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Evidence for the Formation of a Radical-Mediated Flavin-N5 Covalent Intermediate

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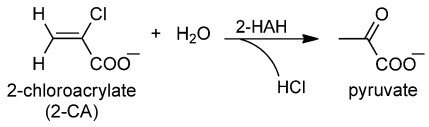
The redox-neutral reaction catalyzed by 2-haloacrylate hydratase (2-HAH) leads to the conversion of 2-chloroacrylate to pyruvate. Previous mechanistic studies demonstrated the formation of a flavin-iminium ion as an important intermediate in the 2-HAH catalytic cycle. Time-resolved flavin absorbance studies were performed in this study, and the data showed that the enzyme is capable of stabilizing both anionic and neutral flavin semiquinone species. The presence of a radical scavenger decreases the activity in a concentration-dependent manner. These data are consistent with the flavin iminium intermediate occurring by radical recombination.

Halogenated hydrocarbons are extensively used in industrial processes as solvents and pharmaceutical precursors. In agriculture, halogenated compounds are also used as pesticides, and there is evidence that some halogenated xenobiotics are toxic to humans.[1] For example, polychlorinated biphenyl and 1,2-dichloroethane have been classified as highly toxic and carcinogenic agents, respectively.[2] The toxicity of halogenated compounds was not realized until they had been extensively used by the chemical industry for decades. Today, the presence of these toxic compounds is an important environmental concern, and dehalogenases have the potential to be used in the bioremediation of environments polluted by these compounds.[3] 2-Haloacrylate hydratase (2-HAH) is a flavin-dependent enzyme that catalyzes the conversion of 2-chloroacrylate to pyruvate (Scheme 1). 2-HAH catalyzes a redox-neutral reaction that is different from those catalyzed by the well-known



Scheme 1. The reaction catalyzed by 2-HAH.

flavin-dependent enzymes in which redox changes in the flavin cofactor are coupled to changes in the redox state of the substrate.

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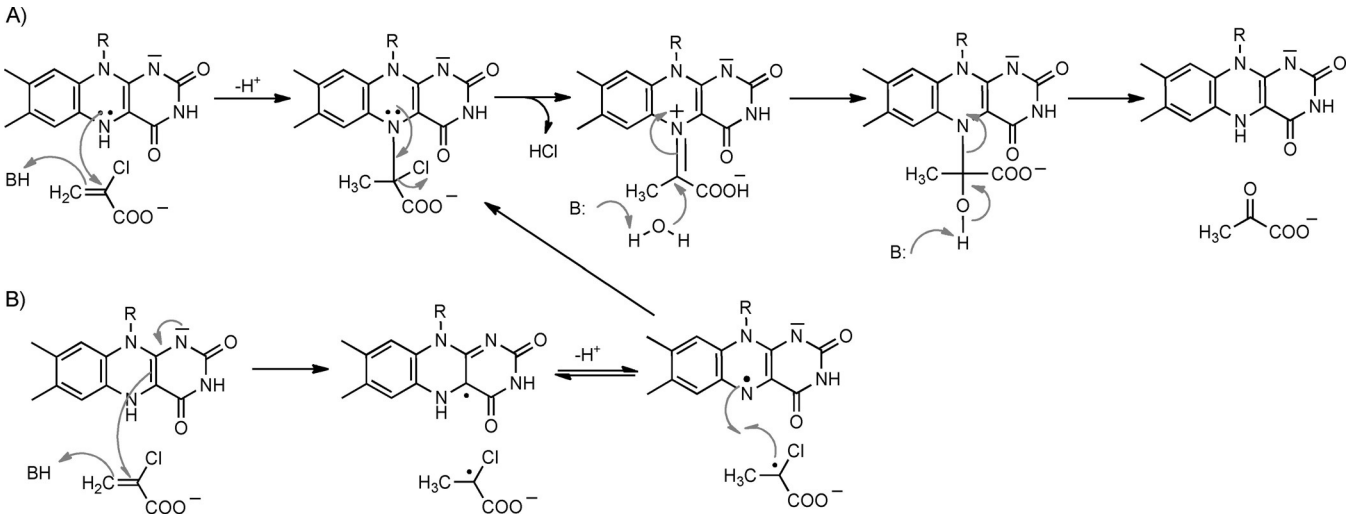
Flavoenzymes that catalyze non-redox or noncanonical reactions are uncommon but play important biological roles. The best-characterized members of this family of enzymes include alkyl-dihydroxyacetonephosphatase (ADAP), type II isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI-2), and UDP-galactopyranose mutase (UGM).[4] ADAP plays a role in the biosynthesis of ether phospholipids and catalyzes the exchange of an acyl chain at the C-1 position of acyl-dihydroxyacetonephosphate (acyl-DHAP) with a fatty alcohol, thus forming an ether linkage in alkyl-DHAP.[5] This mechanism of ADAP has been shown to involve a covalent adduct intermediate that forms between a carbanion of acyl-DHAP and the oxidized flavin. The isomerization reaction catalyzed by IDI-2 is important for isoprenoid biosynthesis. IDI-2 requires the reduced form of the flavin for activity. In this reaction, the flavin functions as an acid/base catalyst.[6] The reaction catalyzed by UGM is essential for the biosynthesis of galactofuranose, which is found in the cell walls of mycobacteria, fungi, and several glycan structures on the cell surface of parasites and nematodes.[7] UGM activity also requires reduced flavin; however, in the sugar isomerization, the flavin functions as a nucleophile, forming a covalent intermediate with galactose.[8] The reaction of 2-HAH has been shown to have an absolute requirement for the reduced form of the flavin for activity.[9] We recently demonstrated the presence of a flavin-acrylate intermediate during turnover, as we and others demonstrated for UGM.[10,11] However, the mechanism by which this intermediate is formed was not elucidated. In principle, this intermediate can form according to the mechanisms shown in Scheme 2. In one mechanism, the covalent adduct could form by a nucleophilic attack of the FAD-N5 atom, similar to what has been shown for UGM (Scheme 2A).[11] Alternatively, a single-electron transfer step could lead to a flavin-acrylate radical pair that can recombine to form the covalent FAD-N5-acrylate adduct (Scheme 2B).

