Different Modes of Binding of Mono-, Di-, and Trihalogenated Phenols to the Hemoglobin Dehaloperoxidase from *Amphitrite ornata*†

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Received August 20, 2008; Revised Manuscript Received January 6, 2009

**ABSTRACT:** The hemoglobin dehaloperoxidase (DHP), found in the coelom of the terebellid polychaete *Amphitrite ornata*, is a dual-function protein that has the characteristics of both hemoglobins and peroxidases. In addition to oxygen transport function, DHP readily oxidizes halogenated phenols in the presence of hydrogen peroxide. The peroxidase activity of DHP is high relative to that of wild-type myoglobin or hemoglobin, but the most definitive difference in DHP is a well-defined substrate-binding site in the distal pocket, which was reported for 4-iodophenol in the X-ray crystal structure of DHP. The binding of 2,4,6-trihalogenated phenols is relevant since 2,4,6-tribromophenol is considered to be the most soluble trihalogenated phenol, 2,4,6-trifluorophenol, acts as a highly soluble structural analogue to the native substrate 2,4,6-tribromophenol. To improve our understanding of substrate binding, we compared the most soluble substrate analogues, 4-bromophenol, 2,4-dichlorophenol, and 2,4,6-trifluorophenol, using 1H and 19F NMR to probe substrate binding interactions in the active site of the low-spin metmyoglobin adduct of DHP. Both mono- and dihalogenated phenols induced changes in resonances of the heme prosthetic group and an internal heme edge side chain, while 1H NMR, 19F NMR, and relaxation data for a 2,4,6-trihalogenated substrate indicate a mode of binding on the exterior of DHP. The differences in binding are correlated with differences in enzymatic activity for the substrates studied.

The terebellid polychaete *Amphitrite ornata* inhabits estuarine mudflats with other marine annelids, such as *Notomastus lobatus*, *Saccoglossus kowalevskii*, and *Thelepus crispus*, which secrete brominated aromatic compounds as a means of territorial protection (1−3). While such repellents would be deterrents for some organisms, *A. ornata* has developed a novel defense mechanism in the hemoglobin dehaloperoxidase (DHP).† DHP is found in the coelom of *A. ornata* and is one of two hemoglobins in the organism (4). Structurally, the DHP monomer is homologous to myoglobin containing the globin fold with eight helices and a heme prosthetic group ligated to the protein backbone via a proximal histidine (5, 6). The novelty of DHP lies in its ability to oxidatively dehalogenate haloaromatics found in its environment while simultaneously maintaining an oxygen storage function consistent with its hemoglobin structure (7−9).

†This project was supported by Army Research Office Grant 52278-LS.

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†Abbreviations: Ap, ampicillin; 4-BP, 4-bromophenol; 2,4-DCP, 2,4-dichlorophenol; DHP, dehaloperoxidase; DHPCN, cyanide-ligated dehaloperoxidase; HRP, horseradish peroxidase; IPTG, isopropyl β-d-thiogalactopyranoside; 2,4,6-TBP, 2,4,6-tribromophenol; 2,4,6-TCP, 2,4,6-trichlorophenol; 2,4,6-TFP, 2,4,6-trifluorophenol.

