Notomastus lobatus Chloroperoxidase and *Amphitrite ornata* Dehaloperoxidase Both Contain Histidine as Their Proximal Heme Iron Ligand†

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ABSTRACT: Two novel heme-containing peroxidases, one able to incorporate halogens into aromatic substrates and the other able to remove them, have recently been isolated from marine sources and initially characterized by Chen et al. [(1991) *J. Biol. Chem. 266,* 23909−23915; (1996) *J. Biol. Chem. 271,* 4609−4612]. The haloperoxidase *Notomastus lobatus* chloroperoxidase (NCPO) is unusual in requiring a flavoprotein component for peroxidase activity. The dehaloperoxidase (DHP), isolated from *Amphitrite ornata*, is the only heme-containing peroxide-dependent dehalogenase known to be capable of removing halogens including fluorine. Both enzymes are also quite atypical in that the molecular weights of their heme-containing subunits are less than 16 000, approximately one-half to one-fifth the size of typical heme-containing peroxidases. Interestingly, we have also found that both enzymes are isolated in their oxyferrous states even though all protein purification was done in the absence of any reductant. In the present study, we have examined these two enzymes with magnetic circular dichroism and UV−visible absorption spectroscopy in order to determine the identity of their proximal heme iron ligand. Four derivatives of each enzyme, cyanoferric, deoxyferrous, oxyferrous, and (carbonmonoxy)ferrous, have been examined and spectroscopically compared to parallel derivatives of myoglobin, a well-studied histidine-ligated heme protein. The spectra observed for each derivative of the two new enzymes are very similar to each other and, in turn, to the spectra of the same derivatives of myoglobin. We conclude that both new heme enzymes contain histidine as their proximal heme iron ligand. This makes NCPO the first histidine-ligated heme-containing peroxidase capable of chlorinating halogen acceptor substrates using chloride as the halogen donor. Further, the novel reactivity of DHP is not the result of an unusual proximal ligand. The present results with NCPO and DHP challenge the current dogma of how heme-containing peroxidases function: one chlorinates substrates without having a thiolate proximal ligand, and the other both oxygenates and dehalogenates haloaromatics and yet has a histidine proximal ligand like numerous other peroxidases that are not capable of such a combined reactivity.

High levels of halogenated, particularly brominated, aromatic compounds have been found in coastal marine sediments (Gribble, 1994; King, 1986; Woodin, 1991; Woodin et al., 1987). These compounds are produced as secondary metabolites by marine polychaetes and hemichordates through the action of haloperoxidases such as the unique chloroperoxidase system from the marine capitellid polychaete *Notomastus lobatus* (NCPO)[1] that requires both flavin *and* heme for activity (Chen et al., 1991). The toxicity of the haloaromatics is well known, and they significantly affect patterns of recruitment (Woodin et al., 1993) and also predation (S. A. Woodin, K. Fielman, C. Richmond, and D. E. Lincoln, unpublished results). Interestingly, organisms resistant to the toxic haloaromatics have also been found in haloaromatic-contaminated marine sediments. Both resistant halometabolite nonproducing organisms and halometabolite producing species contain dehalogenating enzymes which provide a mechanism for haloaromatic detoxification. Working with the marine terebellid polychaete *Amphitrite oronata*, a halometabolite nonproducer which frequently lives in close proximity to halometabolite producers, Chen et al. (1996) have also reported the purification and initial physical characterization of a very unusual heme-containing dehaloperoxidase (DHP). This enzyme removes halogen atoms from halophenols in a H_2O_2-dependent manner.

NCPO belongs to a subfamily of peroxidase enzymes that halogenate organic halogen acceptors with H_2O_2 and halide ions as cosubstrates (eq 1, haloperoxidases) (Dawson & Sono, 1987; Griffin, 1991; Neidleman & Geigert, 1986). The enzyme (M_r = 174 000) has two dissociable protein moieties, a flavoprotein (FAD-bound, M_r = 120 000) with four identical subunits and a heme protein (M_r = 54 000) with two copies each of two subunits (M_r = 15 500 and 11 500) (Chen et al., 1991). The complete system is required for haloperoxidase activity. Halide-independent catalase activity

