

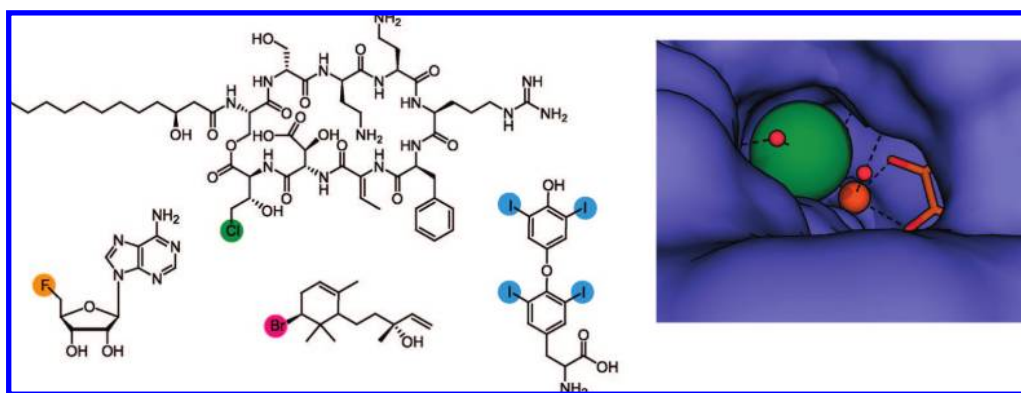
Structural Perspective on Enzymatic Halogenation

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CONSPECTUS



Simple halogen substituents frequently afford key structural features that account for the potency and selectivity of natural products, including antibiotics and hormones. For example, when a single chlorine atom on the antibiotic vancomycin is replaced by hydrogen, the resulting antibacterial activity decreases by up to 70% (Harris, C. M.; Kannan, R.; Kopecka, H.; Harris, T. M. *J. Am. Chem. Soc.* 1985, *107*, 6652–6658). This Account analyzes how structure underlies mechanism in halogenases, the molecular machines designed by nature to incorporate halogens into diverse substrates.

Traditional synthetic methods of integrating halogens into complex molecules are often complicated by a lack of specificity and regioselectivity. Nature, however, has developed a variety of elegant mechanisms for halogenating specific substrates with both regio- and stereoselectivity. An improved understanding of the biological routes toward halogenation could lead to the development of novel synthetic methods for the creation of new compounds with enhanced functions. Already, researchers have co-opted a fluorinase from the microorganism *Streptomyces cattleya* to produce ¹⁸F-labeled molecules for use in positron emission tomography (PET) (Deng, H.; Cobb, S. L.; Gee, A. D.; Lockhart, A.; Martarello, L.; McGlinchey, R. P.; O'Hagan, D.; Onega, M. *Chem. Commun.* 2006, 652–654). Therefore, the discovery and characterization of naturally occurring enzymatic halogenation mechanisms has become an active area of research.

The catalogue of known halogenating enzymes has expanded from the familiar haloperoxidases to include oxygen-dependent enzymes and fluorinases. Recently, the discovery of a nucleophilic halogenase that catalyzes chlorinations has expanded the repertoire of biological halogenation chemistry (Dong, C.; Huang, F.; Deng, H.; Schaffrath, C.; Spencer, J. B.; O'Hagan, D.; Naismith, J. H. *Nature* 2004, *427*, 561–565). Structural characterization has provided a basis toward a mechanistic understanding of the specificity and chemistry of these enzymes. In particular, the latest crystallographic snapshots of active site architecture and halide binding sites have provided key insights into enzyme catalysis.

Herein is a summary of the five classes of halogenases, focusing on the three most recently discovered: flavin-dependent halogenases, non-heme iron-dependent halogenases, and nucleophilic halogenases. Further, the potential roles of halide-binding sites in determining halide selectivity are discussed, as well as whether or not binding-site composition is always a seminal factor for selectivity. Expanding our understanding of the basic chemical principles that dictate the activity of the halogenases will advance both biology and chemistry. A thorough mechanistic analysis will elucidate the biological principles that dictate specificity, and the application of those principles to new synthetic techniques will expand the utility of halogenations in small-molecule development.

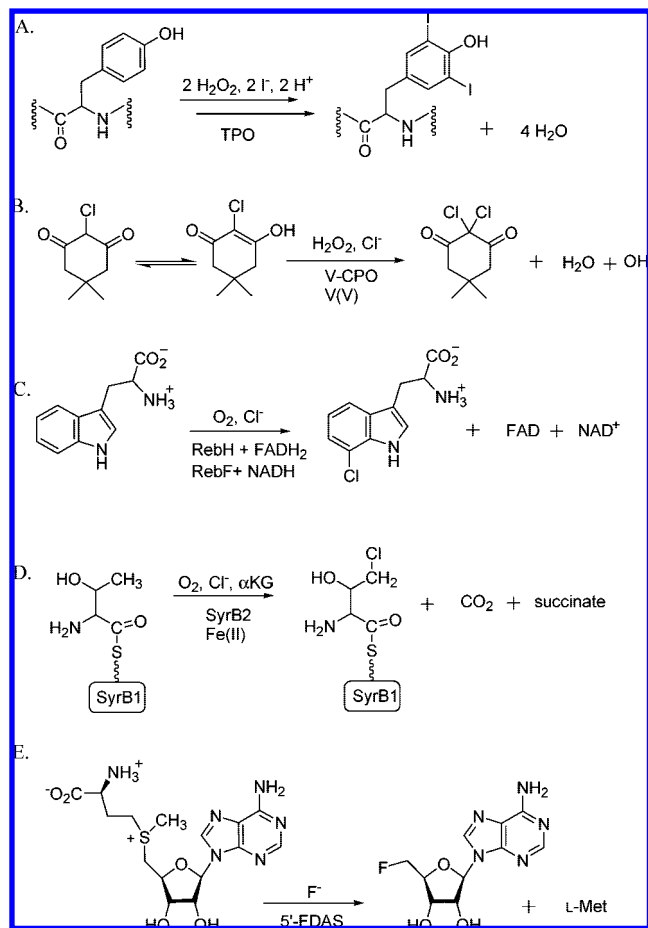
Introduction

Halogenating enzymes can be divided into the hydrogen peroxide (H_2O_2)-requiring haloperoxidases (heme-dependent or vanadium-dependent), the oxygen-dependent halogenases (flavin-dependent or non-heme iron-dependent), and the nucleophilic halogenases.^{1–5} For all classes of halogenases, structural information has proven critical in developing our understanding of mechanism. Here we examine questions of halogenation reactivity and specificity from the primary perspective of enzyme structure, focusing on the recent structures of flavin-dependent, non-heme iron-dependent, and nucleophilic halogenases. Proposed general mechanistic strategies and substrate requirements of each class are shown in Table 1. Additionally, there are other enzymes with known halogenating activity, which will not be discussed in detail here. These include the perhydrolases, whose physiological function is not thought to be halogenation,⁶ and the *S*-adenosyl-L-methionine (AdoMet)-dependent methyl halide transferases for which no structural information is available.⁷

