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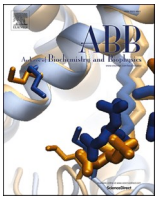

Role of reduced flavin in dehalogenation reactions

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ABSTRACT

Halogenated organic compounds are extensively used in the cosmetic, pharmaceutical, and chemical industries. Several naturally occurring halogen-containing natural products are also produced, mainly by marine organisms. These compounds accumulate in the environment due to their chemical stability and lack of biological pathways for their degradation. However, a few enzymes have been identified that perform dehalogenation reactions in specific biological pathways and others have been identified to have secondary activities toward halogenated compounds. Various mechanisms for dehalogenation of I, Cl, Br, and F containing compounds have been elucidated. These have been grouped into reductive, oxidative, and hydrolytic mechanisms. Flavin-dependent enzymes have been shown to catalyze oxidative dehalogenation reactions utilizing the C4a-hydroperoxyflavin intermediate. In addition, flavoenzymes perform reductive dehalogenation, forming transient flavin semiquinones. Recently, flavin-dependent enzymes have also been shown to perform dehalogenation reactions where the reduced form of the flavin produces a covalent intermediate. Here, recent studies on the reactions of flavoenzymes in dehalogenation reactions, with a focus on covalent catalytic dehalogenation mechanisms, are described.

1. Introduction

Halogenated organic compounds (HOC) are used extensively in the chemical, pharmacological, and agricultural industries as solvents, intermediates in chemical processes, and as pesticides and therapeutic agents [1,2]. Antibiotics such as vancomycin and chloramphenicol are halogenated natural products and represent more than 1000 naturally occurring HOCs [3]. Due to their extensive use and chemical stability, these compounds accumulate in the environment and become major pollutants [4]. Their stability also contributes to accumulation in plants and animals [5]. Several HOCs have been identified as potential carcinogens, such as polychlorinated biphenyls, chlorophenols, and 1, 2-dichoroethane [6,7]. Research in the area of bioremediation, with a focus on identifying bacteria capable of breaking down HOCs, has led to the identification of several enzymes that perform dehalogenation reactions and use HOC as an energy source. Isolation and biochemical studies of some of these enzymes have revealed several strategies for removal of Cl, Br, I, and even removal of F, which represents one of the strongest chemical bonds in nature [8–12]. Biochemical and structural studies have led to the characterization of cofactorless, seleno, B_{12}–Fe–*S*-containing, or flavin-dependent dehalogenases. These enzymes have been shown to catalyzed reductive, oxidative, and hydrolytic dehalogenation mechanisms. Reductive dehalogenation has been well-studied in selenoprotein iodothyronine deiodinases, thioredoxin-like dehalogenase, glutathione *S*-transferase (GST)-dependent dehalogenases, and B_{12}–Fe–S-containing reductive dehalogenase (RDH) [3,11,13,14]. Iodothyronine deiodinases are involved in the regulation of thyroid hormones and are important for proper metabolic control by these hormones, consequently their structure and the mechanism of action has been studied in some detail. Thioredoxin-like dehalogenases are involved in the biosynthesis of microbial natural products but their structure and mechanism have not been studied extensively. However, the mechanism of iodothyronine deiodinases and thioredoxin-like dehalogenase is believed to involve the attack of a thiolate/selenolate to form a covalent cysteinyl/selenyl halide intermediate. Regeneration of the active site of the thiolate/selenolate leads to halide elimination and the formation of a disulfide bond (Fig. 1A) [3,15–17]. The GST-dependent dehalogenation of tetrachlorohydroquinone has been proposed to occur via deprotonation to form a keto species followed by chloride elimination via formation of a diketo intermediate. A covalent intermediate formed with glutathione and removal by an active site cysteine, produces the dehalogenated aromatic product. The active site thiolate is generated by attack of a second glutathione molecule (Fig. 1B) [8,18,19]. The mechanism of B_{12}–Fe–S-dependent dehalogenation is still controversial; however, elimination via formation of a Co-halide bond is surfacing as the favored