FIGURE 1: X-ray crystal structures of the local heme environment and substrate binding residues of DHP shown with (light blue) and without (green) bound substrate 4-iodophenol. Once the substrate binds, the 4-vinyl heme substituent shows slight changes in orientation. More predominant changes are observed in the orientations of His55 and Phe21. DHP has the highest turnover rate for 2,4,6-trihalogenated phenols, which have been shown to be extremely toxic to marine life (10, 11). The first X-ray crystal structure of substrate-bound DHP revealed that 4-iodophenol binds in the distal pocket but is not coordinated to the heme iron (Figure 1) (5, 6). This internal binding site distinguishes DHP not only from other...
bind substrates on the heme periphery (12). According to this X-ray structure (Protein Data Bank entry 1EWA), the average occupancy of the substrate analogue 4-iodophenol in the internal binding site is only $\sim 23\%$ (6). Examination of the substrate-bound DHP crystal structure shows primary structural changes occurring solely at the heme and surrounding residues when substrate is bound. We have also confirmed by X-ray crystallography that para-halogenated (4-bromo- and 4-iodophenol) phenols can occupy this internal binding site (V. de Serrano and S. Franzen, unpublished data). However, 2,4-di- and 2,4,6-trichlorophenols do not readily enter the distal pocket in the crystal form. This is ironic considering the high turnover rate of 2,4,6-trihalophenols, which are the most active substrates for DHP. 2,4,6-Trifluorophenol (2,4,6-TFP) has been shown to enter the distal pocket of the metquo and carbonmonoxy forms of DHP at low temperatures. We have recently shown that 2,4,6-TFP can displace the coordinated water molecule of the metquo form at low temperatures (13). However, it is apparently expelled from the distal pocket in the carbonmonoxy form at room temperature (14). The interaction of diatomic ligands and hydrogen peroxide, in the internal binding site is highly relevant to the function of DHP. The competition for binding in the distal pocket may involve the site occupied by 4-iodophenol in the 1EWA crystal structure.

We have used the carbonmonoxy form of DHP (DHPCO) as a model for the interaction of halogenated phenols and a heme-coordinated diatomic ligand in the distal pocket. 2,4,6-TFP has been observed to enter the distal pocket of the DHPCO at low temperatures at pH 5.5, but not at room temperature (14). We have recently shown that 2,4,6-TFP also binds to the DHPCO form at room temperature at pH 4.7 (50). The functional relevance of this binding is still not clear. Since trihalogenated substrates, such as 2,4,6-tribromophenol (2,4,6-TBP), exhibit the highest turnover rates, it is key from a functional perspective to determine whether oxidation of the substrate can occur in the internal site. The fact that the native substrate, 2,4,6-TBP, and other trihalophenols do not bind in that site under physiological conditions at room temperature and pH 7.4 presents a conundrum for DHP function. These data suggest that the internal binding site is not the active site. Therefore, the binding site for the native substrate, 2,4,6-TBP, has not yet been determined. The well-defined internal binding site for monohalogenated phenols, such as 4-bromophenol, may interfere with the binding of oxygen or hydrogen peroxide and could regulate either the hemoglobin or peroxidase function of DHP.

The X-ray crystal structure of DHP shows that the entire 4-iodophenol molecule binds within 6 Å of the iron center (6). The proximity of the internal molecular binding site to the heme permits the application of paramagnetic NMR experiments in elucidating specific structural changes near the heme iron when molecules bind in the internal binding pocket (15). NMR has been used extensively for active site characterization of high-spin ($S = \frac{5}{2}$) and low-spin ($S = \frac{1}{2}$) forms of many peroxidases and globins (16–23) and provides a sensitive method for probing structural details of the heme prosthetic group. Addition of a strong-field ligand, e.g., cyanide, to the ferric Fe(III) oxidation state of DHP creates a low-spin ($S = \frac{1}{2}$), paramagnetic species. This metcyano form is the focus of the current NMR study, as active site resonances are much sharper and less dispersed in the low-spin form than the high-spin, metquo counterpart.

Herein, we report the first assignment and active site characterization of DHP using NMR spectroscopy and present evidence that there is both an internal and an external binding site for halogenated phenols. Assignment of the resonances was accomplished primarily through natural abundance $^{13}$C–$^{1}$H HSQC (24) and WEFT-NOESY (25) experiments. Differential perturbation of certain active site resonances was observed upon titration of three different halogenated phenols chosen for their high water solubility: 4-bromophenol (4-BP), 2,4-dichlorophenol (2,4-DCP), and 2,4,6-trifluorophenol (2,4,6-TFP). The effects of substrate binding were also compared over a wide pH range.

