Flavin-N5 Covalent Intermediate in a Nonredox Dehalogenation Reaction Catalyzed by an Atypical Flavoenzyme


The flavin-dependent enzyme 2-haloacrylate hydratase (2-HAH) catalyzes the conversion of 2-chloroacrylate, a major component in the manufacture of acrylic polymers, to pyruvate. The enzyme was expressed in *Escherichia coli*, purified, and characterized. 2-HAH was shown to be monomeric in solution and contained a non-covalent, yet tightly bound, flavin adenine dinucleotide (FAD). Although the catalyzed reaction was redox-neutral, 2-HAH was active only in the reduced state. A covalent flavin-substrate intermediate, consistent with the flavin-acrylate iminium ion, was trapped with cyanoborohydride and characterized by mass spectrometry. Small-angle X-ray scattering was consistent with 2-HAH belonging to the succinate dehydrogenase/fumarate reductase family of flavoproteins. These studies establish 2-HAH as a novel noncanonical flavoenzyme.

Flavin cofactors, either as flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN), are essential for reactions catalyzed by flavin-dependent enzymes in numerous cellular processes. Flavin-dependent enzymes are involved in redox reactions such as electron transfer, monoxygenation, dehydrogenation, epoxidation, and light and redox sensing, among others.[1] The structures and chemical mechanisms of many flavoenzymes have been studied extensively over the past several decades, and these efforts have led to a significant increase in our understanding of the mechanistic details of several of these reactions. For example, amino acid oxidation by a hydride transfer mechanism was established and supported by mechanistic, structural, and computational studies.[2] Similarly, the mechanism of oxygen activation and formation of oxygenated flavin intermediates in monoxygenase reactions is now well-understood.[3]

Some flavin-dependent enzymes depart from the classical paradigm by catalyzing redox-neutral reactions. In these enzymes, referred to as noncanonical flavoenzymes, the flavin has an atypical chemical role, such as a nucleophile or general acid/base.[4] 2-Haloacrylate hydratase (2-HAH) is a potential noncanonical flavoenzyme that was recently discovered while screening for enzymes that allowed *Pseudomonas* sp. strain YL and *Burkholderia* sp. strain WS to grow when 2-chloroacrylate (2-CA) was the sole carbon source.[5] 2-HAH was found to catalyze the conversion of 2-CA to pyruvate (Figure 1A). Partial characterization suggested that, although the reaction catalyzed by 2-HAH does not involve a net change in the redox
state of the flavin or substrate, the enzyme nevertheless requires the reduced form of FAD for activity.\(^{[5]}\) Here, we present evidence showing that the 2-HAH mechanism proceeds through a covalent flavin-substrate intermediate, which is a hallmark of noncanonical flavoenzymes.

We generated \(E. \) coli expression systems that enabled the purification of 2-HAHs from \(Pseudomonas\) sp. strain YL (2-HAH\(_{YL}\)) and \(Burkholderia\) sp. strain WS (2-HAH\(_{WS}\)) (85% amino acid sequence identity). Sedimentation velocity analysis showed that both enzymes were monodisperse in solution and had molecular masses of 58.2 kDa (YL) and 61.0 kDa (WS; Figure S1 in the Supporting Information). These values were within 4% of the expected monomeric masses.

2-HAH\(_{YL}\) was used for biochemical and enzymatic characterization. 2-HAH\(_{YL}\) contains a noncovalently bound FAD cofactor, which has a typical flavin spectrum with peaks at 373 and 453 nm and a shoulder at 467 nm (Figure S2). No activity was observed when 2-HAH\(_{YL}\) was in the oxidized form. In contrast, chloride elimination was evident when 2-HAH\(_{YL}\) was reduced with either dithionite (30 mM) or NAD(P)H (2 mM) under anaerobic conditions (Figure 1B). Saturation was observed with increasing 2-CA concentration, and data were fit to the Michaelis–Menten equation to obtain the steady-state kinetic parameters (Table 1 and Figure 1C).

The highest \(k_{\text{cat}}\) value was obtained when dithionite was used as the reductant, followed closely by NADPH and then NADH. The \(K_m\) and \(k_{\text{cat}}/K_m\) values for 2-CA were also relatively insensitive to the source of reducing equivalents (Table 1 and Figure 1C). These results confirmed that the flavin needed to be in the reduced form for 2-HAH activity.