To obtain insight into the steps leading to formation of the covalent adduct in 2-HAH, we performed a series of rapid reaction kinetic analyses. The reaction of oxidized 2-HAH with NAD(P)H was monitored under anaerobic conditions in a stopped-flow spectrophotometer. The change in absorbance as a function of time at 453 nm, characteristic of flavin reduction by NAD(P)H, was selected for kinetic analysis. The experiment was performed at various NAD(P)H concentrations, and the rate constant of flavin reduction (k_{red}) and the K_d values were calculated as previously described.[8b,12] These values are listed in Table 1. The results show that 2-HAH does not have a strong preference for NADPH over NADH. Reduction of 2-HAH with NADH was also performed in the presence of 2-CA. Under this condition, the k_{red} value slightly increased to (0.50 ± 0.01) s^{-1}, whereas the K_d value remained unchanged (Table 1).



Scheme 2. Proposed chemical mechanisms for the 2-HAH-catalyzed reaction.

Table 1. Flavin reduction of 2-HAH by NAD(P)H.[a]

Reductant	k_{red} [s^{-1}]	K_d [mM]	k_{red}/K_d [$mM^{-1}s^{-1}$]
NADH	0.46 ± 0.01	0.67 ± 0.06	0.68 ± 0.30
NADH + 2-CA	0.50 ± 0.01	0.68 ± 0.04	0.72 ± 0.02
NADPH	0.50 ± 0.01	0.56 ± 0.03	0.84 ± 0.02

[a] Conditions: 25 °C in 100 mM sodium phosphate buffer (pH 7.5) in the absence of oxygen. When 2-CA was used, its concentration was 0.5 mM.

2-HAH reduction by NADH alone was accompanied by an absorbance decrease at 453 nm, typical of flavin reduction. In addition, increases and decreases in absorbance at wavelengths >550 nm were observed. This was followed by an increase at 400 nm (Figure 1 A). These features are consistent with a small amount of neutral (or blue) semiquinone forming very fast and then slowly converting to the anionic (or red) semiquinone form.[13] The observation of a flavin semiquinone species resulting from an obligate hydride transfer reductant (NAD(P)H) under anaerobic conditions is unexpected. These results could be explained by single electron transfer from the fully reduced FAD to an aromatic residue in the active site, another oxidized 2-HAH, or a small amount of contaminating oxygen. Regardless the mechanism, these results show that 2-HAH is able to stabilize one-electron reduced forms of its bound FAD.