Heme-Dependent Haloperoxidases. The heme-dependent haloperoxidases require H_2O_2 and halide and catalyze halogenation of aromatic, electron-rich substrates.^{8,9} They can be generally divided into two superfamilies, the plant, fungal, and bacterial peroxidases, including chloroperoxidase (CPO) from *Caldariomyces fumago*,¹⁰ and the “animal” peroxidases, including mammalian enzymes myeloperoxidase (MPO), lactoperoxidase (LPO), eosinophil peroxidase (EPO), and thyroid peroxidase (TPO).^{9,11} The superfamilies differ in sequence, structure, and the attachment of their heme group, which is covalently tethered to the protein in animal peroxidases and unattached in the other enzymes, apart from the proximal iron ligand. While MPO, LPO, and EPO have been implicated in the immune response through production of antimicrobial oxidative species, TPO is involved in biosynthesis of the thyroid hormone thyroxine (Scheme 1A).

Structures of enzymes from both heme-haloperoxidase superfamilies have been solved, including CPO,^{12,13} MPO,^{14,15} and LPO (deposited in the PDB). The structure of one well-characterized heme-haloperoxidase, human MPO, is shown in Figure 1A. MPO is composed of two different protein chains, a short “light” chain and a longer “heavy” chain. The heme is bound in a low-ceilinged cavity ($\sim 5\text{--}6\text{ \AA}$), with a wide channel leading to the distal side of the heme. The general mechanism of heme-dependent haloperoxidases involves initial reaction of H_2O_2 with heme-Fe(III) to produce a heme-Fe(IV)=O species, termed compound I.¹⁶ The exact nature of the halogenating species continues to be a subject of much

SCHEME 1. Examples of Enzymatic Halogenation Reactions



discussion. Compound I first oxidizes the halide by two electrons to produce an enzyme-bound heme-Fe(III)–OX intermediate (where X is a halide),^{16,17} which then can react directly with substrates or produce free hypohalous acid (HOX or XO^-).¹⁸ The halogenating mechanism may depend on the substrates and reaction conditions. For example, MPO has been shown to react directly with the small molecule taurine through an enzyme-bound heme-Fe(III)–OX intermediate^{18,19} but is also capable of producing freely diffusible HOX .^{17,18} Larger substrates that are unable to access heme intermediates likely react with diffusible X^+ outside the active site. Regardless of the exact species responsible for halogenation, the consensus is that haloperoxidases couple reduction of H_2O_2 to oxidation of a halide to produce some form of electrophilic X^+ that can be attacked by an electron-rich substrate (Table 1).^{17,18}

Vanadium-Dependent Haloperoxidases. Vanadium haloperoxidases have been found in fungi, marine algae, and bacteria.^{20–22} They require a vanadate cofactor, H_2O_2 , and a halide to catalyze halogenation of electron-rich substrates (Table 1). H_2O_2 binding to the vanadate produces an activated

TABLE 1. The Five Classes of Halogenating Enzymes

	proposed form of activated halogen	substrate requirements	cofactor and cosubstrate requirements
heme iron-dependent haloperoxidases	X ⁺	aromatic and electron-rich	heme, H ₂ O ₂
vanadium-dependent haloperoxidases	X ⁺	aromatic and electron-rich	vanadate, H ₂ O ₂
flavin-dependent halogenases	X ⁺	aromatic and electron-rich	FADH ₂ , O ₂
non-heme iron-dependent halogenases	X [•]	aliphatic, unactivated	Fe(II), O ₂ , α-ketoglutarate
nucleophilic halogenases	X [−]	electrophilic, good leaving group (AdoMet)	

peroxy intermediate, which is vulnerable to attack by a halide ion. As with the heme-dependent enzymes, there is still some disagreement over the nature of the halogenating species, in particular, whether the substrate reacts predominantly with free hypohalous acid or with some form of enzyme bound V–OX intermediate.^{20,21} Experimental evidence suggests that these enzymes can perform certain reactions with regioselectivity or enantioselectivity supporting reaction with a “trapped” X⁺ equivalent,^{23,24} while in other cases no selectivity is observed.²⁰ Indeed, the *Ascophyllum nodosum* enzyme is purified with a halogen-modified surface tryptophan, which could result from free diffusion of an enzyme self-generated X⁺ equivalent.²⁵ Drawing conclusions is further complicated by the fact that the exact physiological roles and substrates of most vanadium-dependent haloperoxidases are unknown. Thus, enzyme activity is often assayed with a generic monochlorodimedone (MCD) substrate (Scheme 1B). The discovery of three vanadium haloperoxidase genes within a natural product gene cluster in *Streptomyces* sp. CNQ-525

presents an exciting opportunity to characterize haloperoxidases with their physiological substrates.²²

The first crystal structure of a vanadium-dependent haloperoxidase was solved for the chloroperoxidase (V-CPO) from the fungus *Curvularia inaequalis* (Figure 1B).²⁶ The vanadate binding site sits at the bottom of a ~10 Å solvent-accessible channel. More recently, highly homologous structures of three algal bromoperoxidases (V-BPOs) were completed from *Ascophyllum nodosum*, *Corallina officinalis*, and *Corallina pilulifera*.^{20,21}

FADH₂ and O₂-Dependent Halogenases. Many flavin-dependent halogenases have now been characterized, and they can be divided into two main groups: those that catalyze chlorination on free small-molecule substrates (RebH, PrnA)^{27–30} and those that react with substrates tethered to a thiolation domain in a nonribosomal polypeptide synthetase (NRPS) system (PltA, SgcC3).^{1,4} To date, the structures of only two confirmed flavin-dependent halogenases have been solved, PrnA from *Pseudomonas fluorescens*³⁰ and RebH from