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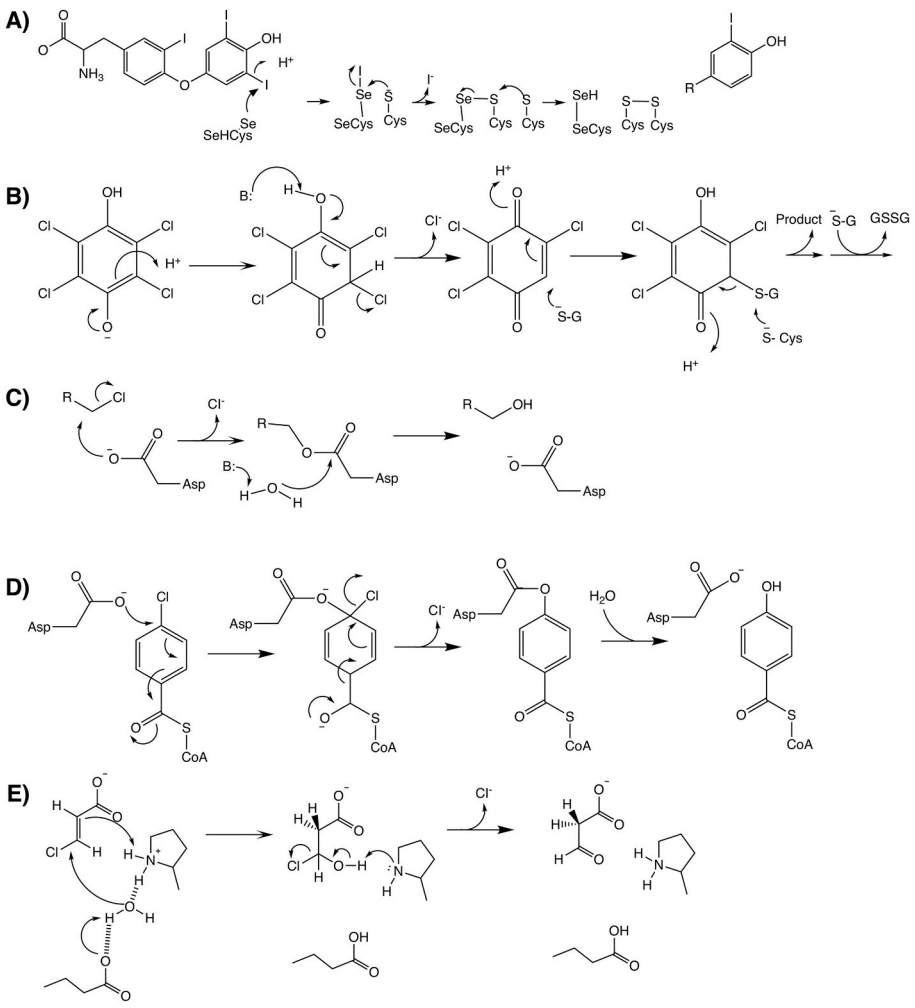

Fig. 1. Reductive dehalogenation reactions catalyzed by seleno-dependent deiodinases (A) and GST-dependent dehalogenases (B). *C*-E show the various mechanism of dehalogenation by hydrolytic dehalogenases.

mechanism [20,21]. Dehalogenation has also been shown to occur through a hydroxylation reaction, where the oxygen atom comes from molecular oxygen or peroxide. Oxidative dehalogenases include the well-studied cytochrome P450 and dehaloperoxidase metalloenzymes (see Ref. [12,22,23] for more details).