**MATERIALS AND METHODS**

**Protein Preparation.** The pET 16b plasmid containing the 6XHisDHP4R DNA insert (7) was transformed into competent BL21(DE3) *Escherichia coli* cells, plated out on LB agar plates with 100 μg/mL ampicillin (Ap), and allowed to
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grow at 37 °C for ~14 h. Single colonies were isolated, and starter growths were used to inoculate 6 L E. coli growths.

The cells were incubated at 37 °C with shaking for 13 h. Expression of 6XHisDHP4R protein was not induced via addition of IPTG. Basal expression of the protein in the nonstringent BL21(DE3) cell line yielded significantly high levels of holoprotein. The cells were collected via centrifugation and then allowed to freeze overnight at ~20 °C. The cells were resuspended in lysis buffer (2 mL/g of cell pellet) [50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole (pH 6)], and lysozyme was added to a final concentration of 1 mg/mL. The cell slurry was allowed to stir at 4 °C for 1 h. The slurry was then sonicated for 30 min, and 200 μL of DNase I (16 mg/mL) and RNase A (10 mg/mL) were added. The cell slurry was again stirred at 4 °C for ~1 h before being frozen overnight at ~20 °C. After rethawing, the cells were centrifuged at 18000 rpm for 45 min, and supernatant His-tagged DHP protein was collected. The crude His-DHP was applied to a Ni-NTA agarose column (Qiagen), and was eluted stepwise between pH 6 with 20 mM KH2PO4 and pH 6 with 150 mM KH2PO4. The concentration of the protein was determined using the Soret band at 406 nm with a molar extinction coefficient of 27000 Hz. Best results were obtained with a mixing time of 100 ms and a delay time of 1.2 s. Gradient-selective COSY spectra were also collected over a spectral width of 27000 Hz. A total of 2048 T2 points and 512 t1 blocks were collected with a delay time of 1.2 s.

UV–Vis Enzymatic Assays. For all experiments, the protein was exchanged into 100 mM potassium phosphate buffer (pH 7). The absorption data were collected using a Hewlett-Packard 8453 multiwavelength spectrometer. The spectra were collected every 5 s over a 60 s time frame. The conditions used for the assays were 5 mM substrate. Substrate turnover was monitored by the disappearance of substrate absorption bands (4-BP, 280 nm; 2,4-DCP, 284 nm; 2,4,6-TFP, 272 nm).

RESULTS

The low-spin metmycyno form of DHP at pH 7.0 and 25 °C exhibits a wide dispersion of hyperfine-shifted heme and active site resonances, from ~12 to 27 ppm as seen in Figure 2 and reported in Table 1.13C–1H HSQC, WEFT-NOESY, gradient-selective COSY, 1D NOE difference, and presaturation NOESY spectra were used to assign the majority of active site resonances. The spectra of resonances pertinent to the substrate binding study are presented.

Table 1. 1H and 13C NMR Assignments and T1 Measurements for Selected Resonances of DHPCN at 25 °C and pH 7.0

<table>
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<th>H δ (ppm)</th>
<th>13C δ (ppm)</th>
<th>T1 (ms)</th>
<th>R2(t) (Å)</th>
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*Calculated using R2(t)/R2(t) = (T1/T1)1/2. Measurements taken from the X-ray structure (5, 6). * Not applicable due to contact shift contribution. d Not accurate measurements due to overlapping resonances.

Hz. A total of 2048 T2 points and 512 t1 blocks were collected with a delay time of 1.2 s.
decrease in the contact shift contribution (28). While the 4β protons are not observed in the 13C−1H HSQC spectra (Figure 4), the 4α resonance was readily assigned at 14 (1H) and 52.8 ppm (13C). The Phe97 side chain was assigned by the natural abundance 13C chemical shifts, CζH at 134.5, CεH at 132.6, and CεH at 132.2 ppm, and scalar coupling between the ring protons (Supporting Information).