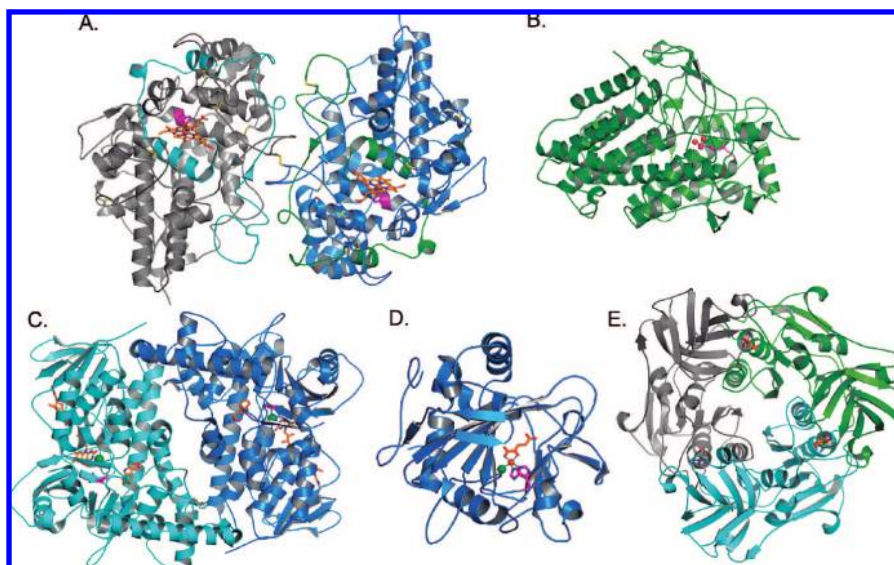


FIGURE 1. The five classes of halogenating enzymes. (A) Human MPO (PDBID 1D2V) dimer with heavy chains in gray and blue and light chains in cyan and green. The heme (orange sticks) is coordinated by the proximal ligand His336 (magenta sticks). Disulfides are shown as yellow sticks. (B) Vanadium-dependent chloroperoxidase from *Curvularia inaequalis* (PDBID 1IDQ). The vanadate cofactor (spheres) is coordinated by His496 (magenta sticks). (C) Flavin-dependent halogenase PrnA from *Pseudomonas fluorescens* (PDBID 2AQJ) dimer showing flavin and L-tryptophan substrate (orange sticks). Lys79 is shown as pink sticks, and chloride is shown as a green sphere. (D) Non-heme iron halogenase SyrB2 from *Pseudomonas syringae* (PDBID 2FCT). αKG is shown as orange sticks and the iron-coordinating ligands (His116 and His235) as magenta sticks. Iron (brown) and chloride (green) are shown as spheres. (E) Trimer of the nucleophilic fluorinase 5'-FDAS from *Streptomyces cattleya* (PDBID 1RQR) with monomers in gray, green, and blue and the 5'-FDA product shown as orange sticks.

Lechevalieria aerocolonigenes,^{29,31} and both enzymes catalyze the chlorination of the free amino acid L-tryptophan on C7 (Scheme 1C).

Flavin-dependent halogenases require reduced flavin (FADH₂), which is provided by a partner-protein reductase (NADH-dependent RebF for RebH). There is clear spectroscopic evidence for reaction of FADH₂ with dioxygen to form a FAD-C4a-OOH intermediate,²⁸ which could react with Cl⁻ to produce hypochlorous acid (HOCl). In the most recently proposed mechanism, HOCl then covalently modifies a protein residue (Lys79 in RebH and PrnA) that in turn acts as the direct chlorinating agent.²⁹ This hypothesis is supported by the observed regioselectivity and the identification of a long-lived, kinetically competent chlorinating intermediate, as well as by structural analysis.²⁹ Flavin-dependent halogenases employ an oxidative strategy of chlorination (with formation of a Cl⁺ equivalent) similar to haloperoxidases and require an electron-rich substrate, although the route through a covalent protein intermediate adds the benefit of regioselectivity (Table 1).

PrnA and RebH share 55% sequence identity and catalyze the same overall reaction. Unsurprisingly, their overall structures are very similar, with a root mean squared deviation (rmsd) of 0.99 Å for 483 aligned C α atoms. Each monomer of the structural dimer consists of an N-terminal flavin binding region with homology to flavin-dependent monooxygenases and a C-terminal substrate binding region (Figure 1C). The PrnA structure provided the first evidence that there was no direct interaction between the tryptophan and flavin intermediates by revealing that >10 Å separate the flavin and tryptophan binding sites.^{29,30} Lys79 (in both RebH and PrnA) is located between the flavin and tryptophan binding sites and ~4 Å from the site of chlorination on tryptophan, which could allow a Lys79 chloramine to direct chlorination to this position.^{29,30} Lys79 is completely conserved across known and predicted flavin-dependent halogenases, and its mutation completely eliminates enzyme activity.^{29,30} Glu357 (RebH numbering, Glu346 in PrnA) appears ideally positioned in the structure for deprotonation of the tryptophan following chlorination.^{29,30}

Non-heme Iron and O₂-Dependent Halogenases. The first *in vitro* activity of a non-heme iron-dependent halogenase was demonstrated in 2005 by Vaillancourt and co-workers.³² Since then at least three other family members have been characterized,⁴ and the structure of the halogenase SyrB2 has been solved.³³ All known members of this class act on a phosphopantetheine-tethered substrate during natural product biosynthesis by NRPS machinery. The structure of SyrB2 revealed an antiparallel β -sandwich or cupin fold com-

mon to mononuclear iron/ α -ketoglutarate (α KG)-dependent enzymes (Figure 1D). The iron coordination in SyrB2 is unusual, with only two histidine ligands coordinating the iron. Most iron/ α KG-dependent enzymes have a third carboxylate ligand to iron, but in the halogenases this is replaced by a chloride. The remaining three coordination sites are occupied by the bidentate α KG and a water molecule.

The overall reaction catalyzed by SyrB2 is shown in Scheme 1D. The proposed mechanism parallels that of non-heme iron dioxygenases and has been discussed in detail.⁴ Briefly, binding of dioxygen to the iron center leads to oxidative decarboxylation of α KG and formation of an Fe(IV)-oxo intermediate, which abstracts a hydrogen from the substrate. The resulting substrate radical then combines with Cl^{*} to give chlorinated product. Involvement of an Fe(IV)-oxo in hydrogen atom abstraction has been shown through Mössbauer spectroscopy in the SyrB2 homologue CytC3.³⁴ The use of a radical halogenation mechanism allows non-heme iron enzymes to regioselectively halogenate substrates at unactivated, aliphatic carbons (Table 1).