Enzymes performing hydrolytic dehalogenation have been

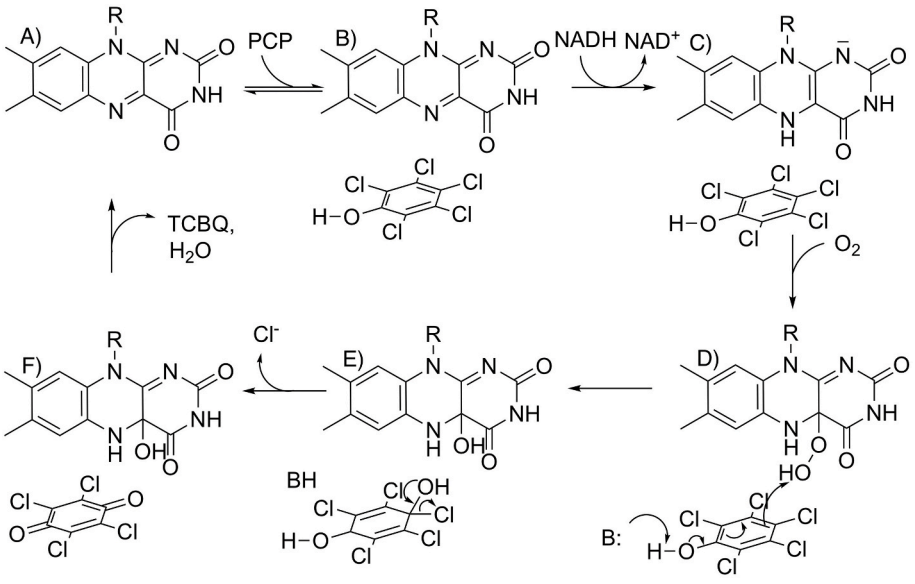

Fig. 2. Oxidative dehalogenation reaction catalyzed by flavin-dependent enzymes. The mechanism is shown for a PcpB, a single component system. In the case of a two-component system, the first two steps (flavin reduction) occur in the reductase component and the reduced flavin is then transferred to the monooxygenase component that reacts with oxygen, forms the Fl_{4a}-OOH, and performs the dehalogenation reaction as shown. In HadA the base that deprotonates the hydroxyl group is a His residue [26].

extensively studied both mechanistically and structurally [8,9,24]. These enzymes catalyze halide elimination via attack of an Asp residue forming a covalent Asp-substrate intermediate that is then hydrolyzed by an activated water molecule (Fig. 1C and D). The dehalogenation of chloroacrylate includes the protonation of the acrylate double bond followed by attack by a water molecule and elimination of the chloride ion (Fig. 1E) [8,9].

Flavin-dependent enzymes have also been shown to catalyze dehalogenation reactions. These enzymes catalyze dehalogenation reactions using reductive, oxidative, and hydrolytic mechanisms using the same cofactor. Below, a short summary of the flavin-dependent mechanisms of oxidative and reductive dehalogenation are presented with a focus on the role of reduced flavin in dehalogenation reactions that involve covalent intermediates.

2. Flavin-dependent oxidative dehalogenases

Dehalogenation reactions by flavin-dependent enzymes that replace a halogen with a hydroxyl group originating from oxygen include single and two-component systems [25]. These enzyme systems have bene isolated from aerobic bacteria and have been shown to degrade halogenated aromatic compounds (HAC) [26]. The reaction requires reducing equivalents from NAD(P)H to produce a reduced flavin cofactor (FAD or FMN), which can activate molecular oxygen by transferring an electron and forming a superoxide and a flavin semiquinone radical pair that recombines, yielding the C4a-hydroperoxyflavin intermediate (Fl_{4a}-OOH) [27,28]. The intermediate is the hydroxylating species. A general reaction for members of this group of enzymes is shown in Fig. 2. The oxidative dehalogenation reaction by PcpB, represents the best characterized single-component system. PcpB is present in *Sphingobium chlorophenolicum* L-1 and catalyzes the conversion of pentachlorophenol (PCP) to tetracholrobenzoquinone (TCBQ) [29]. PcpB is a member of the Class A monooxygenases and as such the catalytic cycle is initiated by binding PCP (Fig. 2A). Binding of PCP is proposed to trigger flavin to move to the *out* position where it can react with NAD(P)H (Fig. 2B). Reduction of the flavin induces conformational changes that place the flavin back into the active site in the *in* position (C). The next step is the formation of Fl_{4a}-OOH and transfer of the distal hydroxyl group to the 4-postion of PCP (D). Re-aromatization of the intermediate and formation of the quinone is coupled to Cl elimination (Fig. 2E) [29]. Dehydration of the flavin and release of TCBQ completes the catalytic cycle (Fig. 2F). Other members of Class A monooxygenases have been shown to catalyze dehalogenation reactions if the right substrate is present. For instance, *p*-hydroxybenzoate hydroxylase catalyzes fluoride elimination from fluorinated-*p*-hydroxylbenzoate [30]. Biochemical and computational studies suggest that the hydroxylation step is the rate limiting step in Class A flavin-monooxygenase catalyzed dehalogenation reactions [31–34].

