Published in final edited form as:

Curr Opin Chem Biol. 2007 October ; 11(5): 553–560.

What's New in Enzymatic Halogenations

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Summary

The halogenation of thousands of natural products occurs during biosynthesis and often confers important functional properties. While haloperoxidases had been the default paradigm for enzymatic incorporation of halogens, via X^+ equivalents into organic scaffolds, a combination of microbial genome sequencing, enzymatic studies and structural biology have provided deep new insights into enzymatic transfer of halide equivalents in three oxidation states. These are: (1) the halide ions (X^-) abundant in nature, (2) halogen atoms ($X^\bullet$), and (3) the X^+ equivalents. The mechanism of halogen incorporation is tailored to the electronic demands of specific substrates and involves enzymes with distinct redox coenzyme requirements.

Introduction

Almost five thousand natural products that contain one or more carbon-halogen bonds have been isolated [1]. The great majority of halogenated metabolites are from prokaryotes and single cell eukaryotes but the tri-iodo (T3) and tetra-iodo (T4) forms of thyronine, the master homeostatic thyroid hormone, remind us of the long reach of halogenation biology [2]. Medicinal chemists have used regio- and stereoselective halogenation of drug candidates to optimize a variety of molecular properties including dipole moment and pKa, to control pharmacokinetics and tissue distribution, and to block or redirect metabolism. Undoubtedly Nature is using equivalent logic in the enzymatic tailoring of natural product scaffolds by halogenation. For example, three common antibiotics, chlortetracycline [3], chloramphenicol [4], and vancomycin [5] are all chlorinated. In vancomycin, the chlorination affects atropisomer distribution and is required to achieve clinically active conformation [6]. Dictyostelium uses chlorinated signaling small molecules [7], and bacteria make antifungal agents with chlorinated heterocyclic units [8].

Dramatic advances in deciphering the logic of halogenation enzymes have occurred in the recent past in part through bacterial genomic and bioinformatic analyses which allow identification of two new classes of halogenases, the flavin-dependent and mononuclear nonheme iron families, collocated with nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) biosynthetic gene clusters [9–21]. Complementary studies of purified proteins in each class have allowed codification of each class as O_2 -consuming halogenases [22,23] and have led to mechanistic and structural studies that have uncovered the molecular logic of catalytic oxidative halogenation during biosynthesis [24–28].

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While most of the enzymatic halogenation reactions are oxidative, recently a new non-enzymatic non-oxidative strategy was elucidated, believed to be responsible for the halogenation of enediyne-derived macrolides isolated from marine actinomycetes [29]. This finding represents an important addition to the substrate diversity of halogenated molecules in nature.

Scope of halogenation reactions in biological molecules

A large variety of aromatic and aliphatic carbon centers are halogenated during natural product biosynthesis, with over 95% of the cases involving chloride or bromide [30]. These include chlorination at positions 4, 5, 6 and 7 of tryptophan-derived rings, chlorination of tyrosines at the ortho position and mono- and di-chlorination of pyrroles [3,31]. In marine organisms where bromide is in higher concentration than in fresh water there is comparable bromination of aromatic and heteroaromatic molecules as well as bromination of isoprenoid metabolites [32, 33]. Halogenations occur adjacent to carbonyl groups, as in the dichloroacetyl moiety of chloramphenicol [4]. Several amino acid side chains in nonribosomal peptides are chlorinated at the γ or δ carbons. For example, the barbamide series of marine metabolites exhibit $-\text{CHCl}_2$ and $-\text{CCl}_3$ substituents derived from a methyl group in leucine [34]. Most remarkable are natural product functional groups such as vinyl chlorides and bromides, alkynyl chlorides and bromides, and bromoallenes [20,35–37]. Iodination is relatively rare with approximately 120 known examples and organofluorination is even less prevalent with only 30 fluorinated natural products known to date [30]. While the paucity of organoiodides may reflect the low natural abundance of iodide, the scarcity of fluorinated metabolites is most likely due to a different, nonoxidative mechanism for fluoride incorporation compared to the oxidative halogenation enzymology open to chloride, bromide, and iodide.

