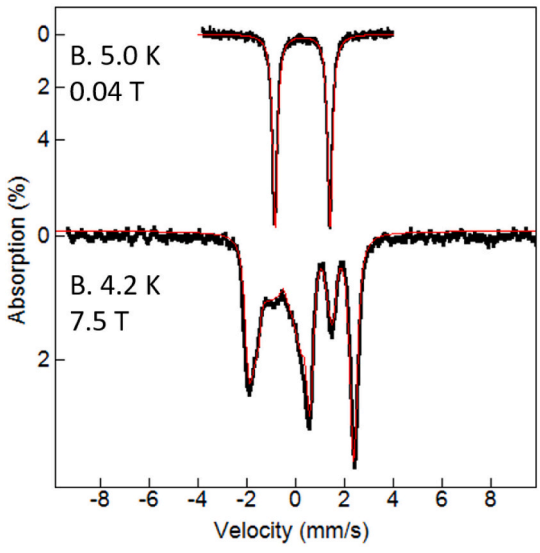

Fig. 4. Mössbauer spectra of ^{57}Fe-DHP in the oxyferrous state (black line) and spectral fits (red line) with the parameters in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

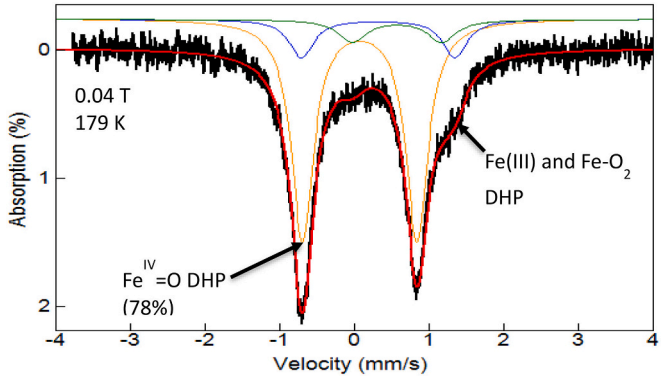

Fig. 5. High-temperature (179 K), 0.04 T-spectrum of the Fe^{IV}═O state of DHP obtained by reacting a 1.3 mM ferric DHP sample with H_2O_2. The data are shown in black, and the solid colored lines are spectral simulations with the parameters in Table 2: yellow, Fe(IV)-oxo; blue, Fe(II)-O_2 and green; Fe(III). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

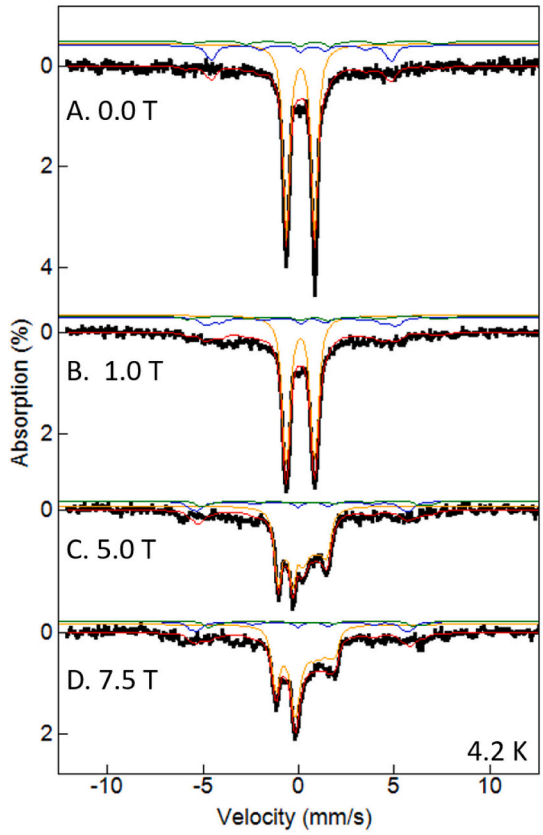

Fig. 6. Variable-field spectra of the Fe^{IV}-oxo state of DHP obtained at 4.2 K. (A) 0.0 T, (B) 1.0 T, (C) 5.0 and (D) 7.5 T magnetic fields applied parallel with respect to the observed gamma radiation. The data are shown in black, and the solid colored lines are spectral simulations with the parameters in Table 2: yellow, Fe(IV)-oxo; blue, Fe(II)-O_2 and green Fe(III). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

an isomer shift at 0.10(2) mm/s, characteristic for Fe(IV)-oxo sites in heme proteins [41]. In addition, the spectrum of H_2O_2-treated DHP also exhibits a shoulder at higher velocity, comprised of two other species: i) a small doublet distinguished by its large quadrupole splitting of ΔE_Q = 2.28 mm/s and an isomer shift of 0.27 mm/s, a value suggesting the presence of a low-spin species similar to the Fe—O_2 complex of DHP, and ii) a doublet with an isomer shift of 0.43 mm/s attributable to unreacted ferric DHP. Regarding this latter species, we cannot rule out that it also represents the H_2O_2-inactivated form of DHP, termed Compound RH. Compound RH is a ferric complex which was proposed to form by reaction of DHP with H_2O_2 in absence of substrates, in which the ferric heme crosslinks with Tyr 34, thus prevents the enzyme from returning to an active form, such as the oxyferrous or the ferric resting state, to begin a new catalytic cycle [20,24,47].