Members of the two-component monooxygenase systems have also been shown to catalyze oxidative dehalogenation reactions of HAC. These enzyme systems require two enzymes, a flavin reductase, and a monooxygenase. The reductase catalyzes the NAD(P)H dependent reduction of FAD or FMN to produce a reduced flavin that is transferred to the monooxygenase component to perform the dehalogenation reaction [25,26]. The best characterized two-component system is HadA from *Ralstonia pickettii* DTP0602, which is involved in dehalogenation of 4-Cl-phenol and other derivatives. The mechanism of action of HadA has been studied using biochemical and computational approaches by Chaiyen and colleagues [36–39]. HadA is specific for reduced FAD, which reacts with oxygen and forms the Fl_{4a}-OOH. Similar to the monooxygenase of the single component system, it has been shown that HadA catalyzes oxidative dehalogenation via hydroxylation followed by halide elimination (Fig. 2) [40]. The reductase component has also been identified as HadX. This enzyme was isolated with bound FAD, is specific for NADH, and transfers $FADH^-$ to HadA via a free diffusion mechanism

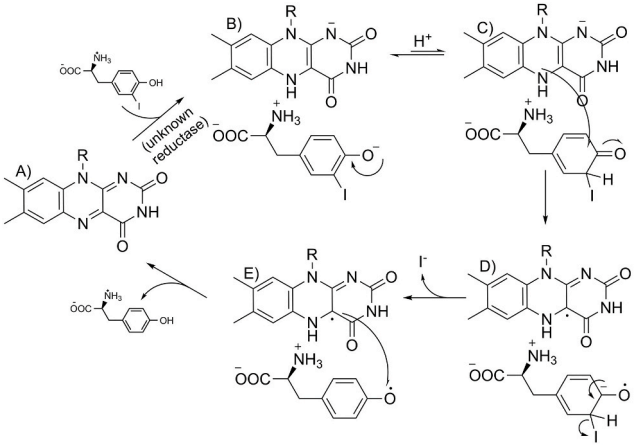

Fig. 3. Mechanism of reductive dehalogenation by iodotyrosine deiodinase (IYD) [35].

[41]. Density functional theory (DFT) calculations and kinetic studies with substrates having various pK_a values suggest that deprotonation of the phenol group is the rate-limiting step [26,40]. This is different from what has been determined for single component monooxygenases where hydroxylation is the rate-limiting step. The difference in the rate-limiting step between these enzyme systems is not obvious because the hydroxylating species (Fl_{4a}-OOH) is the same and the substrates are structurally related. It is possible that because HadA, a two-component system, must orchestrate not only substrate binding but also binding of the $FADH^-$, the active site is not optimally pre-organized for proton abstraction to form the phenolate species.

3. Flavin-dependent reductive dehalogenation

The best structurally- and biochemically-characterized enzyme in this group is iodotyrosine deiodinase (IYD). IYD is an FMN-dependent enzyme and is structurally related to nitroreductases, bacterial flavin reductases, and flavin reductases [42–44]. IYDs are membrane bound via a single *N*-terminus membrane anchor domain, but the redox partner has not been identified. The chemical mechanism of IYD has been elucidated by the Rokita group and involves two single electron transfer steps (Fig. 3). The reaction starts with reduction of IYD by an unknown reductase (Fig. 3A). I-Tyr binds to the reduced IYD in the deprotonated form (Fig. 3B). The next step is the protonation of the phenolate, leading to loss of aromaticity and weakening of the *C*-halide bond (Fig. 3C). The first electron transfer step forms a ketyl anion radical and neutral flavin semiquinone (Fig. 3D). Halide elimination yields a phenoxy radical that can accept a second electron from FMNsq to regenerate FMN_{ox} and produce tyrosine (Fig. 3E) [35]. Support for this mechanism comes from a number of biochemical studies. pH studies showed that the substrate preferentially binds to the enzyme active site in the phenolate form [42]. IYD is capable of dehalogenating I-Tyr, Cl-Tyr, and Br-Tyr, however, it is inactive with F-Tyr [45]. Stopped-flow experiments that monitored the reduction of the flavin in IYD with I-Tyr did not show stabilization of the FMNsq, possibly due to a fast second electron transfer step. However, using F-Tyr as a substrate analog, stabilization of neutral FMNsq was observed during reduction in the stopped-flow spectrophotometer [46, 47]. IYD represents one of the few mammalian dehalogenases.