The 4αH resonance also exhibits an NOE cross-peak to the 5-CH3 heme methyl at 4.3 ppm. The 4.3 ppm resonance is assigned as the 5-CH3 resonance due to the degree of hyperfine shifting in the 13C dimension (−14.8 ppm) and NOEs to a four-proton spin system indicative of the 6-propionate chain. This four-proton spin system at 11.3, 9.4, 1.2, and 0.3 ppm exhibits mutual dipolar coupling with the 5-CH3 heme methyl. Only the two 6βH resonances of the propionate chain [1.2 and 0.3 ppm (84.4, 13C)] were found in the 13C−1H HSQC map. NOE cross-peaks of the two 6αH resonances (11.3 and 9.4 ppm) are observed at higher contour levels. Additional NOE connectivity between the 6-propionate resonances can be found in the Supporting Information.

The remaining downfield heme methyl at 26.8 ppm is assigned as the 8-CH3 resonance on the basis of NOE connectivity to two hyperfine-shifted methylene groups at 12.5 and 10.2 ppm (−32.7, 13C) and 0.4 and −0.2 ppm (118.8, 13C) indicative of the 7-propionate chain. Inspection of the COSY data (Supporting Information) shows both methylene groups are scalar coupled, thus confirming their assignment as the 7αH and 7βH resonances, respectively.

The order of heme methyl shifts in metcyan DHP is 8 > 3 > 5 with the 1-CH3 methyl not yet assigned. The 1-CH3 methyl in low-spin metcyan heme proteins, at ambient temperatures, typically occurs at frequencies lower than those of the 5-CH3 heme methyl. The relative order of heme methyl shifts is correlated to the angle, Φ, of the proximal histidine projection onto the N2-Fe-N4 axis of the heme plane (23, 29–33). According to the X-ray structure of DHP, the axial histidine Φ is ~113° as seen in Scheme 1A (5, 6). Models and experimental data have shown the 113° angle corresponds...
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Effects of Addition of Halogenated Phenols to DHPCN.

The effects of three different substrates on the active site of DHPCN can be seen in Figure 5. The molecules 4-BP, 2,4-DCP, and 2,4,6-TPF were chosen for their high water solubility and/or structural similarities to substrates that have high rates of product turnover in DHP. The native substrate 2,4,6-TBP and commonly used model substrate, 2,4,6-trichlorophenol (2,4,6-DCP) cannot be used in the NMR because of their poor solubility. However, 4-BP is structurally comparable to 4-iodophenol, which was shown to bind in the internal distal cavity of DHP in current X-ray structures (6), while 2,4,6-TPF provides a comparison to the native substrate, 2,4,6-TBP, and the most active laboratory substrate, 2,4,6-TCP. Figure 5 shows there are two different effects induced by substrate binding. The mono- and dihalogenated 4-BP and 2,4-DCP cause similar effects within the active site of DHPCN. In both cases, the 3-CH₃ heme methyl and internal Phe97 exhibit significant line broadening when substrate is added in excess. The Phe97 side chain and heme 3-CH₃ methyl are separated by ~4.5 Å according to the X-ray structures (5, 6), with the Phe side chain located slightly proximal to the 3-CH₃ heme methyl. Introduction of either 4-BP or 2,4-DCP also causes a slight change in the chemical shift of the 7α propionate resonances. The 8-CH₃ resonance exhibits a slight ~0.2 ppm shift in the presence of 4-BP and a 0.1 ppm shift in the presence of 2,4-DCP. On the other hand, the interaction of 2,4,6-TPF with DHP does not create frequency perturbations and/or line broadening of active site resonances in DHPCN at the pH values studied. Comparing the effects of substrate titrations provides initial evidence for different modes of binding for substrates 2,4-DCP and 4-BP compared to 2,4,6-TPF. The differences in the nature of binding interactions are observed even when a significant molar excess of 2,4,6-TPF is used in DHPCN solutions (Supporting Information).