The requirement of reduced FAD for activity in the non-redox reaction of 2-HAH suggests the flavin might play a novel role in catalysis. Our group and other studies the flavoprotein UDP-galactopyranosyl mutase (UGM). UGM catalyzes the conversion of UDP-galactopyranosyl (UDP-Galp) to UDP-galactofuranosyl (UDP-Galf). In this non-redox reaction, the reduced form of the flavin is also required for catalysis. The mechanism of UGM involves nucleophilic attack of NS of the reduced FAD on the anomeric C1 atom of UDP-Galp to form a covalent FAD-galactose adduct, which facilitates ring opening and contraction steps during the formation of UDP-Galf (Scheme 1A).\(^{[5]}\) Because both 2-HAH and UGM require the reduced flavin to catalyze a non-redox reaction, we wondered whether these enzymes shared a similar chemical mechanism. It is possible that in 2-HAH, the reduced flavin functions as a nucleophile, attacking the C2 atom and leading to the formation of a flavin-chloroacylate adduct. Chloride elimination would be facilitated by formation of a flavin-acrylate iminium ion. The hydroxylation and release of pyruvate could be assisted by an active site base (Scheme 1B). To test this possibility, the spectral changes upon mixing reduced 2-HAH\(_{WS}\) with 2-CA were monitored by a stopped-flow spectrophotometer. When the reduced enzyme was mixed with pyruvate, no spectral changes were detected (Figure S3A). Similar results were obtained when the same experiment was performed with buffer alone (not shown). In contrast, after mixing with 2-CA, the difference spectra of 2-HAH displayed a decrease in absorbance at 390 nm, accompanied by a very small increase at \(\sim 530\) nm (Figure S3B). The changes in absorbance of the reduced flavin upon mixing with 2-CA were similar to the changes in absorbance that we and others have observed in the reaction of reduced UGM with UDP-Galf, which were attributed to the formation of an N5-FAD-Gal iminium adduct.

To provide evidence of the formation of an FAD-NS-acrylate iminium ion intermediate, an activity assay with 2-HAH\(_{YL}\) was performed in the presence of sodium cyanoborohydride, which can reduce the iminium ion during turnover, thus trapping the intermediate. The reacted flavin was extracted and analyzed by HPLC. We were unable to find conditions in which a covalent flavin adduct was separated from the free flavin peak. To test whether a flavin adduct coeluted with unreacted flavin, the extract was subjected directly to LC-MS analysis. The control experiment, which consisted of extracted flavin from the reaction of dithionite-reduced enzyme alone with cyanoborohydride showed two major peaks at \(m/z\) 786.15 and 808.11, corresponding to the protonated and sodiated FAD ions, respectively (Figure 2A). However, including 2-CA in the assay resulted in the appearance of two additional peaks at \(m/z\) 876.24 and 898.20. These masses were consistent with the molecular weight of the protonated and sodiated ion forms of the C4a-hydroxyflavin adduct shown in Figure 2B. To confirm the presence of this adduct, cyanoborodeuteride was used instead of cyanoborohydride as the trapping reductant. Under these conditions, peaks were observed at \(m/z\) 877.20 and 899.16, consistent with the structure of a deuterated C4a-hydroxyflavin adduct (Figure 2C). A hydroxylated C4a adduct was also previously reported for UGM.\(^{[7]}\) Isolation of a hydroxylated product is consistent with the C4a position becoming more nucleophilic when the flavin iminium ion is formed.

The mechanism that led to the formation of this intermediate might occur by direct nucleophilic attack, as shown to occur with UGM. Alternatively, a mechanism involving a single electron transfer (SET) step and formation of flavin semiquinone and acrylate radical would also lead to formation of the covalent adduct after radical recombination. A SET mechanism was also initially proposed for UGM but later disregarded.\(^{[8]}\)

The solution structural properties of 2-HAH were investigated by using SAXS. Data collected from 2-HAH\(_{WS}\) at three enzyme concentrations indicated an average radius of gyration \((R_g)\) of \((24.9 \pm 0.1)\) Å. (Figure 3A). The molecular mass from the

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**Table 1. Steady-state kinetic parameters for 2-HAH, with 2-CA as the variable substrate.**

<table>
<thead>
<tr>
<th>Reductant</th>
<th>(k_{\text{cat}}) [s(^{-1})]</th>
<th>(K_m) [mM]</th>
<th>(k_{\text{cat}}/K_m) [mM(^{-1})·s(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium dithionite</td>
<td>1.8 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>NADPH</td>
<td>1.5 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>NADH</td>
<td>1.3 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
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</table>