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[1] Abbreviations: NCPO, chloroperoxidase from *Notomastus lobatus*; DHP, dehaloperoxidase from *Amphitrite oronata*; MCD, magnetic circular dichroism; CCPO, chloroperoxidase from *Caldariomyces fumago.*

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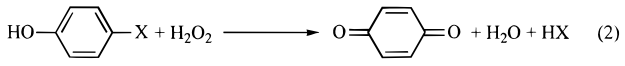
has been observed both for the heme protein and for the complete system. The NCPO heme protein is unusual in its need for the flavoprotein component for peroxidase activity and in the small subunit size (M_r = 11 500−15 500), one-half to one-fifth of the size of other known haloperoxidase enzymes (Marshall & Wright, 1996; Rush et al., 1995; Itoh et al., 1994, 1992; Krenn et al., 1989).

$$A{-}H + H_2O_2 + H^+ + X^- \rightarrow A{-}X + 2H_2O \quad (1)$$

Chloroperoxidase from *Caldariomyces fumago* (CCPO) is the best understood heme-containing haloperoxidase (Dawson & Sono, 1987; Dawson, 1988; Griffin, 1991). Its crystal structure has recently been reported by Poulos and coworkers (Sundaramoorthy et al., 1995). Like the heme-containing monooxygenase cytochrome P450 (Poulos et al., 1995), CCPO is unusual among heme proteins in having a thiolate proximal heme ligand. Unlike other histidine-ligated heme-containing haloperoxidases that are best at brominating or iodinating substrates [see, for example, Renganathan et al. (1987)], CCPO will also chlorinate substrates using chloride as the halogen source in the presence of H_2O_2 (Dawson & Sono, 1987; Dawson, 1988; Griffin, 1991; Hollenberg et al., 1974).

DHP (Chen et al., 1996) catalyzes the H_2O_2-dependent conversion of mono-, di-, or trihalophenols (X = Br, Cl, or F) into quinones. The halide removed is replaced by an oxygen atom, possibly from H_2O_2, with concomitant oxidation of the aromatic substrate to a quinone (eq 2, dehaloperoxidases). The H_2O_2-dependent defluorination reactions catalyzed by DHP are unprecedented. Lignin peroxidase is capable of oxidatively dechlorinating *p*-chlorophenols (Hammel et al., 1988) but has not been shown to be capable of defluorinating fluorophenols. The DHP dehalogenation reaction is distinct from known O_2-dependent and hydrolytic aromatic dehalogenation reactions where the halide is replaced by a hydroxyl group or from reductive aromatic dehalogenation where the halide is replaced by hydrogen. Almost all known dehalogenases are bacterial, do not contain heme, and function by a hydrolytic mechanism. In the best studied case, the dehalogenase from *Xanthobacter autotrophicus* proceeds via nucleophilic attack of an aspartate residue (Verschueren et al., 1993). DHP contains two identical subunits (M_r = 15 530), the small size of which is again unusual compared to known heme-containing peroxidases.



(2)

Horseradish peroxidase, the prototypical histidine-ligated peroxidase, catalyzes some oxygen transfer reactions (Kedderis et al., 1986; Ortiz de Montellano et al., 1987; Kobayashi et al., 1986, 1987). In general, however, the source of the incorporated oxygen atom is not H_2O_2; for this reason, the reaction is not thought to proceed through direct interaction between the substrate and the oxo-iron group. CCPO, on the other hand, has been shown to catalyze the incorporation of an oxygen atom from H_2O_2 into olefins and sulfides (Kedderis et al., 1986; McCarthy & White, 1983; Ortiz de Montellano et al., 1987; Kobayashi et al., 1986).

In an effort to characterize the heme iron coordination structures of the two novel heme-containing peroxidases, we have examined both enzymes with magnetic circular dichroism (MCD) spectroscopy in the UV−visible region. MCD spectroscopy is a powerful method for determining the identity of axial ligands in structurally uncharacterized heme proteins through comparison of their spectral properties with those of structurally defined heme proteins and model complexes (Dawson & Dooley, 1989). The method has been applied in this manner to study numerous heme proteins including cytochrome P450, CCPO, secondary amine monooxygenase, indoleamine dioxygenase, nitric oxide synthase, guanylyl cyclase, and heme oxygenase (Dawson & Dooley, 1989; Alberta et al., 1989; Sono et al., 1995; Burstyn et al., 1995; Hawkins et al., 1996). The results presented herein reveal that both NCPO and DHP contain histidine as their proximal ligand. As histidine is the most common axial ligand in heme proteins, the novel reactivities of these two peroxidase enzymes are clearly not the result of an atypical proximal ligand.