Halogenases vs haloperoxidases

From the original discovery of a fungal chloroperoxidase in the 1960s the paradigm of H_2O_2 and chloride ion giving an Fe- OCl equivalent in a heme protein active site was the knowledge base for chlorination, bromination, and iodination enzymology [38]. The finding that bromoperoxidases from marine algae are vanadium-containing enzymes, using a V- OBr brominating species for bromoterpene biosynthesis expanded the scope of H_2O_2 -dependent biological halogenation machinery [32]. The view that haloperoxidases were the full story changed with the genetic demonstration that the chlorination step in chlortetracycline biosynthesis was encoded by a flavoprotein homolog [9]. Bioinformatic analysis of the biosynthetic gene clusters for other polyketides and nonribosomal peptides identified additional flavin-based halogenases. Biochemical characterization of some representative members has demonstrated that they use O_2 to form a flavin- OOH intermediate which oxidizes halide ions [25,31,39]. Given that flavoprotein oxygenases are not powerful enough to hydroxylate unactivated aliphatic carbons, it was anticipated that flavoprotein halogenases would similarly not be up to the task for such halogenations. Indeed, bioinformatic analysis of NRPS gene clusters for syringomycin, barbamide, and coronatine indicated the absence of flavoenzymes. Instead, researchers identified mononuclear nonheme iron enzymes of the O_2 and α -ketoglutarate-dependent family that constitute a novel types of halogenases [15,17–19]. The sections that follow categorize the scope of substrates halogenated by whether the enzymes use X^- , $\text{X}^\bullet$, or X^+ as proximal halogen donors, titrated to the electronic demand of the cosubstrates. This in turn determines which cofactors are required to affect catalysis.

Halogenation via X^-

The best example for use of ground state halide ions as nucleophiles in C-X bond formation has been the bacterial enzyme fluorinase [40]. The enzyme has two obvious requirements for enabling catalysis. First, it must provide a route to desolvation of F^- so that this electronegative

anion can function as nucleophile. A serine side chain in the active site is proposed to provide an alternate hydrogen bond to assist solvation [41]. Second, there must be an electrophilic carbon site in an organic cosubstrate for a halide ion to attack. This is provided by S-adenosylmethionine (SAM) where the 5' carbon of the ribose moiety is electrophilic and the methionine serves as a good leaving group (Figure 1a) as 5-F-adenosine is formed. Subsequent metabolism generates fluoroacetaldehyde, a precursor to fluoroacetate and 4-fluorothreonine. The *Streptomyces cattleya* fluorinase will use Cl^- slowly as alternate nucleophile [42].

Recently, a non-enzymatic pathway for the incorporation of halide anions into organic substrates has been postulated for the biosynthesis of halogenated polycyclic nuclei of sporolides A and B, and cyanosporosides A and B, isolated from marine actinomycetes *Salinispora tropica* and *Salinispora pacifica*, respectively [43,44] (Figure 1b). These molecules are proposed to be biosynthetically derived from an enediyne PKS [44,45], the presence of which was recently confirmed by genome sequencing of *S. tropica* [46]. The unique halogenation event is postulated to occur during the cycloaromatization of the enediyne unit. The feasibility of this proposal has been demonstrated in a model system, where it was found that slight heating of the enediyne cyclodeca-1,5-dien-3-ene, in the presence of lithium chloride, bromide or iodide and a weak acid, is converted to 1-halotetrahydronaphthalene [29]. The kinetic parameters of the reaction are consistent with rate-limiting formation of *p*-benzyne intermediate. Trapping of this intermediate by halide nucleophile and the protonation of the resulting aryl anion leads to the formation of monohalogenated products (Figure 1b).

In general enzymatic halogenation via X^- attack on electrophilic carbon sites is rare. Fluoride is restricted to this nonoxidative route because its high electronegativity makes it resistant to oxidative chemistry. Chloride, bromide and iodide could be incorporated this way but thus far only few metabolites – SAM and *p*-benzyne biradical intermediates – were demonstrated to be viable substrates.