[a] Reactions were incubated at room temperature for 7 min in 100 mM phosphate buffer, pH 7.5, and were terminated by addition of HCO\(_3\)\(^{-}\). Reactions were performed by using saturating amounts of reductant (30 mM sodium dithionite, 2 mM NADPH, or 2 mM NADH).
SAXS Q invariant was \((55.6 \pm 0.9)\) kDa, which was within \(6\%\) of the expected mass of a monomer. The SAXS distance distribution function was unimodal, with a maximum particle dimension of \(\approx 85\) Å (Figure 3B). This type of distribution is consistent with a particle having a single lobe. Previous analysis of the amino acid sequence of 2-HAH suggested similarity to the flavoproteins succinate dehydrogenase and fumarate dehydrogenase.\(^5\) Consistent with this observation, current homology modeling servers identified members of the succinate dehydrogenase/fumarate reductase family\(^9\) as structural templates for 2-HAH. The fold of 2-HAH was predicted to contain an N-terminal Rossmann fold FAD-binding domain (residues 1–246, 352–414), a capping domain (residues 247–351), and a helical C-terminal domain (residues 415–547; Figure 3C). The theoretical scattering curve calculated from the model showed reasonable agreement \((\chi = 0.88–2.1)\) with the experimental curves (Figure 3A), and the model was consistent with the SAXS shape reconstruction (Figure 3C).

The data presented here demonstrate that 2-HAH should be added to the growing list of flavoenzymes with new flavin functionalities, which are centered on the reactivity of the N5 atom of the flavin.\(^10\) Some of these new functionalities include the role of the oxidized flavin in the reaction catalyzed by alkyl-dihydroxyacetone phosphate synthase, in which flavin N5 acts as an electrophile and forms a covalent intermediate.\(^11\) Similarly, the oxidized N5-FAD forms a covalent adduct upon reaction with carbanions in nitroalkane oxidase.\(^12\) In the reduced form, the reactivity of flavins includes several forms of N5 adducts. Formation of a prenylated FMN in the enzyme UbiD is proposed to involve the formation of an N5-prenyl adduct. The prenylated FMN is required for decarboxylation reactions.\(^13\)

Formation of an N5-oxide species was also demonstrated in a Favorovski-type rearrangement in the biosynthesis of the antibiotic enterocin by EncM.\(^17\) Formation of an N5-iminium ion was also recently shown to be involved in the nucleotide methylation reaction catalyzed by flavin-dependent thymidylate synthase and in RNA methylation by TrmFO and RimFO.\(^18\)

In summary, 2-HAH catalyzed the dehalogenation and hydration of 2-CA through formation of an N5-flavin iminium

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Scheme 1. Role of reduced flavin in non-redox reactions. A) Reported chemical mechanism for UGM. B) Proposed chemical mechanisms for 2-HAH-catalyzed reaction.
adduct, similar to the intermediate reported for UGM and other noncanonical flavoenzymes. SAXS and homology model-

Figure 2. Trapping of a covalent flavin intermediate. A) HRMS results of the HPLC peak obtained from the reaction of dithionite-reduced 2-HAH, with cyanoborohydride (without 2-CA), which served as the control experiment. B) Cyanoborohydride and C) Sodium cyanoborodeuteride trapping of the product of the reaction of reduced 2-HAH, with 2-CA.

Figure 3. SAXS analysis of 2-HAH. A) Scattering curves and Guinier analysis (inset). Black circles show experimental data for 2-HAH_{ex}, collected at enzyme concentrations of 3, 6, and 9 mg mL^{-1} (50–150 μM). Smooth curves were calculated by FoXS using a homology model of 2-HAH_{ex} generated by SWISS-MODEL. The goodness-of-fit parameter (q) for each curve is 0.88 (3 mg mL^{-1}), 1.3 (6 mg mL^{-1}), and 2.1 (9 mg mL^{-1}). For clarity, the SAXS curves were offset by applying an arbitrary scale factor. B) Distance distribution functions calculated from the SAXS data shown in (A). Red, green, and blue curves correspond to enzyme concentrations of 3, 6, and 9 mg mL^{-1}, respectively. The inset shows the normalized P(r) curves. C) Superimposition of the SAXS shape reconstruction and a homology model of 2-HAH. Surface represents the averaged filtered dummy atom model from GASBOR. The predicted domains are colored as follows: Rossmann FAD-binding, red; capping, blue; helical C-terminal domain, green. FAD is shown in yellow.

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