Nucleophilic Halogenases. Thus far, only one native fluorinase has been characterized, the 5'-fluoro-5'-deoxyadenosine synthase (5'-FDAS) from *Streptomyces cattleya*, whose structure was solved in 2004.³⁵ The enzyme employs a nucleophilic mechanism of fluorination and uses AdoMet as a substrate (Table 1). In a unique reversal of the typical methylation reaction catalyzed by AdoMet-dependent enzymes, 5'-FDAS exploits L-Met as an excellent leaving group for an S_N2-type reaction.^{35,36} The fluorinase catalyzes attack of F⁻ on AdoMet to produce 5'-fluoro-5'-deoxyadenosine (5'-FDA) (Scheme 1E). 5'-FDAS can also catalyze chlorination, although the reverse reaction (dechlorination) is thermodynamically favored.³⁶ Recently, a homologue of 5'-FDAS, SalL from the marine bacterium *Salinispora tropica*, was characterized and shown to catalyze nucleophilic chlorination under native conditions.³⁷ SalL, which has 35% sequence identity with 5'-FDAS, converts AdoMet to 5'-chloro-5'-deoxyadenosine (5'-CIDA) and cannot perform fluorination.

The fold of the nucleophilic halogenases is unique (Figure 1E), and structures of 5'-FDAS and SalL can be superimposed with an rmsd of 1.37 Å for 248 C α atoms.^{35,37} The monomer is composed of two domains, an N-terminal Rossmann-like domain and a C-terminal β -sandwich domain. The structure is arranged as a trimer, with three active sites located between the N-terminal domain and the C-terminal domain of the adjacent monomer. The physiological oligomer is thought to be a hexamer that is created in the crystal lattice by stacking two trimers.

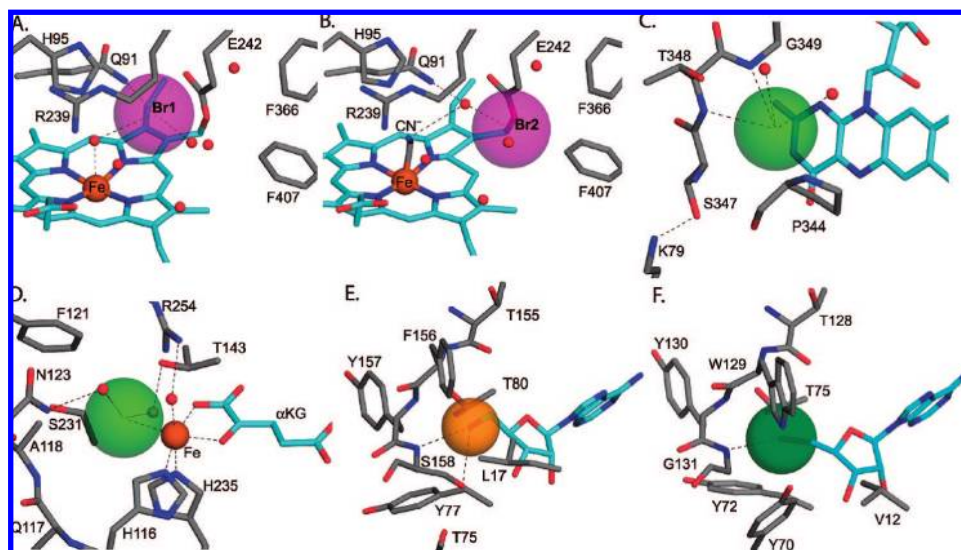


FIGURE 2. Comparison of halide binding sites. Halides shown as translucent spheres representing the halide ionic radius with Br in purple, Cl in green, and F in orange. Water is shown as small red spheres, and iron as a brown sphere. (A) MPO with bound Br⁻ (Br1, PDBID 1D2V). The heme is shown as cyan sticks. (B) MPO with bound Br⁻ and CN⁻ (Br2, PDBID 1D7W) in the same orientation as panel A. The heme is shown as cyan sticks. (C) PrnA (PDBID 2AQJ) with bound FAD (cyan sticks) and Cl⁻. (D) SyrB2 with bound Cl⁻ (PDBID 2FCT). The cosubstrate α KG is shown as cyan sticks. (E) 5'-FDAS (PDBID 1RQP) with bound 5'-FDA shown as cyan sticks. (F) SalI (PDBID 2Q6I) with 5'-CIDA and L-Met shown as cyan sticks.

Halide Binding Sites

Identification of halide binding sites in halogenase crystal structures can lead to new insight about the enzyme mechanism and halide specificity. Halide binding may play a particularly important role in the highly regioselective halogenation reactions catalyzed by flavin-dependent, non-heme iron-dependent, and nucleophilic halogenases. One difficulty can be definitively assigning the identity of an electron density peak as a halide. Both Br⁻ and I⁻ are heavier atoms with accessible anomalous signals, which can aid in their assignment. Cl⁻ can often be assigned due to its higher σ electron density than can be explained by oxygen, but smaller F⁻ is very difficult to distinguish from water at typical structure resolutions. Even after a halide binding site is identified, its relevance to the enzyme mechanism must still be established. Halides are typical additions to crystallographic conditions, and they are commonly found in protein structures. In fact, more than 2500 structures in the Protein Data Bank (PDB) contain a bound chloride ion, representing greater than 5% of the currently deposited structures. For the majority of these structures, the halide is likely an artifact of the crystallization conditions. The relevance of a halide binding site can be established in several ways, including the observation of a bound halogenated product. In other cases, as for heme-dependent MPO, the importance of the observed halide binding sites remains unclear.

Heme-Dependent Haloperoxidases. Structures of CPO and MPO with bound halides are currently available.^{13–15} When CPO crystals were soaked with high concentrations (> 50 mM) of Br⁻ and I⁻, seven partially occupied halide binding sites were identified in anomalous difference maps.¹³ However, none of the halide binding sites are located near the active site, and they are therefore not likely to be relevant to the reaction with heme iron intermediates. Although chloride-soaked CPO structures have been solved, no chloride binding sites were identified.¹³

As with CPO, soaking of MPO with Br⁻ results in multiple halide ion binding sites.^{14,15} MPO crystals soaked with 20 mM NaBr have two partially occupied surface sites and one fully occupied site on the proximal side of the heme.¹⁵ Additionally, one Br⁻ site (site Br1) at 44% occupancy is observed in the distal-heme cavity at 5 Å from the heme iron (Figure 2A). The bromide is only 3.1 Å from the water binding site above the distal iron. This halide site could represent a site of competitive inhibition with H₂O₂ binding, or it may be a productive halide binding site in the presence of compound I. In order to mimic the structure of compound I, a bromide-soaked structure with the inhibitor CN⁻ was obtained (Figure 2B).¹⁴ In this case, the Br⁻ binding site (site Br2) is shifted away from the heme iron, and site Br1 instead contains a water molecule (Figure 2A). The difference in the bromide binding site upon CN⁻ binding may be explained simply by steric effects; larger Br⁻ is unable to bind near the heme once CN⁻ is

bound. Site Br2 is $>7 \text{ \AA}$ from the iron, which is too far away for direct reaction with compound I. Since MPO can catalyze iodination, even larger I^- must be able to access the compound I intermediate. CN^- is an imperfect mimic of compound I (with two atoms coordinated to the iron versus one oxygen), so potentially the larger halides could bind productively at site Br1 after compound I is formed. Alternatively, a halide binding site may not be necessary, with the halides directly approaching and reacting with the compound I intermediate.