Previously, the reaction of fully reduced 2-HAH and 2-CA was monitored under anaerobic conditions on a stopped-flow spectrophotometer. The only flavin spectral changes observed were consistent with the formation of a covalent flavin iminium ion.[10] The lack of any transient semiquinone forming

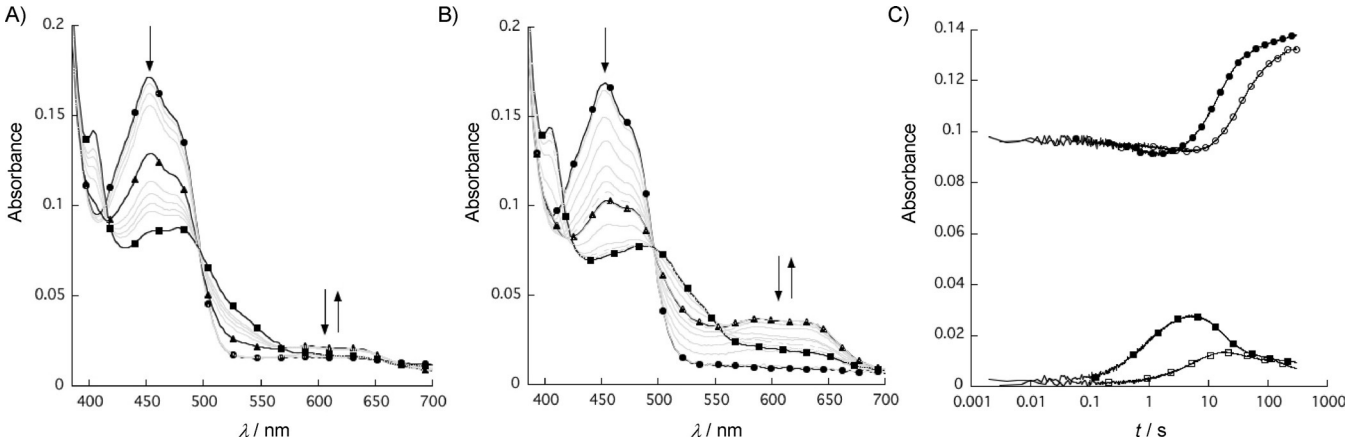


Figure 1. Spectral changes during flavin reduction with NADH (0.1 mM) measured in a stopped-flow spectrophotometer under anaerobic conditions. A) The reaction of oxidized 2-HAH (●) with NADH in the absence of 2-CA shows typical bleaching of the absorbance peak at 453 nm. The spectra also show an increase and subsequent decrease in a wide absorption band (>550 nm), consistent with the transient formation of a neutral semiquinone (▲). The appearance of a peak at 400 nm, characteristic of an anionic semiquinone, is observed at later time points (■). B) In the presence of 2-CA, the early time points of the reduction process also include the transient formation of a neutral semiquinone (▲); however, significantly more neutral semiqinione is observed than in the absence of 2-CA. The neutral semiquinone is slowly converted to the anionic semiquinone (■);● = 2-HAH. C) Time evolution of the formation of the anionic semiquinone species in the absence (○) and presence (●) of 2-CA monitored at 400 nm, and of a transient neutral semiquinone in the presence (■) and absence (□) of 2-CA.