4. Covalent catalysis by flavoenzymes

Formation of covalent adducts during flavin synthesis, reaction with inhibitors, or alternative substrates has been known for decades [48,49]. In flavoenzymes, formation of the C4a-(hydro)peroxyflavin, C4a-hydroxyflavin, or a C4a-thiol adduct in flavin monooxygenases and flavoprotein disulfide reductases have been very well-established [50,

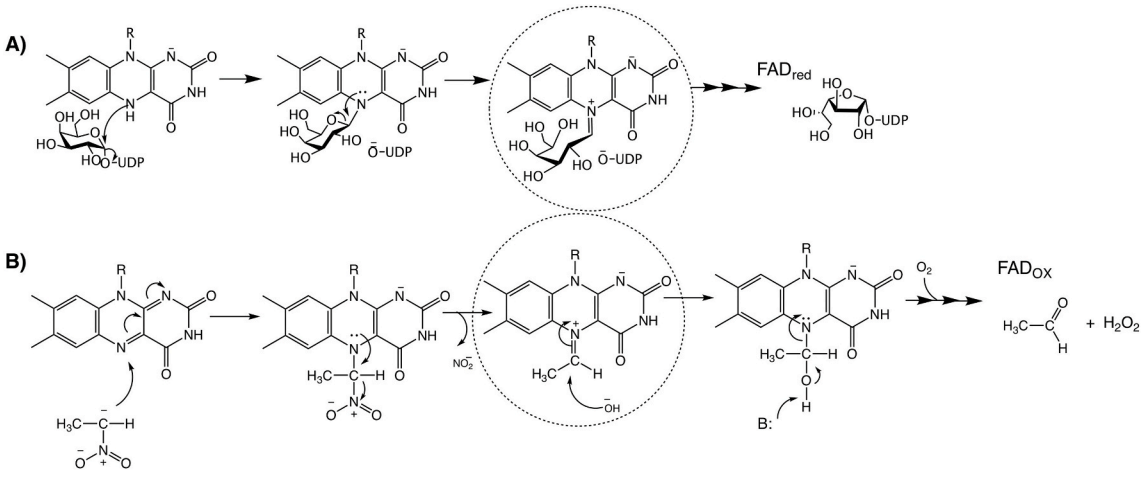

Fig. 4. A) Role of reduced flavin in the mechanism of UDP-galactopyranose mutase. B) Role of oxidized flavin in the reaction catalyzed by nitroalkane oxidase. The iminium ion intermediate is shown with dashed circles.

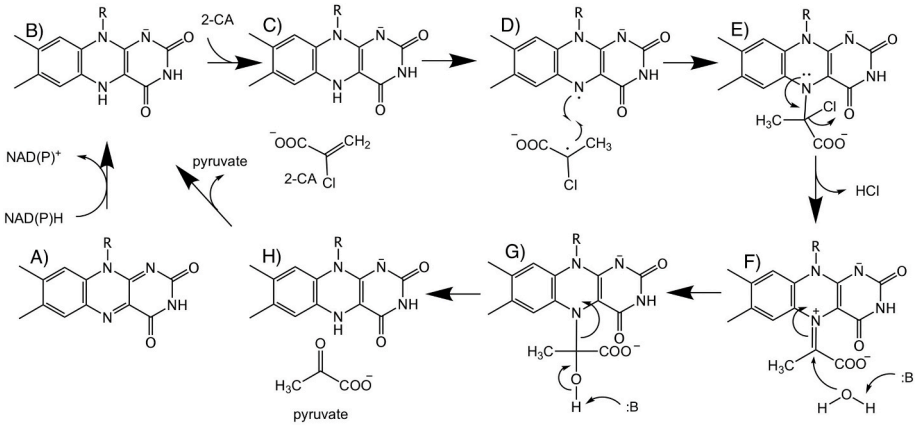

Fig. 5. Mechanism of dehalogenation by 2-HAH.