Vanadium-Dependent Haloperoxidases. Thus far, a definitive halide binding site has not been established for vanadium haloperoxidases. There are no crystal structures that include a bound halide, although soaking experiments have been attempted.³⁸ Further, no K_D 's have been determined for halide binding to these enzymes. The halide may not interact strongly with the enzyme until the peroxo intermediate has formed, which would make crystallographic characterization challenging because adding halide to crystals in the V-peroxo form would result in turnover.

Flavin-Dependent Halogenases. Flavin-dependent halogenase PrnA has a chloride binding site that packs against the *re*-face of the flavin cofactor (Figure 2C).³⁰ At least three other flavin-dependent enzymes, AhpF (PDBID 1HYU), vanillyl alcohol oxidase (PDBID 1VAO), and L-aspartate oxidase (PDBID 1KNR), contain remarkably similar chloride binding sites against the same face of the flavin. None of these enzymes have been observed to have chlorinating activity. The presence of such similar halide binding sites in non-halogenases could indicate that this halide binding site is an artifact of crystallization. Alternatively, flavin halogenases could have evolved by exploitation of this favorable halide binding motif in order to do halogenation. The PrnA halide binding site involves backbone amide residues (347–349) that curve around Cl^- . Chloride interacts with the amide NH groups of Gly349 and Thr348, and the hydrophobic ring of Pro344 completes the binding pocket. Cl^- is not completely desolvated; it forms interactions with two waters at 3.1 and 3.3 \AA .

Non-heme Iron and O_2 -Dependent Halogenases. The halide binding site of non-heme iron halogenase SyrB2 is unique because the halide coordinates directly to the mononuclear iron (Figure 2D).³³ The halide site is largely hydrophobic and is formed by side chains of Ala118, Ser231, and Phe121. The side-on interaction with the aromatic ring of Phe121 is similar to interactions we will see at the halide binding site of nucleophilic halogenases. As with flavin-dependent halogenases, Cl^- in SyrB2 makes two interactions with waters (3.2 and 3.9 \AA), although one or both of these may be displaced upon substrate binding. Arg254 hydrogen bonds

the apical water on the iron and is located 3.9 \AA above Cl^- . In a structure of SyrB2 with bromide bound in place of chloride, the position of the bromide almost exactly overlays the location of the chloride, and no changes are observed in the surrounding protein structure.³³

Nucleophilic Halogenases. The F^- binding site in 5'-FDAS can be inferred from the position of fluorine in the enzyme product complex (Figure 2E).³⁵ The fluorine sits in a largely hydrophobic pocket composed of the backbones of residues 156–158. Two interactions are formed to the hydroxyl and amide NH of Ser158. F^- is only 3.5 \AA from the edge of the ring of Phe156, which has been shown to be crucial for activity, likely because it helps promote desolvation of the fluoride. The remainder of the binding pocket is formed by Thr80 and Tyr77 and the 5'-FDA molecule. 5'-FDAS can catalyze chlorination as well as fluorination,³⁶ and a 5'-CIDA product complex structure is available. The fluorine and chlorine binding sites overlay one another, with no changes in the surrounding backbone structure. However, the chlorine atom of the 5'-CIDA product is shifted slightly away from Ser158, lengthening the distances to the amide backbone and side chain hydroxyl group, perhaps due to its larger size.

The crystal structure of nucleophilic chlorinase SalL was also solved with bound 5'-CIDA product (Figure 2F). The major change in the active site compared with 5'-FDAS is replacement of Ser158 (131 in SalL) with a glycine. This substitution eliminates one interaction to the halide and slightly increases the size of the halide binding pocket. The edge of the aromatic ring of Tyr70 (Thr75 in 5'-FDAS) fills in the space left by the missing serine. Additionally, a side-on interaction with Phe156 in 5'-FDAS is replaced with a similarly positioned Trp129 in SalL.

Comparison of Halide Binding Sites. There are several interesting trends in the composition of the observed halide binding sites. In no cases do the negatively charged halides form close interactions with positively charged amino acids, such as arginine or lysine. In both the flavin-dependent halogenase and the fluorinase structures, the halides are coordinated by a curved stretch of backbone and make interactions with amide NHs. The halides are typically bound in a largely hydrophobic pocket, and side-on interactions with phenylalanine rings are commonly observed. The edges of aromatic rings bear a partial positive charge and therefore are thought to favor interaction with small anions.³⁹ Similar amide nitrogen interactions and ring-edge aromatic interactions have also been observed in halide binding sites of dehalogenase enzymes.⁴⁰ We also note that only the nucleophilic haloge-

TABLE 2. Trends in the Halides

ionic radius	$F^- < Cl^- < Br^- < I^-$
electronegativity	$I^- < Br^- < Cl^- < F^-$
basicity	$I^- < Br^- < Cl^- < F^-$
nucleophilicity (protic solvent)	$F^- < Cl^- < Br^- < I^-$
nucleophilicity (gas phase)	$I^- < Br^- < Cl^- < F^-$
good leaving group	$F^- < Cl^- < Br^- < I^-$
standard redox potential of HOX/X ⁻ couple	$I^- < Br^- < Cl^- < F^-$

nases bind a completely desolvated halide, which could allow fluorination by activating usually hydrated F^- for nucleophilic attack.

Halogenation Specificity

To understand how and why halogenases are selective for particular halides, several factors must be considered. The relative electronegativity, nucleophilicity, and size of the halides (Table 2) can influence their suitability for different halogenation strategies. Additionally, when a clear halide binding site exists, steric factors may limit the size of the accepted halogen (ionic radii (Å): fluoride, 1.33; chloride, 1.81; bromide, 1.96; iodide, 2.20).⁴¹ Here we discuss what is known about halide selectivity for the different halogenases and describe what is currently understood about the basis for this specificity. Many questions concerning halide selectivity remain, and further investigation would help validate our current mechanistic understanding of halogenation.