MATERIALS AND METHODS

Materials. DHP was purified from *Amphitrite oronata* according to the procedure of Chen et al. (1996). NCPO was purified from *Notomastus lobatus* by the method of Chen et al. (1991). Activity measurements on both enzymes carried out at various stages of purification were comparable to the values previously published by Chen et al. (1991, 1996). Carbon monoxide was obtained from Matheson Co. Potassium cyanide and potassium ferricyanide were purchased from Aldrich. Sodium dithionite was obtained from Sigma.

Preparation of Samples. All samples were handled at 277 K in concentrations of 40−50 μM in 100 mM potassium phosphate buffer, pH 5.0. Concentration determinations and identification of the heme as iron protoporphyrin IX were accomplished by the pyridine hemochrome method (Fuhrhop & Smith, 1975). DHP and NCPO are both isolated in the oxyferrous state despite the absence of reductants in buffers during the purification process. The deoxyferrous species of both enzymes was prepared in a cuvette sealed with a rubber septum by first exchanging the atmosphere in the cuvette with nitrogen followed by addition of solid sodium dithionite. The (carbonmonoxy)ferrous adduct of both enzymes could be prepared from either the oxyferrous or the deoxyferrous species simply by bubbling either solution with carbon monoxide. The ferric state of both enzymes was formed by a titration of the native oxyferrous species with microliter volumes of a solution of 12 mM potassium ferricyanide until no further spectral changes occurred in the UV−visible absorption spectrum. Attempts to generate the ferric species by addition of excess solid potassium ferricyanide followed by desalting on a P6DG (Bio-Rad) gel filtration column failed to completely oxidize both enzymes. The cyanoferric adduct of both enzymes was formed by addition of microliter volumes of excess potassium cyanide from a 1 M solution. (**Caution:** *Addition of acid to solutions containing cyanide generates poisonous HCN gas.*)

Spectroscopic Techniques. UV−visible absorption spectra were recorded with a Cary 210 spectrophotometer interfaced to an IBM PC. MCD spectra were measured at 277 K and 1.41 T with a JASCO J500-A spectropolarimeter equipped with a JASCO MCD-1B electromagnet and interfaced to a Gateway 2000 4DX2-66V PC through a JASCO IF-500-2

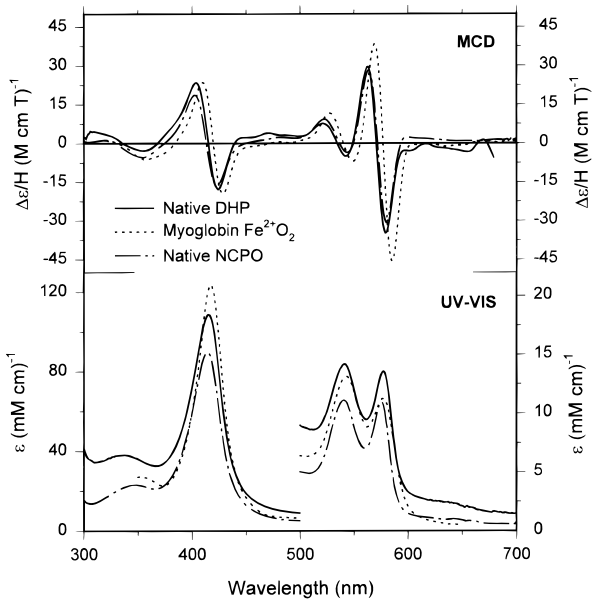


FIGURE 1: Magnetic circular dichroism (MCD) and UV−visible absorption (UV−VIS) spectra of native (as-isolated) DHP in 100 mM potassium phosphate buffer, pH 5.0 (—), native (as-isolated) NCPO in 100 mM potassium phosphate buffer, pH 5.0 (— - —), and oxyferrous myoglobin in 100 mM potassium phosphate buffer, pH 7.0 (---).