Halogenation via X^+

The more common mechanism for enzymatic halogenation has been oxidative conversion of X^- to enzyme-bound $-\text{OX}$, where the hypohalites act as delivery agents for “ X^+ ” equivalents. These are the now-classic cases of the heme-iron haloperoxidases and the vanadyl bromoperoxidases [32,47]. The oxygen-based oxidant cosubstrate is hydrogen peroxide as the enzyme nomenclature reflects. The metal-OOH species proceed to form metal-OX forms as proximal halogenating agents [48–50]. Halogenation reactions with OX^- equivalents require the reacting carbon center in the substrate to have the opposite polarity from the case described above. Now the reacting carbon site must be electron rich. Thus halogenation adjacent to phenolic oxygens, and bromination of pyrroles are typical outcomes (Figure 2a) [8,32,51]. Similarly π -electrons in isoprenoid metabolites can yield cyclic bromonium ions as evidenced by the conversion of nerolidol to snyderol regioisomers by a vanadium-dependent bromoperoxidase [33] (Figure 2a). Finally, the bromoallene in the natural product laurallene is derived via bromonium-ion promoted cyclization of prelaureatin catalyzed by a bromoperoxidase (Figure 2a)[52].

A second class of enzymes generating $-\text{OX}$ equivalents are the flavin-dependent halogenases found in PKS and NRPS biosynthetic clusters [31,39]. In this case molecular oxygen, not hydrogen peroxide is the oxidizing cosubstrate. The dihydroflavin (FADH_2) oxidation state is the reactive form of the coenzyme and, by one electron pathways and radical recombination, yields the prototypic FAD-4a-OOH that is typical in all flavoproteins that react with O_2 . At this point a chloride (or bromide) ion in the active site fragments the O-O single bond to generate HO-Cl [25,27]. While this could be the proximal donor of “ Cl^+ ”, the FAD cofactor is positioned 10 Å away from the bound substrate and it is likely that the $\epsilon\text{-NH}_2$ of an active

site lysine, conserved in all flavin-dependent halogenases, intervenes and yields a chloramine [24,53]. The --NH--Cl species would have higher kinetic stability than HOCl and allow for regiospecific placement of the “ Cl^+ ” equivalent near the bound substrate, thus explaining the ability of three different flavoenzyme halogenases to act as 5-, 6-, or 7-chlorotryptophan forming enzymes (Figure 2b) [54–57]. As in the haloperoxidases that generate “ X^+ ” equivalents, the substrates for O_2 - and FADH_2 -dependent halogenases are electron rich scaffolds such as phenols, indoles, and pyrroles, which provide the nucleophilic character on the reactive carbon in halogenation transition states [39]. While some members of this halogenase class work on small molecules such as free tryptophan [54,56], others halogenate polyketide and nonribosomal peptide intermediates, tethered as *S*-pantetheinyl thioesters to carrier protein domains, being built on enzymatic assembly lines [58].

Recently, a systematic genetic profiling approach has been applied to the discovery of new halogenated metabolites [59]. Conserved primers that target flavin-dependent halogenases have been used in a genomic screening of a large library of actinomycete strains. This strategy led to the discovery of novel halogenating enzymes in 20% of screened genomes. Subsequent analysis of the genetic content of a gene upstream and a gene downstream of the corresponding halogenase gene allowed for the prediction of the compound class putatively produced by each of the strains. Products of two of the gene clusters harboring halogenases were isolated and structurally characterized, resulting in two novel chlorinated metabolites, glycopeptide antibiotic CB2364-I and a novel polycyclic xanthone CBS40 (Figure 2c). These findings demonstrate that screening of large strain collections with halogenase probes is a valuable approach not only for genome-guided discovery of novel halogenated compounds, but also allows for the rapid pre-selection of strains that may produce therapeutically useful metabolites.

Halogenation via $\text{X}^\bullet$

The discovery of mononuclear iron enzymes using O_2 and α -ketoglutarate to effect chlorination rather than hydroxylation of unactivated methyl groups in substrates has been made recently [21,23,60,61]. The first example was the enzyme SyrB2, which generates a 4-chloro-L-threonine residue incorporated into the framework of the nonribosomal lipopeptidolactone syringomycin produced by *Pseudomonas syringae* (Figure 3a)[23]. This initial discovery has been followed up by characterization of comparable NRPS-associated nonheme iron halogenases active in the biosyntheses of coronatine, barbamide, and dichloroaminobutyrate [21,60,61]. These chloromethyl products can in turn in some cases undergo enzyme-mediated intramolecular elimination to the cyclopropane ring as it is the case in coronamic acid biosynthesis (Figure 3a)[60]. This may be a likely route to the methylcyclopropane ring in the cyanobacterial metabolite curacin [16].