Heme-Dependent Haloperoxidases. Heme-haloperoxidases can oxidize all halides with an electronegativity below a given threshold.⁹ Thus, if the enzyme can catalyze chlorination, it can also catalyze bromination and iodination. None of these enzymes have the redox power to oxidize highly electronegative F^- , and only MPO can efficiently catalyze chlorination at neutral pH. Enzyme-dependent halide specificity can be at least partially explained in terms of a simple redox argument.^{9,42,43} The standard redox potentials for conversion between HOX and X^- follow the trend $Cl^- > Br^- > I^-$. Likewise, the redox potentials for conversion from compound I to Fe(III) decrease from MPO > EPO > LPO, which parallels the decreasing oxidative power of these enzymes. Thus, MPO readily catalyzes chlorination, EPO can oxidize Br^- and I^- , and LPO only reacts with I^- . The electron-withdrawing effects of the covalent sulfonium linkage between Met243 of MPO and the heme likely play an important role in tuning the redox potential of the two-electron reduction of compound I.^{9,44} This sulfonium linkage is not present in EPO or LPO, which may help explain their lower redox potentials.

Vanadium-Dependent Haloperoxidases. The vanadium haloperoxidases are named for the most electronegative halogen they can readily oxidize, and only chloro-, bromo-, and

iodoperoxidases are known.²⁰ As with heme-dependent haloperoxidases, the vanadium–peroxo intermediate is not powerful enough to oxidize electronegative F^- . However, there remains an interesting question of selectivity between Cl^- , Br^- , and I^- . Why do some haloperoxidases readily catalyze chlorination, while others can only oxidize iodide? Comparison of the crystal structures of bromoperoxidases with the chloroperoxidase reveals that the vanadate binding residues are largely conserved.^{20,21} There are a few residues near the active site that differ between V-CPO and V-BPOs: Arg395 in V-BPOs is a tryptophan in V-CPO, and His480 in V-BPOs is a phenylalanine in V-CPO. In one exciting experiment, BPO from *C. pilulifera* was endowed with chlorination activity by mutating Arg395 to either tryptophan or phenylalanine.⁴⁵ The mutant enzyme still retained bromination activity. Given that all haloperoxidases are capable of oxidizing the less electronegative and larger halides (for example, chloroperoxidases can also brominate and iodinate), it seems unlikely that halide specificity is mediated by a selective halide binding event. Rather, changes in hydrogen bonding capabilities and the presence of charged residues around the active site may tune the redox properties of the vanadium–peroxo intermediate and influence its ability to react with increasingly electronegative halides.

FADH₂-Dependent Halogenases. Several flavin-dependent halogenases can catalyze bromination reactions, as well as chlorination, although with lower efficiency.^{27,46,47} Thus far, no flavin-dependent halogenases are known to catalyze fluorination or iodination. The proposed mechanism^{1,4,29} involves halide oxidation to an X^+ equivalent, and thus it would be highly unexpected for these enzymes to have the redox potential to oxidize electronegative F^- . The lack of iodination activity could be explained by the larger size of I^- impeding binding to the correct site. Alternatively, the relatively high instability of a lysine iodamine adduct could play a role, resulting in competition between productive halogenation and nonproductive hydrolysis of a lysine iodamine intermediate. The observed halide binding site is quite small, with several enzyme– Cl^- distances of <3.5 Å, which would favor Cl^- binding over Br^- or I^- , but it will be mechanistically interesting to determine whether I^- can access this site.

Non-heme Iron and O₂-Dependent Halogenases. Non-heme iron halogenases can catalyze both chlorination and bromination, although the bromination reactions are less efficient.⁴⁸ No fluorination or iodination activity has been observed. The halide binding site can accommodate a bromide ion, as evidenced by the bromide-bound structure.³³

The higher redox potential for conversion of F^- to the radical could again be responsible for the lack of fluorination activity. Alternatively, binding of F^- to the iron at the correct site might be disfavored by the high cost of desolvation of the fluoride. The lack of activity with I^- likely results from a steric inability of this larger ion to bind the correct coordination site, although as yet no I^- binding studies have been reported.

Nucleophilic Halogenases. 5'-FDAS catalyzes chlorination as well as fluorination, but no evidence for either bromination or iodination has been observed.³⁶ The chlorination activity was initially overlooked, because the reaction equilibrium greatly favors the reverse reaction. As described above, the halide binding site easily accommodates the larger Cl^- ion. Therefore, the fluorination specificity depends on the larger relative nucleophilicity of the desolvated fluoride versus chloride. Although when solvated in water the nucleophilicity of the halides is reversed from their basicity, the halide binding site of 5'-FDAS is designed to fully desolvate the fluoride. When unencumbered by protic solvent, the order of nucleophilicity is expected to follow $F^- > Cl^- > Br^- > I^-$. The small size of the halide binding site may sterically prevent the fluorinase from catalyzing bromination or iodination, while, as discussed below, the larger binding site of the chlorinase SaLL permits these reactions.

The exciting discovery of SaLL, the nucleophilic chlorinase, has helped illuminate the underlying causes of halide specificity.³⁷ SaLL can catalyze chlorination, bromination, and iodination, but not fluorination. The main factors appear to be a slightly larger halide binding pocket and the loss of an interaction with the hydroxyl of Ser158 (5'-FDAS numbering, Gly131 in SaLL). A Gly131Ser SaLL mutant is inactive, but a Tyr70Thr/Gly131Ser double mutant actually improves SaLL's chlorination and bromination activity while eliminating the iodination activity.³⁷ The size of the halide binding pocket in the SaLL double mutant is smaller than in the wild-type enzyme, explaining the lack of iodination. However, the double mutant SaLL is still unable to perform fluorination, probably because the halide binding pocket remains larger and more open than that in the fluorinase. Thus, comparison of SaLL and 5'-FDAS have permitted evaluation of subtle active site changes that create a binding site optimized for a particular halide.

Conclusions

Nature has devised a range of halogenation mechanisms tailored to different halide, cofactor, and substrate reactivities, and crystallographic structure determination has proven essen-

tial in gaining an initial understanding of these mechanisms. Structural analysis has allowed visualization of metal centers and cofactors, led to identification of key residues involved in catalysis, pinpointed the location (or absence) of halide binding sites, and provided insight into halogenase regioselectivity and specificity.