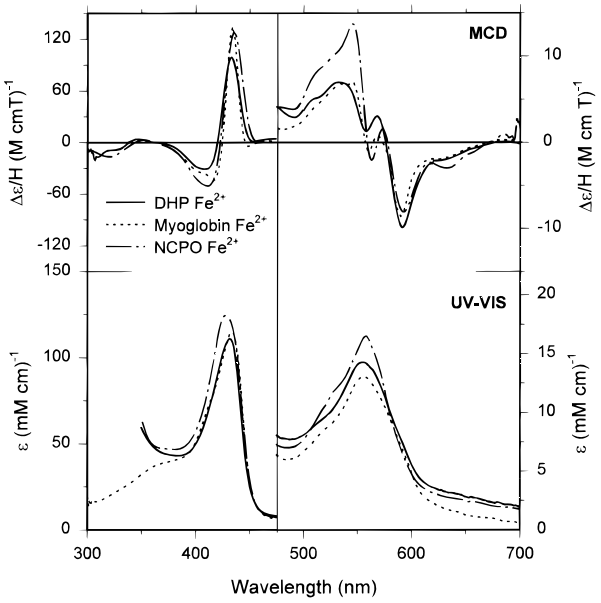


FIGURE 2: Magnetic circular dichroism (MCD) and UV−visible absorption (UV−VIS) spectra of deoxyferrous DHP in 100 mM potassium phosphate buffer, pH 5.0 (—), deoxyferrous NCPO in 100 mM potassium phosphate buffer, pH 5.0 (— - —), and deoxyferrous myoglobin in 100 mM potassium phosphate buffer, pH 7.0 (---).

interface unit. Data acquisition and manipulation has been described elsewhere (Huff et al., 1993). UV−visible absorption spectra were re-recorded following the MCD measurements to verify sample integrity. The spectra of NCPO and DHP presented here are overplotted with spectra of parallel derivatives of myoglobin. The myoglobin data have been presented elsewhere (Dawson et al., 1992) and are in good agreement with earlier published work (Vickery et al., 1976).

RESULTS

Pyridine hemochrome assays of both NCPO and DHP yielded UV−visible spectra with maxima at 418, 524, and 555 nm (data not shown). These values match those of pyridine hemochrome spectra of iron protoporphyrin IX (Fuhrhop & Smith, 1975) and indicate that NCPO and DHP both contain this most common iron porphyrin as do myoglobin and most heme-containing peroxidases.

In order to establish the identity of the proximal ligand to the heme iron in *Notomastus lobatus* chloroperoxidase (NCPO) and *Amphitrite ornata* dehaloperoxidase (DHP), we have examined both enzymes in their oxyferrous, deoxyferrous, (carbonmonoxy)ferrous, and cyanoferric states using magnetic circular dichroism (MCD) and UV−visible absorption spectroscopy (Figures 1−4). The spectra of NCPO and DHP have been obtained at pH 5.0 since this is the pH optimum for each enzyme. In each case, the data have been overplotted with the spectra of parallel derivatives of myoglobin, which has been well established to have a proximal histidine ligand (Takano, 1977). The respective MCD spectra of each of the four derivatives of NCPO and DHP (Figures 1−4) are quite similar to each other as well as to the parallel states of myoglobin. These four heme protein states have been chosen for the present study because in each case either the identity of the distal ligand is known [cyanoferric, oxyferrous, and (carbonmonoxy)ferrous] or there is no distal ligand (deoxyferrous). Thus, the only