The amino acid moiety to be chlorinated is presented to a halogenase active site as an *S*-pantetheinyl carrier protein. X-ray analysis of SyrB2 revealed that the normal two His, one Asp/Glu “facial triad” ligand set found in related dioxygenases [62] is replaced by a two His set with chloride ion replacing the Asp/Glu due to a mutation of that residue to Ala (Figure 3b) [26]. The reaction path for both hydroxylases and halogenases in this nonheme iron enzyme family proceeds through comparable $\text{Fe}^{\text{IV}}=\text{O}$ intermediates that can abstract an $\text{H}^\bullet$ from the substrate methyl group [28,63]. In hydroxylases there is an $\text{OH}^\bullet$ rebound while for halogenases $\text{Cl}^\bullet$ rebound is proposed (Figure 3c) to capture a transient primary radical on the substrate. Stopped flow absorbance and rapid freeze quench Mössbauer spectroscopies have allowed for trapping of the high valent oxo iron species in both hydroxylases and halogenases of this class [28,63], confirming the conserved mechanism of hydrogen-atom abstraction by these enzymes.

Conclusions and Unsolved Problems

The combination of microbial genome sequencing, bioinformatic analysis, halogenase purification and mechanistic study, mechanistic organic chemistry and structural biology efforts in the past five years have dramatically changed the paradigms for how halogens are incorporated into natural products. Three new classes of enzymes, fluorinase [40], FADH₂- and O₂-dependent halogenases [22,24,25,27,39,54,56,58], and nonheme Fe^{II} α -ketoglutarate- and O₂-dependent halogenases [21,23,28,60], have been characterized. These findings have led to a codification of the kinds of halogenating enzymes and their associated coenzymes that are matched to substrate electronic demand in C-X bond formation. In addition, a nonenzymatic nucleophilic addition of halides has recently been elucidated in a model system [29].

There are still halogenated functional groups in natural products whose origin is as yet unclear. These include the vinyl chloride and alkynyl bromide groups in jamaicamide (Figure 4) [20]. The vinyl chloride group could arise by a convergence of the logic and enzymatic machinery of mononuclear nonheme iron chlorination and the formation of Δ^2 -isopentenyl-S-carrier proteins-bound intermediates, found in bacillaene [64,65], curacin [66] and myxovirescin A [67] gene clusters. The bromoalkyne origin is mysterious although perhaps it could arise from double hydrogen bromide elimination of a terminal tribromohexanoyl group.

Acknowledgements

We thank Dr. Christopher S. Neumann for careful proofreading of the review. This work was supported in part by NIH grants GM 20011 and GM 49338 to C.T.W. and the Damon Runyon Cancer Research Foundation Fellowship to D.G.F (DRG-1893-05)

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Figure 1. Halogenations with halide anions. **(A)** Halide ions as nucleophiles toward carbon electrophiles: *Streptomyces cattleya* fluorinase [40]. **(B)** Non-enzymatic halogenation via trapping of *p*-benzyne biradical intermediate in Bergman cyclization of marine enediynes [29].

Figure 2.

Hypohalites as electrophilic halogens toward carbon nucleophiles. **(A)** Marine haloperoxidases [32,33,50]. **(B)** Halogenation of tryptophan by flavin-dependent halogenases.[24,54–57] PrnA – Trp-7 halogenase in pyrrolnitrin biosynthesis (*Pseudomonas fluorescens*); RebH- Trp-7 halogenase in rebeccamycin biosynthesis (*Lechevalieria aerocolonigenes*); Thal- Trp-6 halogenase in thienodolin biosynthesis (*Streptomyces albogriseolus*); PyrH- Trp-5 halogenase in pyrroindomycin biosynthesis (*Streptomyces rugosporus*). **(C)** Novel halogenated metabolites discovered by flavin halogenase genetic screen [59].

Figure 3.

Halide atom incorporation by mononuclear nonheme iron halogenases. (A) Chlorination in syringomycin and coronamic acid biosyntheses [23,60]. (B) Mononuclear nonheme iron dioxygenases vs mononuclear nonheme iron halogenases [28,63]. (C) Mononuclear nonheme iron halogenases utilize high-valent iron(IV)-oxo intermediates to activate the substrate via hydrogen atom abstraction [28].

Figure 4. Jamaicamide A, halogenated natural product with unsolved carbon-halogen bond-forming mechanisms [20].