The presence of an Fe^{IV}-oxo species was further confirmed by probing the electronic ground state in variable large magnetic fields between 1.0 T and 7.5 T (Fig. 6). The spectra in high-field exhibited a majority species with hyperfine splittings characteristic for species with an S = 1 electronic ground state and isomer shift corresponding to the majority species observed in the low-field spectra. By simultaneously fitting the high-field data set we determined that this S = 1 species has an axial zero-field splitting of 21 cm^{-1} and an axial magnetic hyperfine coupling tensor with $A_{xx,yy} = -19.5$ T and $A_{zz} = -1.0$ T (Table 2). From the dependence of the spectral splittings on magnetic field we can conclude that there are no spin couplings affecting this S = 1 species. The good fits to the data also exclude presence of multiple species, which contrasts with the spectra of the ferric DHP at pH 7, in which we observed two species with distinct ZFS. The characteristics of this species are comparable with published parameters for the Fe(IV)-oxo compound HRP I observed by Schulz et al. [10] and a corresponding form of myoglobin [12] shown in Table S-3 in the Electronic Supplementary Information. The isomer shift and the S = 1 ground state provide compelling evidence that this represents the catalytically-active Fe^{IV}═O species invoked in the peroxidase and peroxygenase cycles of DHP.

4. Conclusion

In this study we presented the Mössbauer spectra of *A. ornata* dehaloperoxidase in its ferrous (deoxy), oxyferrous (Fe—O_2), ferric, and Fe(IV)-oxo forms. MB parameters for hemes are clustered within narrow ranges for each oxidation and spin state. For example, the six-line pattern in low-filed and the 5 K – isomer shift of 0.42 mm/s and quadrupole splitting of 1.27 mm/s are reliable indicators of the high-spin Fe (III) state for DHP, as seen in haemoglobins, myoglobin and heme enzymes [10,11,41,45]. (Tables 1 and S-2, S-3).

Our MB data show that the electronic structure of the ferric heme in DHP is very sensitive to pH. Changes in the splittings and line-shape of the spectra in large applied magnetic fields can be attributed to changes in the zero-field splitting, D. The presence of species with different D-values is reasonable considering the dependence of D on the strength of the ligand field at the iron ion. The pH change from pH 5 to 7 is documented to cause a shift in H-bonding around the heme, including the protonation equilibrium of the coordinated H_2O. Spectroscopic studies in the literature discussed here also show that hydrogen bond shifts can change the Fe-N_{His} bond length, which directly affect the electron-donating properties of the N-atom of the proximal His residue. Thus, the pH-dependence of the ZFS, while it is subtle and only observable at high applied magnetic fields, shows that the electronic structure of DHP is different from other peroxidases. As discussed in this study, pH effects on ZFS are traceable to modulation of the ligand field strength, which in turn has been invoked as a valid explanation for DHP's peroxygenase function on haloindoles [22].

Accessible from the Fe(III) enzyme at pH 7 are the oxyferrous (haemoglobin-like) and ferryl (peroxidase-like reactive intermediate) states. Both of these states were obtained in high yield, which allowed us to obtain the electronic parameters that converge with the respective states of myoglobin [12], horseradish peroxidase [10] and cytochrome *c* peroxidase [28]. Thus, apart from the pH-dependent heterogeneity in the electronic parameters observed in the Fe(III) state, the other three states studied here do not show major between DHP and myoglobin and peroxidases and other heme enzymes.

In conclusion, our MB investigation demonstrates that although DHP

shows no sequence similarity with haemoglobins and peroxidases and–unlike them–is multifunctional, has a heme site that shares some spectroscopic similarity (isomer shift, quadrupole splitting) to both, which was expected, given that isomer shifts and quadrupole splittings for heme proteins do not show and observable correlation with their functions. However, the zero-field splittings of DHP are finely tuned by pH, likely through electronic tuning of the ligand field at the iron ion, through hydrogen bonding. The flexible hydrogen bonding in the active site cofactor in DHP, is also known to be a factor in the accessibility of substrates: sterically accessible substrates are oxidized via a peroxygenase mechanism, sterically hindered substrates proceed via a peroxidase pathway (as O-atom transfer is blocked), and activated substrates (e.g., dimethylindole) reduce the heme leading to a radical-based oxygenase activity. Our MB data suggest that the multifunctionality of DHP is ultimately due in part to the intrinsic electronic structure of the heme iron and its direct surroundings, which govern the Fe^{3+} electron-donating power and which are more sensitive to pH than other heme proteins. Future theoretical studies may help bring a firmer footing for the causal relation between the subtle tuning of the iron's electronic structure and the multiple functions of DHP.

Author contribution statement

C. V. Popescu, conceptualization, methodology, data curation, formal analysis, project administration. C. V. Popescu and R. A. Ghiladi, funding acquisition, supervision, validation, writing, editing. Thanh-minh Dinh, Anastasia Washburn investigation, writing; Hongli Chen, Danielle Miller investigation, analysis. Ashlyn McGuire, Rania Dumarieh, and Jennifer D'Antonio investigation, methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2022.111867.

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