51]. The chemical properties of flavoenzymes have recently been shown catalyze reactions that include the formation of flavin-N5 covalent intermediates. Accumulated evidence in support of these intermediates come from mechanistic and structural studies and computational biology (see Ref. [52–54] for more details). One interesting observation from these studies is the nucleophilic role of the flavin-N5 atom in the reduced state. In the UDP-galactopyranose mutase (UGM) (Fig. 4A) and UbiX (formation of prenyl-FMN) reactions, nucleophilic attack of the flavin-N5 atom lead to formation of a flavin-N5 adduct [53,55,56]. In the case of UGM, formation of the flavin-N5-iminium adduct is coupled to breaking of *C*–O bond. Other examples of flavin-N5-adducts have also been reported for nitroalkane oxidase (NAO) and alkyl-dihydryoxyacetonephosphate synthase; however, in these cases the nucleophilic substrate attacks the flavin-N5 in the oxidized form. Here, formation of the flavin-N5-iminium adduct is coupled with elimination of nitrate (Fig. 4B) or fatty acid [57,58].

As described in the previous sections, flavoenzymes perform dehalogenation reactions forming covalent intermediates in oxidative dehalogenations. In this case, the C4a position is the reactive center or "hot spot," where the covalent bond between the aromatic substrate and the distal oxygen of the Fl_{4a}-OOH occur. In cases where the reduced flavin is the catalytic active form, an alternative hot spot for catalysis is used, the N5-atom [52,59]. Implementation of covalent flavin-N5-intermediates in the dehalogenation of HOCs is an alternative mechanistic possibility of flavoenzyme catalysis. The next sections highlight two mechanisms by which flavoenzyme catalyzed dehalogenation reactions utilizing the reduced form of the flavin.

4.1. Covalent flavin catalysis with halo-acrylates: the case of 2-haloacrylate hydratase

Borrowing from approaches used to determine the mechanism of UGM, a new mechanism of dehalogenation was proposed for the enzyme 2-halo-acrylate hydratase (2-HAH). 2-HAH is a flavin-dependent enzyme that catalyzes the conversion of 2-chloroacrylate to pyruvate (Fig. 5). This enzyme was identified while screening for proteins that allowed *Pseudomonas* sp. strain YL and *Burkholderia* sp. WS to grow using 2-chroloacrylate as the sole carbon source [60,61].

Two proteins that are 91% identical were identified: 2-HAH_{YL} (from *Pseudomonas* sp. strain YL) and 2-HAH_{WS} (from *Burkholderia* sp. WS). Amino acid sequence analysis of 2-HAH_{YL} and 2-HAH_{WS} showed that these proteins contain the GxGxxG motif, suggesting that they might bind flavin cofactors. Indeed, expression and partial characterization of the recombinant enzymes showed that they bind FAD. Although the overall reaction does not involve a net redox change of substrate or cofactor, the enzymes have an absolute requirement for the reduced form of the flavin for activity [61]. Our initial work with 2-HAH supported the previous report that this enzyme required the reduced flavin for activity and uses either NADPH or NADH with equal efficiencies [62]. Since 2-HAH catalyzes a redox neutral reaction utilizing a reduced flavin, we reasoned that perhaps the mechanism involved the formation of a flavin-substrate adduct, as previously shown for UGM [55,56,63].