19F NMR and Relaxation Data for Substrate 2,4,6-TPF in DHPCN. Utilization of 19F NMR permits direct observation of the substrate as a probe of binding. In general, any binding interactions between the smaller 2,4,6-TPF substrate and DHP will result in a decrease in T₂ and, therefore, an increase in line width due to a slower molecular tumbling rate. Alternatively, depending on the rate of exchange, separate resonances may be visible for the fluorinated substrate, one for both the bound and free state of the substrate (36, 37).

Figure 6 shows the 19F NMR spectra of substrate 2,4,6-TPF and titration of 2,4,6-TPF to the fully formed DHPCN complex. A significant change in chemical shift is observed in the para (F4) resonance as it is titrated to DHP. The F4 resonance of 2,4,6-TPF, at -123.2 ppm, shifts to -124.4 ppm when the substrate is introduced at a 1:1 ratio. Additionally, the ortho (F2/F6) resonance shifts from -131.2 to -132.1 ppm. During the course of the titration, the F4 and F2/F6 resonances shift to slightly higher frequencies and approach the chemical shifts of 2,4,6-TPF without the presence of protein. Slight broadening of both resonances is observed at 1:1 and 3:1 ratios of 2,4,6-TPF to DHPCN. The F4 resonance...
FIGURE 7: (A) $T_1$ relaxation curves and (B) $T_2$ relaxation curves for the meta protons of 2,4,6-trifluorophenol, alone (black), and in the presence of DHPCN (red) at a 15:1 molar ratio.

The dehalogenation of a variety of halogenated phenols, including bromo-, chloro-, and even fluorophenols, by DHP was first reported in 1996 (2). It was subsequently revealed that the original turnover numbers are not accurate (8). The X-ray crystal structure revealed the binding of 4-iodophenol in the distal pocket, which was assigned as the active site (3). The native substrate, however, is considered to be 2,4,6-TBP, and there has been no report of dehalogenation of 4-halophenols by DHP. This study uses soluble halogenated phenols to systematically test the differences in binding and correlated activity of three types of halogenated phenols. The molecules, 4-BP and 2,4-DCP, appear to bind or at least interact at an interior site in DHPCN and are poor substrates in a typical ferric DHP peroxidase assay using H$_2$O$_2$. On the other hand, 2,4,6-TFP interacts with the protein at an exterior site and has activity comparable to that of the native substrate, 2,4,6-TBP. In retrospect, this result is not surprising, since most known peroxidases have exterior substrate binding sites (12).

Binding of the monohalogenated (4-BP) and dihalogenated (2,4-DCP) substrates demonstrated comparable effects on the $^1$H NMR spectrum of the metcyano adduct of DHP. The effects include perturbations along the internal heme edge near the 3-CH$_3$ heme methyl and Phe97 side chain. The broadening of these resonances appears to be greatest when the solution pH < pK$_a$ of the substrate (Supporting Information). However, even at alkaline pH (pH 9.9), there is still observable broadening associated with the addition of 4-BP and 2,4-DCP. The effect of 4-BP and 2,4-DCP binding is localized to an internal region near the heme 3-CH$_3$ methyl and Phe97 residue. There is no broadening of other active site resonances, such as the 8-CH$_3$ methyl. Broadening of specific resonances of the heme and its substituents has been observed previously in substrate binding studies of horseradish peroxidase (HRP). Titration of the substrate benzyohdroxamic acid to HRPCN resulted in significant broadening of the 8-CH$_3$ heme methyl and a 7a-propionate resonance, in a manner similar to the observed broadening of the 3-CH$_3$ and Phe97 resonances in DHPCN (39–41). Substrate binding is known to be external to the distal pocket in HRP. However, the 3-CH$_3$ and Phe97 resonances are more deeply buried than the 8-CH$_3$ heme methyl and 7a protons in DHP. Consequently, the binding interactions between 4-BP/2,4-DCP and DHPCN appear to occur at the internal site in the distal pocket. On the other hand, WEFT-NOESY and presaturation NOESY experiments did not reveal NOEs between DHPCN and the substrate analogues, 4-BP and 2,4-DCP. The lack of NOEs may be due to fast exchange of the substrate between bound and free states. These experiments suggest that the binding of 4-BP and 2,4-DCP in the distal pocket of DHPCN may involve rapid exchange with solvent.