The haloperoxidases are optimized for electrophilic activation of halides but lack clear specificity and regioselectivity. Redox factors, rather than selective halide binding, appear to play a large role in the reactivity of haloperoxidases with different halides. Flavin-dependent halogenases also employ a peroxy intermediate to form an X^+ equivalent, but regioselectivity is apparently achieved through trapping the halogenating species as a covalent intermediate. A wider range of nonaromatic substrates is accessed through use of a powerful non-heme iron cofactor and radical halogenation strategy. Thus far, the only known fluorinase employs a nucleophilic mechanism that takes advantage of the excellent nucleophilicity of desolvated F^- . The fluorinase also exploits the properties of its substrate (AdoMet) to allow this unusual nucleophilic reaction. The recent discovery of halogenases that employ a nucleophilic mechanism with the less electronegative halogens highlights the rapid pace of development in our understanding of biological halogenation. The structural characterization of the nucleophilic chlorinase illustrated how subtle changes in a halide binding site can tune enzyme selectivity for particular halides. Further detailed structural and biochemical investigation of halide binding and selectivity in flavin-dependent and non-heme iron-dependent halogenases is clearly required and would help validate our current mechanistic understanding.

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BIOGRAPHICAL INFORMATION

Leah C. Blasiak received a B.A. in chemistry from Grinnell College in 2003 and a Ph.D. in biological chemistry from MIT in 2008. Her graduate research with Drennan focused on using X-ray crystallography to investigate enzymatic halogenation of natural products.