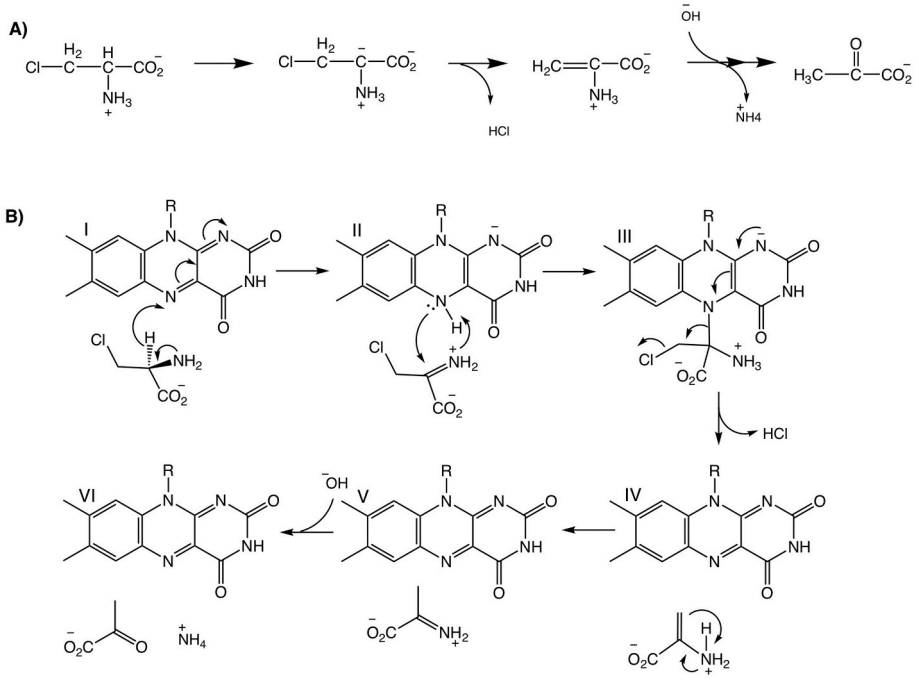

Fig. 6. A)Proposed mechanism of dehalogenation by D-amino acid oxidase involving a carbanion intermediate. B) Covalent dehalogenation reaction catalyzed by DAAO.

Specifically, the presence of a flavin-N5-iminium ion was tested by performing the reaction in the present of cyanoborohydride (Fig. 4). After HPLC and MS analysis of the extracted flavin, it was demonstrated that an FAD-pyruvate intermediate was isolated. Rapid reaction kinetic analysis of the reduced enzyme with 2-chloroacrylate, under anaerobic conditions, showed changes in absorbance at ~530 nm, which were interpreted as formation of a transient flavin-N5 iminium ion, as previously shown for UGM [55,56,62,64,65].

Stopped-flow analysis of the reaction of NAD(P)H with 2HAH in the presence of 2-chloroacrylate under anaerobic conditions showed the presence of a transient neutral semiquinone species. Addition of a radical trapping scavenger (i.e., DEPMPO) inhibits the activity of 2-HAH [66]. Identification of an FAD-N5-iminium ion and the observation of a transient semiquinone suggest a new mechanism for 2-HAH catalysis. These data support the mechanism shown in Fig. 5. The catalytic cycle starts with the oxidized enzyme binding and reacting with NAD(P)H (Fig. 5, A and B). Upon binding of 2-chloro acrylate (2-CA), the reduced flavin transfers one electron to the substrate forming a radical pair that can recombine to form a flavin-N5-adduct (Fig. 5C and D). Formation of a flavin N5-iminum ion leads to chloride elimination. Formation of this adduct also activates the C2-atom for attack by water molecules (Fig. 5E and F). Proton abstraction from the resulting alcohol leads to release of pyruvate and regeneration of the reduced flavin (Fig. 5G and H).

Initial work with 2-HAH was confronted with significant inconsistency on the activity of the protein when reduced with NAD(P)H. It was shown that variation of the activity of the protein was not due to protein stability, because the activity values were very consistent when the protein was reduced with dithionite. This issue might explain that, although the enzyme was identified in 2010 and that it could be recombinantly expressed and purified in *E. coli*, new publications were available only 8-years later. Serendipitously, we discovered that the enzyme was highly active when exposed to light, specifically to blue light. Further experiments showed that the mechanism of light activation was related to binding/reactivity with NAD(P)H. When the protein is not exposed to blue light, i.e., "activated", the enzyme does not rapidly react with NAD(P)H. Only after exposure to blue light (~30 s) does the enzyme react, ~1000-fold faster, with NAD(P)H (unpublished data). The structure of 2-HAH is not currently available; however, amino acid sequence analysis suggests that the 3-dimensional structure is related to members of the succinate dehydrogenase family of flavoenzymes. Efforts to solve the 3-dimensional structure to obtain insight into the mechanism of regulation of this enzyme are currently underway.

4.2. *Covalent flavin catalysis with halo-amino acids: the case of amino acid oxidase*

D-amino acid oxidase (DAAO) is one of the most studied flavoenzymes, with initial reports published in the early 70's [49,67]. DAAO catalyzes the oxidation of amino acids to keto acids, with the exception of acidic amino acids. DAAO has recently been involved in the learning and memory by regulation of D-serine, a coagonist of *N*-methyl D-aspartate receptors [68]. Early studies on the chemical mechanism of DAAO focused on *C*–H bond cleavage. An early mechanistic proposal was the formation of a carbanion intermediate. This mechanism was supported by experiments with β-Cl-Alanine (β-Cl-Ala), where formation of pyruvate, ammonia, and Cl elimination was observed in addition to production of β-Cl-pyruvate and ammonia. It was also reported that Cl elimination did not involve redox change of the flavin and did not occur in the presence of saturating concentrations of oxygen [67]. The data was analyzed in support of the formation of a carbanion intermediate by abstraction of a proton from the α-C position. Cl elimination is induced by formation of an amino acrylate intermediate followed by imino acid formation and non-enzymatic deamination (Fig. 6A).