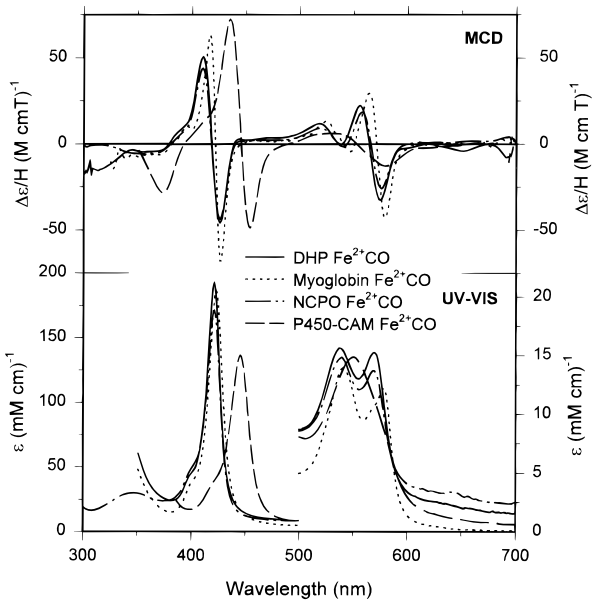


FIGURE 3: Magnetic circular dichroism (MCD) and UV−visible absorption (UV−VIS) spectra of (carbonmonoxy)ferrous DHP in 100 mM potassium phosphate buffer, pH 5.0 (—), (carbonmonoxy)ferrous NCPO in 100 mM potassium phosphate buffer, pH 5.0 (— - —), (carbonmonoxy)ferrous myoglobin in 100 mM potassium phosphate buffer, pH 7.0 (---), and (carbonmonoxy)ferrous cytochrome P450-CAM in 100 mM potassim phosphate buffer, pH 7.0 (— —), the latter taken from Sono et al. (1996).

unknown is the identity of the proximal ligand to the heme iron of NCPO and DHP. This same strategy has been used to establish the identity of the proximal ligand of secondary amine monooxygenase, for example (Alberta et al., 1989). These four derivatives also represent examples of low-spin ferric, high-spin ferrous, and low-spin ferrous states of both enzymes, three of the four commonly encountered spin states for ferric and ferrous heme proteins. The remaining spin state not examined, the high-spin ferric state, will be discussed below.

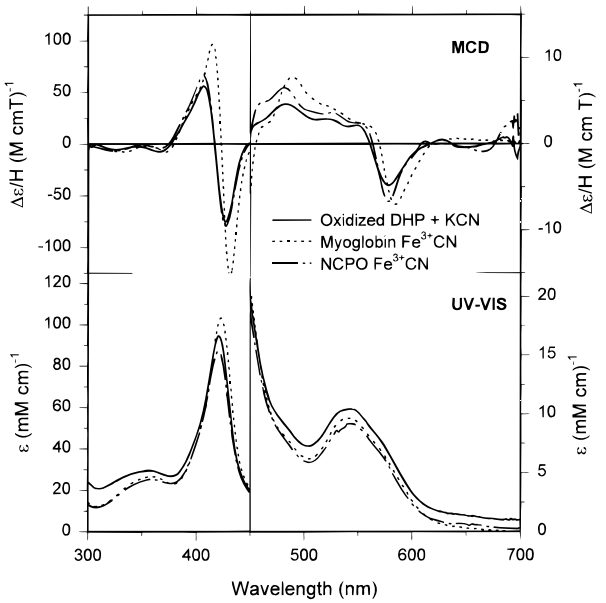


FIGURE 4: Magnetic circular dichroism (MCD) and UV−visible absorption (UV−VIS) spectra of cyanoferric DHP in 100 mM potassium phosphate buffer, pH 5.0 (—), cyanoferric NCPO in 100 mM potassium phosphate buffer, pH 5.0 (— - —), and cyanoferric myoglobin in 100 mM potassium phosphate buffer, pH 7.0 (---).

The spectra of NCPO and DHP in Figure 1 that are overplotted with those of oxyferrous myoglobin are obtained with the enzymes as-isolated. It therefore appears that both enzymes are actually isolated in their oxyferrous states, a highly unusual observation for heme-containing peroxidases in that such enzymes usually only function in their ferric and higher oxidation states. Furthermore, all protein purification was carried out without the presence of any reductant to generate the ferrous state. The possible involvement of the oxyferrous states of both enzymes in their catalytic activities will be the object of future mechanistic studies. Confirmation that each enzyme is isolated in its ferrous state was also obtained through the observation that it is possible to prepare the (carbonmonoxy)ferrous derivative by simply adding carbon monoxide to the as-isolated enzyme to generate the species whose spectra are displayed in Figure 3. As carbon monoxide only binds to ferrous heme proteins (Antonini & Brunori, 1971a), this observation provides direct evidence that NCPO and DHP are isolated in their ferrous states. MCD and UV−visible absorption spectra of the (carbonmonoxy)ferrous cytochrome P450-CAM are shown in Figure 3 to highlight the substantial spectral differences observed for a *thiolate-ligated* (carbonmonoxy)-ferrous heme complex relative to DHP, NCPO, and myoglobin.