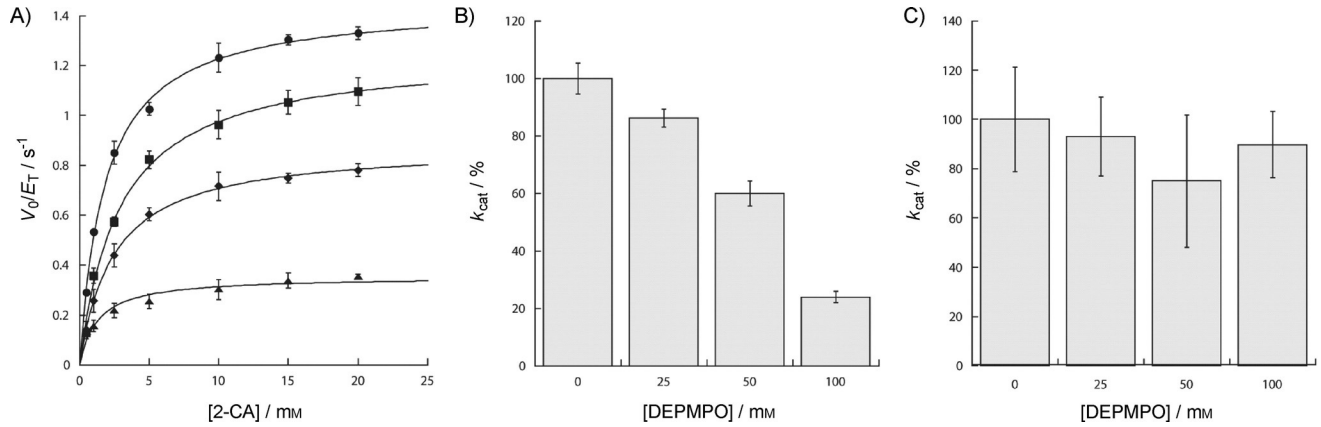


Figure 2. Effect of the free radical scavenger DEPMPO on 2-HAH activity. A) Activity of 2-HAH at various concentrations of DEPMPO (0–100 mM). B) Decrease in k_{cat} normalized to the value obtained in the absence of DEPMPO. C) Reaction of NADPH-reduced UGM in the presence of DEPMPO.

under these conditions suggests that this intermediate might form and decay very rapidly. We probed the rate-limiting step under steady-state conditions by measuring the kinetic solvent isotope effect (KSIE). A KSIE value of 2 was calculated for k_{cat}, thus indicating that there is a proton in flight in the rate limiting step (Figure S2 in the Supporting Information). We believe that this step is the hydrolysis of the iminium adduct (Scheme 2).

To further characterize the reaction of 2-HAH, the changes in the flavin spectra during reaction with NADH (0.10 mM) were also monitored in the presence of 2-CA. These changes were similar to those observed in the absence of 2-CA; however, formation of the flavin semiquinone species was accelerated, and the formation of the neutral semiquinone was significantly augmented (Figure 1B). Absorbance changes at 600 and 400 nm were analyzed to determine the relative increase in the rate of formation caused by 2-CA. In the presence of 2-CA, the rate of formation of the neutral semiquinone is approximately four times greater, whereas formation of the anionic semiquinone is approximately two times faster (Figure 1C).

The stopped-flow experiment showed that 2-HAH is capable of stabilizing the anionic flavin and forming a transient neutral semiquinone in the presence of 2-CA (Figure 1B). If the flavin covalent adduct forms through a radical rearrangement (Scheme 2B), the activity of 2-HAH would be affected by radical trapping agents. To test this possibility, the reaction was performed with various concentrations of the free radical scavenger 5-(diethylphosphono)-5-methyl-1-pyrroline *N*-oxide (DEPMPO).[14] As the concentration of DEPMPO increased, the k_{cat} value decreased in a linear fashion (Figure 2B). As a control experiment, the effect of DEPMPO was tested on the reaction of NADPH-reduced UGM. It was determined that DEPMPO did not reduce the activity of UGM (Figure 2C). Similar results were obtained if UGM was reduced by dithionite (Figure S1). The data are consistent with the presence of free radicals in the reaction of 2-HAH and inconsistent with a radical mechanism in the reaction catalyzed by UGM, as expected. Furthermore, EPR analysis of the 2-HAH reaction when using 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS) showed evidence of radical species; however, no detailed characterization of the observed species was provided.[15] It was postulated that the radical intermediates would donate an electron back to the flavin semiquinone, thereby forming the reduced flavin and a cation intermediate that would be attacked by a hydroxy group leading to chloride elimination and the formation of pyruvate.[15] Our previous characterization of a covalent substrate flavin adduct is not consistent with this proposed mechanism.