Several decades after these observations, experimental data became available that were inconsistent with the carbanion mechanism and, rather, supported a direct hydride transfer mechanism. It was shown that replacement of the flavin 5-deaza-flavin did not support chloride elimination, although the flavin was not predicted to play a role in the reaction [69]. Elucidation of the 3-dimensional structure did not show an amino acid capable of abstracting the αC proton to form the proposed carbanion intermediate [70]. Kinetic isotope effects and structural studies with substrate analogs were consistent with a direct hydride transfer mechanism and discarded the carbanion mechanism [71–73]. In an effort to explain the observed β-Cl-elimination, Ghisla et al.

performed a detailed analysis of the reaction of DAAO with β-Cl-Ala using stopped-flow spectroscopy, isotope effects, and by monitoring product formation [74]. The authors concluded that the reaction occurs as shown in Fig. 6. In this mechanism, the reaction is initiated by direct hydride transfer of the α-*C*-atom to form the reduced flavin and the iminium ion intermediate, which was observed as a charged transfer complex (Fig. 6B–I). The presence of the halide promotes a direct attack by the flavin-N5 atom to the α-C of Ala. It was proposed that protonation of the amino group could be catalyzed by the flavin-N5 atom, since the structure does not show other residues that could act as a base (Fig. 6 B-II). Elimination of the chloride ion occurs concomitant to oxidation of the flavin and decay of the intermediate to an amino acrylate (Fig. 6 B-III). This complex was also observed in the stopped flow experiments. The acrylate intermediate can be protonated by the amino group to form the imino acid, which gets deaminated by hydration (Fig. 6 B-IV-VI). Proton transfer from the amino group is consistent with studies using α-^{2}H/^{3}H-β-Cl-Ala, which shows that ~30% retention of the label is in the product (pyruvate) [67,74]. This mechanism not only provides an explanation of how DAAO achieves amino acid dehalogenation, but also explains how the reaction occurs in the absence of an active site base/acid and how the redox state of the flavin does not change during the reaction.

5. Conclusions

Few enzymes dedicated to dehalogenation in biological reactions have been identified and characterized. The best studied enzymes are the mammalian deiodinases. Another example is the thioredoxin-like reductive debrominase, Bmp8, which is present in the biosynthetic gene cluster for the production of pentabromopseudilin [17,75]. On the other hand, the other enzymes have gained dehalogenase function due to the presence and prevalence of HOC, which has provided selective pressure for these reactions. Many of the enzyme that have been shown to perform dehalogenation reactions contain flavin cofactors. This is not surprising since flavin cofactors are thought to be the most versatile cofactors in nature [28,76]. The mechanisms of dehalogenation include the formation of covalent intermediates involving a thiol group, from Cys or glutathione, or an oxygen atom, from Asp or a Fl_{4a}-OOH intermediate. These are observed in enzymes performing both reductive and oxidative dehalogenations. Because the flavin cofactor contains a number of different functionalities, flavoenzymes have developed additional mechanisms for carbon-halogen bond cleavage. In the reductive dehalogenation reaction, IYD performs single electron transfer steps to facilitate iodine elimination instead of forming a covalent intermediate. In redox neutral reactions, flavoproteins utilized the reduced form of the flavin to form a covalent intermediate with the flavin-N5 atom. Formation of a flavin-N5-iminium adduct is coupled to halide elimination. This is consistent to what has been shown for UGM and NAO, where formation of the flavin-iminium ion is coupled to an elimination step. Covalent flavin intermediates have additional functions, such as protonation of intermediates in the catalytic pathway (e.g., Fig. 6 B-II and possible for Fig. 5C). It is reasonable to believe that flavoenzymes will be amenable to being engineered to catalyze the dehalogenation of diverse environmental pollutants. Future screening and protein engineering experiments should include testing the function of the reduced flavin in dehalogenation reactions.

Acknowledgements

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