The MCD spectrum of deoxyferrous NCPO exhibits some dissimilarities to those of deoxyferrous myoglobin and DHP in the visible region which are most likely attributable to a very small percentage (approximately 5%) of low-spin character induced by ligation of an endogenous ligand in that state of NCPO. This phenomenon has been observed to a greater extent in deoxyferrous secondary amine monooxygenase where 30% of low-spin content was estimated (Alberta et al., 1989; Sono et al., 1996).

All derivatives of DHP and NCPO, with the exception of the deoxyferrous state, exhibit MCD and UV−visible absorption spectra which are slightly blue-shifted relative to the corresponding derivatives of myoglobin. This is most likely due to subtle differences in the heme environments of DHP and NCPO relative to myoglobin.

Native oxyferrous NCPO and DHP can be oxidized by addition of potassium ferricyanide to yield high-spin ferric species whose MCD and UV-visible spectra bear some resemblance to those of high-spin aquo-ferric myoglobin (data not shown). However, the spectra are clearly the result of a mixture of high- and low-spin components, indicating that the extent of occupancy of the distal ligand binding site and identity of the distal ligand(s) are uncertain.

DISCUSSION

Establishing the heme iron coordination structure of new heme proteins is, at a minimum, a two-stage process. The first stage is to determine the type of heme prosthetic group that is present in the protein. This is generally done through use of the pyridine hemochrome assay (Fuhrhop & Smith, 1975). The second stage involves the use of spectroscopic methods to identify the axial ligands to the heme iron. In the case of NCPO and DHP, the pyridine hemochrome assay has shown that the heme prosthetic group is protoheme (iron protoporphyrin IX), the same heme group as is found in a majority of heme proteins and enzymes including myoglobin. MCD spectroscopy is a very powerful method for use in determining the axial ligands present in heme proteins in both ferric and ferrous oxidation states (Dawson & Dooley, 1989). The close similarity between the MCD spectra of the individual four derivatives of NCPO and DHP (Figures 1−4), first to each other and then to the appropriate parallel state of myoglobin, leads us to conclude that both enzymes have a histidine proximal ligand in both ferric and ferrous states. This conclusion is further supported by comparison of the UV−visible absorption spectra within each of the four sets of three protein derivatives (Figures 1−4). While it is not possible at this time to completely rule out all other possible proximal ligands (such as, for example, an alternative nitrogenous ligand as in lysine), the consistent similarity of the MCD *and* UV−visible absorption spectra of NCPO and DHP to myoglobin in four different derivatives covering three different oxidation/spin state combinations argues strongly for histidine as the proximal ligand in the two new enzymes.

While the MCD spectra of the cyanoferric adducts of NCPO and DHP in Figure 4 strongly suggest that histidine is the proximal ligand in both enzymes, the identity of the ligand trans to histidine in the exogenous ligand-free ferric state is much less clear. The samples prior to addition of cyanide appear to be a mixture of high- and low-spin ferric species. The low-spin component in the MCD spectrum prevents us from making a coordination assignment for the high-spin species; i.e., we cannot determine whether the high-spin species is a five-coordinate or six-coordinate complex. The identity of the ligand(s) trans to histidine will be the subject of future investigations.

The determination that two new heme-containing peroxidase enzymes contain histidine as their proximal ligands is not surprising. Nearly all peroxidase enzymes are histidine-ligated. However, what makes the present results quite unusual is that the types of reactivities that NCPO and DHP display have typically been seen in heme enzymes that have a thiolate proximal ligand provided by cysteine. The only previously studied heme-containing peroxidase capable of

using a combination of chloride ion and hydrogen peroxide to enzymatically chlorinate halogen acceptor substrates has been the chloroperoxidase from *Caldariomyces fumago* (CCPO).[2] Extensive spectroscopic studies of CCPO led to the prediction that it contained a cysteinate thiolate proximal ligand (Dawson & Sono, 1987), a fact recently borne out in the X-ray crystal structure reported by Poulos and co-workers (Sundaramoorthy et al., 1995). Other heme-containing haloperoxidases have been shown to contain a histidine proximal ligand and to only be capable of brominating and iodinating substrates using the halide ion as the source of the incorporated halogen. The fact that NCPO can chlorinate halogen acceptor substrates while possessing a histidine proximal ligand is clearly unexpected.