While the addition of 4-BP and 2,4-DCP induced observable changes in active site resonances, 2,4,6-TEP essentially had no effect on the $^1$H NMR spectrum. The $^{19}$F NMR data, however, show that the F2/F6 and F4 fluorine resonances of 2,4,6-TEP exhibit slight broadening and perturbations in chemical shifts when present at a 1:1 molar ratio with DHPCN. Resonance broadening and/or changes in chemical shift are commonly used to identify binding of small molecule protein ligands (42–44). The line width of the F2/F6 and F4 resonances at a 1:1 molar ratio of DHPCN to 2,4,6-TEP increases by 33 and 17%, respectively. This broadening translates to a decrease in the apparent transverse...
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relaxation time \( T_2 \) from 17.7 to 13.3 ms for F2/F6 and from 13.3 to 11.4 ms for F4 (45) as discussed in the Supporting Information. The decrease in \( T_2 \) suggests a longer rotational correlation time \( (\tau_r) \) of the molecule (46). On the basis of the Stokes–Einstein equation, \( \tau_r \approx 34 \) ns for DHPCN assuming that the radius \( r = 20 \) Å at a viscosity of 1 cP, whereas free 2,4,6-TFP would be expected to have a \( \tau_r \) on the picosecond time scale (see the Supporting Information).

To elucidate the binding of 2,4,6-TFP to DHPCN, \( T_1 \) and \( T_2 \) relaxation experiments were used to observe changes in the rotational correlation time, \( \tau_r \). The \( T_1 \) relaxation time of the 2,4,6-TFP meta protons decreased from 8.1 to 5.8 s and the \( T_2 \) relaxation time of these same resonances from 6.4 to 1.5 s when 2,4,6-TFP was present at a 15:1 molar ratio (Figure 7). The high ratio of TFP to DHPCN was needed to fully resolve the 2,4,6-TFP signal over the background of the protein resonances. Because the same value for \( T_1 \) is consistent with two separate rotational correlation times in the limits \( \omega_0 \tau_r \approx 1 \) and \( \omega_0 \tau_r \gg 1 \), \( T_2 \) values were analyzed to determine whether the decrease in \( T_1 \) can be attributed to a significant decrease in the \( \tau_r \) of 2,4,6-TFP in the presence of DHPCN (47). The analysis of the decrease in \( T_1 \) and \( T_2 \) times corresponds to a \( \tau_r \) of \( \approx 35 \) ps for free 2,4,6-TFP and a \( \tau_r \) of \( \approx 53 \) ns for 2,4,6-TFP in the presence of DHPCN. This increase in \( \tau_r \) is in accord with the expected value for complexation of 2,4,6-TFP to DHPCN. Further details of the analysis are supplied in the Supporting Information. Although the analysis is based on a single dipolar interaction of an \(^{13}C^1\) nucleus that is 2.47 Å from the meta hydrogen nucleus, we recognize that the relaxation may be more complicated in the bound state. The point of the analysis is to establish the 2,4,6-TFP binds at an external site and exhibits behavior different from that of the other two phenols studied, 4-BP and 2,4-DCP.