In summary, 2-HAH catalyzes the dehalogenation and hydration of 2-CA through the formation of an N5-flavin iminium adduct, similar to the intermediate reported for UGM and other enzymes. The data presented here support the formation of this intermediate by radical recombination. This is a variation of the formation of the iminium ion through the nucleophilic mechanism shown for the reduced flavin in UGM. Thus, 2-HAH should be added to the growing list of flavoenzymes with new flavin functionalities that are centered on the reactivity of the N5-atom of the flavin.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: covalent adducts · flavin · non-redox reaction · radicals · semiquinones

[1] S. Fetzner, F. Lingens, *Microbiol. Rev.* **1994**, *58*, 641–685.
[2] S. H. Safe, *Crit. Rev. Toxicol.* **1994**, *24*, 87–149.
[3] R. M. de Jong, B. W. Dijkstra, *Curr. Opin. Struct. Biol.* **2003**, *13*, 722–730.
[4] a) P. Sobrado, *Int. J. Mol. Sci.* **2012**, *13*, 14219–14242; b) V. Piano, B. A. Palfey, A. Mattevi, *Trends Biochem. Sci.* **2017**, *42*, 457–469.
[5] A. Razeto, F. Mattiroli, E. Carpanelli, A. Aliverti, V. Pandini, A. Coda, A. Mattevi, *Structure* **2007**, *15*, 683–692.
[6] C. J. Thibodeaux, H. W. Liu, *Arch. Biochem. Biophys.* **2017**, *632*, 47–58.

[7] a) F. Pan, M. Jackson, Y. Ma, M. McNeil, *J. Bacteriol.* **2001**, *183*, 3991 – 3998; b) S. M. Beverley, K. L. Owens, M. Showalter, C. L. Griffith, T. L. Doering, V. C. Jones, M. R. McNeil, *Eukaryotic Cell* **2005**, *4*, 1147 – 1154; c) M. Oppenheimer, A. L. Valenciano, P. Sobrado, *Enzyme Res.* **2011**, *2011*, 415976; d) B. Kleczka, A. C. Lamerz, G. van Zandbergen, A. Wenzel, R. Gerardy-Schahn, M. Wiese, F. H. Routier, *J. Biol. Chem.* **2007**, *282*, 10498 – 10505.

[8] a) M. Soltero-Higgin, E. E. Carlson, T. D. Gruber, L. L. Kiessling, *Nat. Struct. Mol. Biol.* **2004**, *11*, 539 – 543; b) M. Oppenheimer, A. L. Valenciano, K. Kizjakina, J. Qi, P. Sobrado, *PLoS One* **2012**, *7*, e32918; c) R. Mehra-Chaudhary, Y. Dai, P. Sobrado, J. J. Tanner, *Biochemistry* **2016**, *55*, 833 – 836.

[9] A. M. Mowafy, T. Kurihara, A. Kurata, T. Uemura, N. Esaki, *Appl. Environ. Microbiol.* **2010**, *76*, 6032 – 6037.

[10] Y. Dai, K. Kizjakina, A. C. Campbell, D. A. Korasick, J. J. Tanner, P. Sobrado, *ChemBioChem* **2018**, *19*, 53 – 57.

[11] J. J. Tanner, L. Boechi, J. A. McCammon, P. Sobrado, *Arch. Biochem. Biophys.* **2014**, *544*, 128 – 141.

[12] a) R. Dhatwalia, H. Singh, L. M. Solano, M. Oppenheimer, R. M. Robinson, J. F. Ellerbrock, P. Sobrado, J. J. Tanner, *J. Am. Chem. Soc.* **2012**, *134*, 18132 – 18138; b) R. M. Robinson, P. J. Rodriguez, P. Sobrado, *Arch. Biochem. Biophys.* **2014**, *550 – 551*, 58 – 66.

[13] a) K. Crozier-Reabe, G. R. Moran, *Int. J. Mol. Sci.* **2012**, *13*, 15601 – 15639; b) P. Sobrado, P. F. Fitzpatrick, *Biochemistry* **2003**, *42*, 13833 – 13838.

[14] H. Karoui, F. Chalier, J. P. Finet, P. Tordo, *Org. Biomol. Chem.* **2011**, *9*, 2473 – 2480.

[15] A. M. Mowafy, T. Kurihara, Y. Ohashi, T. Watanabe, W. Buckel, N. Esaki, in *Flavins and Flavoproteins 2011* (Eds.: S. Miller, R. Hille, B. Palfey), Lulu Press, Berkeley, **2011**, pp. 75 – 82.

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