The ability of DHP to dehalogenate haloaromatics in a hydrogen peroxide-dependent manner is an infrequently observed dehalogenation reaction. The incorporation of an oxygen atom, possibly from hydrogen peroxide, into the product with concomitant oxidation of the aromatic substrate to a quinone product is another unique feature of the DHP reaction. Kobayashi et al. (1986) and Ortiz de Montellano et al. (1987) have shown that the incorporation of oxygen atoms into substrates in a hydrogen peroxide-dependent reaction by chloroperoxidase involves direct transfer of an oxygen atom from hydrogen peroxide. This has led to the use of the term "peroxygenation" for oxygen atom transfer from hydrogen peroxide to a substrate. If the DHP-catalyzed dehalogenation of haloaromatics does involve direct incorporation of an oxygen atom from hydrogen peroxide, then the enzyme should be renamed dehaloperoxygenase. In any event, it is noteworthy that the unusual reactivity of DHP, especially its ability to defluorinate fluorophenols, does not require an unusual proximal ligand and must therefore be brought about by a unique protein environment on the distal side of the heme, where the substrate likely binds.

Another highly unusual property of both new peroxidases is the observation that they are each isolated in their oxyferrous states. In the peroxidase vernacular, this complex is known as compound III because it is the third species to form upon addition of excess hydrogen peroxide to ferric horseradish peroxidase, the most extensively studied peroxidase (Dunford, 1991). The first and second intermediates generated upon addition of hydrogen peroxide to horseradish peroxidase are known as compounds I and II and are oxo-Fe(IV) adducts. Further addition of hydrogen peroxide to compound II of horseradish peroxidase leads to formation of the oxyferrous (compound III) derivative (Dunford, 1995). Therefore, it is possible that the reason NCPO and DHP are isolated in their oxyferrous states is that sufficient amounts of hydrogen peroxide were present *in vivo* to produce compound III and the enzymes have remained in that state during purification. Normally, however, the oxyferrous derivatives of heme proteins autoxidize to the ferric state at sufficiently fast rates (Antonini & Brunori, 1971b) that it would not be possible for the protein to remain in that state throughout the time necessary for purification. A stable oxyferrous derivative of cytochrome *c* peroxidase, for example, has never been reported, and efforts in our lab to prepare it have been unsuccessful even at sub-zero temperatures (M. Sono and J. H. Dawson, unpublished results). These observations suggest that both enzymes may have distal heme environments that are atypical of heme-containing peroxidases and that the heme iron may have an unusually high reduction potential. Electrochemical studies of NCPO and DHP are being planned.

The molecular weights of the heme-binding subunits of NCPO and DHP are both less than 16 000 (Chen et al., 1991, 1996), one-half to one-fifth the size of other known heme-containing peroxidases (Dunford, 1991; English & Tsaprailis, 1995). These two new heme enzymes therefore appear to be the smallest known heme enzymes. The crystallization and very preliminary spectroscopic characterization of DHP have been reported (Zhang et al., 1996)

In summary, *Notomastus lobatus* chloroperoxidase (NCPO) and *Amphitrite ornata* dehaloperoxidase (DHP) both contain histidine as their proximal heme iron ligand. NCPO is therefore the first histidine-ligated heme-containing peroxidase capable of chlorinating halogen acceptor substrates using chloride as the halogen donor. DHP is unique in being the first heme-containing peroxidase capable of removing halogens including fluorine from haloaromatics; the results presented herein demonstrate that it is also histidine-ligated. Thus, the novel reactivity of DHP is not the result of an unusual proximal ligand.

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[2] H_2O_2-dependent chlorinations have been observed in reactions involving myeloperoxidase (Klebanoff, 1991) and eosinophil peroxidase (Weiss et al., 1986). However, the active halogenating species in both cases is hypochlorous acid (HOCl). The chlorinations are thus nonenzymatic.

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