Although the relaxation data are consistent with an external binding site for 2,4,6-TFP, the location of that site has not been determined on the basis of the data obtained here. FTIR studies at cryogenic temperatures showed that 2,4,6-TFP can bind in the internal substrate binding pocket of the CO form of ferrous DHP, which is isoelectric with DHPCN (14). However, at physiologically relevant pHs, there was no binding of 2,4,6-TFP above 260 K in the internal site (14). At room temperature, 2,4,6-TFP binding in the distal pocket is also observed in the CO form using FTIR, but only at pH < 4.7 (50). It is difficult to study DHPCN at pH < 5.0 to investigate the relevance of this observation using NMR due to the protonation of CN\(^-\) to form HCN. The \(^1\)H NMR data corroborate the FTIR data in that no observable internal pocket perturbations are observed for the trihalogenated substrate at ambient temperatures in the pH range from 5.5 to 9.9.

The origin of the alteration of the axial histidine torsion angle shown in Scheme 1 requires further explanation. The value of the torsion angle \( (\Phi) \) of \( \approx 113^\circ \) shown in Scheme 1A was obtained by X-ray crystallography. The X-ray structures further indicate stabilization of the proximal histidine H89 by a strong hydrogen bond of the imidazole NH group to the carbonyl oxygen of L83 with a N····O distance of 2.74 Å (5, 6). The \( \Phi \) value of \( \approx 90^\circ \) in solution (Scheme 1B) is based on the well-established correlation of the ordering of heme methyl hyperfine shifts. The discrepancy in these data may arise from the dynamics of H89 and associated amino acid residues. The average thermal factors for amino acid residues 87–92, a region which includes the proximal histidine H89, were observed to be 50% larger than the average value in DHP as a whole (5). The region near H89 has the largest thermal motion in the molecule despite the fact that it is buried in the interior of the protein and bonded to the heme cofactor. The flexibility of H89 could be the reason for the decrease in the axial histidine torsion angle \( (\Phi) \) from 113° to 90°, which was determined by the spacing and \( 8 > 3 > 5 > 1 \) order of heme methyl chemical shifts in the \(^1\)H NMR spectrum. Similar deviations in the proximal histidine plane orientation between X-ray and solution structures have been observed in mouse neuroglobin (38), where higher thermal factors in the region of the proximal histidine indicated greater flexibility.

CONCLUSION

This study has shown that substrates interact with the six-coordinate DHPCN form in two different ways. Binding of both 4-BP and 2,4-DCP affects internal amino acid residues and heme group substituents in a pH-dependent manner, with the greatest affinity occurring under acidic conditions. The NMR data suggest that the binding has a strong and specific effect on the heme, which may result from an internal binding site where the substrate analogue is in rapid exchange with solvent. The molecules 4-BP and 2,4-DCP are inactive and greatly reduced in activity when compared to 2,4,6-trihalogenated phenols (7, 48), including 2,4,6-TFP (Supporting Information and ref 49). Binding of 2,4,6-TFP, which is a structural homologue of the native substrate 2,4,6-TBP, did not induce effects on the internal amino acid residues, as indicated by \(^1\)H NMR. However, broadening and chemical shift perturbations of the fluorine resonances are consistent with an external binding interaction. In the presence of DHPCN, the rotational correlation time of 2,4,6-TFP was found to increase from the picosecond to nanosecond time scale, consistent with the hypothesis that there is an external binding site. Although 2,4,6-TBP is not sufficiently soluble to permit a \(^1\)H NMR study, the conclusion that 2,4,6-TFP binds at an external site clearly implies that the native substrate, 2,4,6-TBP, also binds at an external site. The similar reactivity of both of these substrates leads us to conclude that the active site for substrate oxidation in DHP is an external site, as observed in other heme peroxidases.

SUPPORTING INFORMATION AVAILABLE

1D NOE, gradient-selective COSY, presaturation NOESY data, variable-temperature \(^1\)H NMR spectra, and hyperfine-shifted resonances in response to varying pH; additional titrations of substrate 2,4,6-TFP at alternative pHs; equations used for analysis of relaxation data; and spectroscopic data for enzyme assays of 4-BP, 2,4-DCP, and 2,4,6-TFP. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


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BI801568S