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FOOTNOTES

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REFERENCES

- Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; Garneau-Tsodikova, S.; Walsh, C. T. Nature's inventory of halogenation catalysts: Oxidative strategies predominate. *Chem. Rev.* **2006**, *106*, 3364–3378.
- van Pée, K. H.; Dong, C.; Flecks, S.; Naismith, J.; Patallo, E. P.; Wage, T. Biological halogenation has moved far beyond haloperoxidases. *Adv. Appl. Microbiol.* **2006**, *59*, 127–157.
- Anderson, J. L.; Chapman, S. K. Molecular mechanisms of enzyme-catalysed halogenation. *Mol. Biosyst.* **2006**, *2*, 350–357.
- Fujimori, D. G.; Walsh, C. T. What's new in enzymatic halogenations. *Curr. Opin. Chem. Biol.* **2007**, *11*, 553–560.
- Neumann, C. S.; Fujimori, D. G.; Walsh, C. T. Halogenation strategies in natural product biosynthesis. *Chem. Biol.* **2008**, *15*, 99–109.
- Kirk, O.; Conrad, L. S. Metal-free haloperoxidases: Fact or artifact. *Angew. Chem., Int. Ed.* **1999**, *38*, 977–979.
- Wuossmaa, A. M.; Hager, L. P. Methyl chloride transferase: A carbocation route for biosynthesis of halometabolites. *Science* **1990**, *249*, 160–162.
- Hofrichter, M.; Ullrich, R. Heme-thiolate haloperoxidases: Versatile biocatalysts with biotechnological and environmental significance. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 276–288.
- Zederbauer, M.; Furtmuller, P. G.; Brogioni, S.; Jakopitsch, C.; Smulevich, G.; Obinger, C. Heme to protein linkages in mammalian peroxidases: Impact on spectroscopic, redox and catalytic properties. *Nat. Prod. Rep.* **2007**, *24*, 571–584.
- Welinder, K. Superfamily of plant, fungal and bacterial peroxidases. *Curr. Opin. Struct. Biol.* **1992**, *2*, 388–393.
- Kimura, S.; Ikeda-Saito, M. Human myeloperoxidase and thyroid peroxidase, two enzymes with separate and distinct physiological functions, are evolutionarily related members of the same gene family. *Proteins* **1988**, *3*, 113–20.
- Sundaramoorthy, M.; Terner, J.; Poulos, T. L. The crystal structure of chloroperoxidase: A heme peroxidase-cytochrome P450 functional hybrid. *Structure* **1995**, *3*, 1367–1377.
- Kuhnel, K.; Blankenfeldt, W.; Terner, J.; Schlichting, I. Crystal structures of chloroperoxidase with its bound substrates and complexed with formate, acetate, and nitrate. *J. Biol. Chem.* **2006**, *281*, 23990–23998.
- Blair-Johnson, M.; Fiedler, T.; Fenna, R. Human myeloperoxidase: Structure of a cyanide complex and its interaction with bromide and thiocyanate substrates at 1.9 Å resolution. *Biochemistry* **2001**, *40*, 13990–13997.
- Fiedler, T. J.; Davey, C. A.; Fenna, R. E. X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 Å resolution. *J. Biol. Chem.* **2000**, *275*, 11964–11971.
- Wagenknecht, H. A.; Woggon, W. D. Identification of intermediates in the catalytic cycle of chloroperoxidase. *Chem. Biol.* **1997**, *4*, 367–372.
- Furtmuller, P. G.; Obinger, C.; Hsuanyu, Y.; Dunford, H. B. Mechanism of reaction of myeloperoxidase with hydrogen peroxide and chloride ion. *Eur. J. Biochem.* **2000**, *267*, 5858–5864.
- Ramos, D. R.; García, M. V.; Canle, M. L.; Santaballa, J. A.; Furtmuller, P. G.; Obinger, C. Myeloperoxidase-catalyzed taurine chlorination: Initial versus equilibrium rate. *Arch. Biochem. Biophys.* **2007**, *466*, 221–233.
- Marquez, L. A.; Dunford, H. B. Chlorination of taurine by myeloperoxidase. Kinetic evidence for an enzyme-bound intermediate. *J. Biol. Chem.* **1994**, *269*, 7950–7956.
- Butler, A.; Carter-Franklin, J. N. The role of vanadium bromoperoxidase in the biosynthesis of halogenated marine natural products. *Nat. Prod. Rep.* **2004**, *21*, 180–188.
- Littlechild, J.; Garcia-Rodriguez, E.; Dalby, A.; Isupov, M. Structural and functional comparisons between vanadium haloperoxidase and acid phosphatase enzymes. *J. Mol. Recognit.* **2002**, *15*, 291–296.
- Winter, J. M.; Moffitt, M. C.; Zazopoulos, E.; McAlpine, J. B.; Dorrestein, P. C.; Moore, B. S. Molecular basis for chloronium-mediated meroterpenoid cyclization: cloning, sequencing, and heterologous expression of the napyradiomycin biosynthetic gene cluster. *J. Biol. Chem.* **2007**, *282*, 16362–16368.
- ten Brink, H. B.; Schoemaker, H. E.; Wever, R. Sulfoxidation mechanism of vanadium bromoperoxidase from *Ascophyllum nodosum*. Evidence for direct oxygen transfer catalysis. *Eur. J. Biochem.* **2001**, *268*, 132–138.
- Carter-Franklin, J. N.; Butler, A. Vanadium bromoperoxidase-catalyzed biosynthesis of halogenated marine natural products. *J. Am. Chem. Soc.* **2004**, *126*, 15060–15066.
- Feiters, M. C.; Leblanc, C.; Kupper, F. C.; Meyer-Klaucke, W.; Michel, G.; Potin, P. Bromine is an endogenous component of a vanadium bromoperoxidase. *J. Am. Chem. Soc.* **2005**, *127*, 15340–15341.
- Messerschmidt, A.; Prade, L.; Wever, R. Implications for the catalytic mechanism of the vanadium-containing enzyme chloroperoxidase from the fungus *Curvularia inaequalis* by X-ray structures of the native and peroxide form. *Biol. Chem.* **1997**, *378*, 309–315.
- Yeh, E.; Garneau, S.; Walsh, C. T. Robust in vitro activity of RebF and RebH, a two-component reductase/halogenase, generating 7-chlorotryptophan during rebeccamycin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3960–3965.
- Yeh, E.; Cole, L. J.; Barr, E. W.; Bollinger, J. M., Jr.; Ballou, D. P.; Walsh, C. T. Flavin redox chemistry precedes substrate chlorination during the reaction of the flavin-dependent halogenase RebH. *Biochemistry* **2006**, *45*, 7904–7912.
- Yeh, E.; Blasiak, L. C.; Koglin, A.; Drennan, C. L.; Walsh, C. T. Chlorination by a long-lived intermediate in the mechanism of flavin-dependent halogenases. *Biochemistry* **2007**, *46*, 1284–1292.
- Dong, C.; Flecks, S.; Unversucht, S.; Haupt, C.; van Pee, K. H.; Naismith, J. H. Tryptophan 7-halogenase (PrnA) structure suggests a mechanism for regioselective chlorination. *Science* **2005**, *309*, 2216–2219.
- Bitto, E.; Huang, Y.; Bingman, C. A.; Singh, S.; Thorson, J. S.; Phillips, G. N., Jr. The structure of flavin-dependent tryptophan 7-halogenase RebH. *Proteins* **2008**, *70*, 289–93.
- Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; O'Connor, S. E.; Walsh, C. T. Cryptic chlorination by a non-haem iron enzyme during cyclopropyl amino acid biosynthesis. *Nature* **2005**, *436*, 1191–1194.
- Blasiak, L. C.; Vaillancourt, F. H.; Walsh, C. T.; Drennan, C. L. Crystal structure of the non-haem iron halogenase SyrB2 in syringomycin biosynthesis. *Nature* **2006**, *440*, 368–371.
- Galonic, D. P.; Barr, E. W.; Walsh, C. T.; Bollinger, J. M., Jr.; Krebs, C. Two interconverting Fe(IV) intermediates in aliphatic chlorination by the halogenase CytC3. *Nat. Chem. Biol.* **2007**, *3*, 113–116.
- Dong, C.; Huang, F.; Deng, H.; Schaffrath, C.; Spencer, J. B.; O'Hagan, D.; Naismith, J. H. Crystal structure and mechanism of a bacterial fluorinating enzyme. *Nature* **2004**, *427*, 561–565.
- Deng, H.; Cobb, S. L.; McEwan, A. R.; McGlinchey, R. P.; Naismith, J. H.; O'Hagan, D.; Robinson, D. A.; Spencer, J. B. The fluorinase from *Streptomyces cattleya* is also a chlorinase. *Angew. Chem., Int. Ed.* **2006**, *45*, 759–762.
- Eustáquio, A. S.; Pojor, F.; Noel, J. P.; Moore, B. S. Discovery and characterization of a marine bacterial SAM-dependent chlorinase. *Nat. Chem. Biol.* **2007**, *4*, 69–74.
- Weyand, M.; Hecht, H.; Kiess, M.; Liaud, M.; Vilter, H.; Schomburg, D. X-ray structure determination of a vanadium-dependent haloperoxidase from *Ascophyllum nodosum* at 2.0 Å resolution. *J. Mol. Biol.* **1999**, *293*, 595–611.
- Burley, S. K.; Petsko, G. A. Weakly polar interactions in proteins. *Adv. Protein Chem.* **1988**, *39*, 125–89.
- de Jong, R. M.; Dijkstra, B. W. Structure and mechanism of bacterial dehalogenases: Different ways to cleave a carbon–halogen bond. *Curr. Opin. Struct. Biol.* **2003**, *13*, 722–730.
- Shannon, R. D.; Prewitt, C. T. Effective ionic radii in oxides and fluorides. *Acta Crystallogr.* **1969**, *B25*, 925–946.
- Arnhold, J.; Monzani, E.; Furtmuller, P. G.; Zederbauer, M.; Casella, L.; Obinger, C. Kinetics and thermodynamics of halide and nitrite oxidation by mammalian heme peroxidases. *Eur. J. Inorg. Chem.* **2006**, 3801–3811.
- Senthilmohan, R.; Kettle, A. J. Bromination and chlorination reactions of myeloperoxidase at physiological concentrations of bromide and chloride. *Arch. Biochem. Biophys.* **2006**, *445*, 235–244.
- Kooter, I. M.; Moguilevsky, N.; Bollen, A.; van der Veen, L. A.; Otto, C.; Dekker, H. L.; Wever, R. The sulfonium ion linkage in myeloperoxidase. Direct spectroscopic detection by isotopic labeling and effect of mutation. *J. Biol. Chem.* **1999**, *274*, 26794–802.
- Ohshiro, T.; Littlechild, J.; Garcia-Rodriguez, E.; Isupov, M. N.; Iida, Y.; Kobayashi, T.; Izumi, Y. Modification of halogen specificity of a vanadium-dependent bromoperoxidase. *Protein Sci.* **2004**, *13*, 1566–1571.
- Lin, S.; Van Lanen, S. G.; Shen, B. Regiospecific chlorination of (S)-β-tyrosyl-S-carrier protein catalyzed by SgcC3 in the biosynthesis of the enediyne antitumor antibiotic C-1027. *J. Am. Chem. Soc.* **2007**, *129*, 12432–12438.
- Dorrestein, P. C.; Yeh, E.; Garneau-Tsodikova, S.; Kelleher, N. L.; Walsh, C. T. Dichlorination of a pyrrolyl-S-carrier protein by FADH₂-dependent halogenase PItA during pyoluteorin biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13843–13848.
- Vaillancourt, F. H.; Vosburg, D. A.; Walsh, C. T. Dichlorination and bromination of a threonyl-S-carrier protein by the non-heme Fe(II) halogenase SyrB2. *ChemBioChem* **2006**, *7*, 748–752.