On the Stereochemistry of 2-Hydroxyethylphosphonate Dioxygenase

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§Supporting Information

ABSTRACT: Stereochemical investigations have shown that the conversion of 2-hydroxyethylphosphonate to hydroxymethylphosphonate by the enzyme HEPD involves removal of the pro-S hydrogen at C2 and, surprisingly, the loss of stereochemical information at C1. As a result, the mechanisms previously proposed for HEPD must be re-evaluated.

N atural-product phosphonates and phosphinates have found wide use in medicine and agriculture. 1 One such compound, phosphinothricin (PT), 2–5 is the active component in commercial herbicides (Liberty, Basta, and Ignite) that are used in conjunction with transgenic crops such as corn, cotton, soy, and canola. 6–9 Recent genetic and biochemical studies on PT biosynthesis have shown that 2-hydroxyethylphosphonate (2-HEP) is converted to hydroxymethylphosphonate (HMP) by hydroxymethylphosphonate dioxygenase (HEPD), the gene product of phpD (Scheme 1). 7,8

Investigations of this unique carbon−carbon bond cleavage reaction have shown that HEPD is a non-heme mononuclear iron-dependent dioxygenase that requires only ferrous iron and molecular oxygen for activity. 8 Studies using 2-HEP isotopologues, 18O2 and H2,18O identified formate as an additional product and led to two proposed mechanisms (Scheme 2). 8 In each case, O2 activation is postulated to generate a ferric superoxo species. 9 After H-atom abstraction from C2 of 2-HEP to generate substrate radical I, the mechanisms differ in that the substrate is either hydroxylated with formation of an Fe(IV)=O intermediate or hydroperoxylated to form Criegee intermediate III. Experiments in which substrate analogues were incubated with HEPD resulted in the direct observation of Criegee rearrangement products. 10 These results, in combination with the expected high pKa of the anion in intermediate II, suggesting a high-energy intermediate, 11,12 previously led us to favor the hydroperoxylolation mechanism. 10 However, this proposal requires a very unusual hydrolysis of the intermediate formyl ester to account for the partial incorporation of oxygen from the solvent into the hydroxyl group of HMP. 8 To further investigate the mechanism of the reaction, in this study we examined the stereochemical course of catalysis at C1 and C2 of 2-HEP. If the reaction proceeds by a hydroperoxylolation mechanism, net inversion of configuration should occur at C1 with retention during the Criegee rearrangement 13 and inversion in the hydrolysis step.

A previous investigation showed that both hydrogen atoms at C1 were retained in HMP when 2-[1-2H2]-HEP was used as a substrate. 8 To complement this experiment, 2-[2-2H2]-HEP was synthesized (Scheme S1 in the Supporting Information) and incubated with HEPD. The HMP and formate produced were characterized as previously described, 8,10 and it was found that the formate contained deuterium but the HMP did not (Figure S1 in the Supporting Information), thus confirming that the putative hydrogen atom abstraction is regioselective. To test the stereospecificity of this step, (R)- and (S)-2-[2-2H2]-HEP were synthesized 16 and incubated with HEPD. The formate produced from (R)-2-[2-2H2]-HEP contained deuterium, whereas that produced with the S enantiomer did not (Figure S2). These observations show that the pro-S hydrogen is abstracted, which agrees well with the active-site geometry and bidentate substrate binding observed in the cocrystal structure of Cd(II)−HEPD with 2-HEP. 8

We next prepared (R)- and (S)-2-[1-2H1]-HEP (84 and 86% ee, respectively; 99% isotopic purity for both compounds) 15 as substrates for HEPD. Moreover, authentic (R)- and (S)-[1,2H1]-HMP with 99% ee were prepared from (R)- and (S)-disopropyl hydroxy-[1,2H1]-methylphosphonate 18 (Scheme S2). The compounds were deprotected using TMSBr/allylsilane 19 followed by hydrolysis of the formed silyl esters. For storage and later use, the free phosphonic acids were converted to the ammonium salts. To prepare the (R)-Mosher esters (i.e., the esters of (R)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA)), the free acids were esterified at the phosphate group with a distilled ethereal solution of diazomethane to produce dimethyl hydroxy-[1,2H1]-methylphosphonates, and the hydroxyl groups were subsequently esterified with (S)-MTPACl/pyridine.

Scheme 1. 2-Hydroxyethylphosphonate (2-HEP) Is Converted to Hydroxymethylphosphonate (HMP) by HEPD during Phosphinothricin (PT) Biosynthesis

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Synthetic (R)- and (S)-2-[1-2H1]-HEP were incubated with HEPD, and the produced [2H1]-HMP was derivatized to the dimethyl ester, which was purified to a few milligram quantities of dimethyl [2H1]-HMP. The stereochemistry of this product was assessed by conversion to the corresponding Mosher’s ester as described above and subsequent 1H NMR analysis. Unexpectedly, comparison with the Mosher’s esters prepared from authentic (R)- and (S)-[2H1]-HMP showed that HEPD converted both (R)- and (S)-2-[1-2H1]-HEP into near-racemic [2H1]-HMP (Figure 1 and Scheme 3a). Because of this unexpected outcome, further experiments were conducted to ensure that the stereochemical information was not lost during conversion of HMP to its phosphonate dimethyl ester or its Mosher’s ester.

Previous experiments demonstrated that HMP is also a substrate for HEPD that is slowly converted to phosphate and formate.10 We first determined the stereochemistry of this reaction. When (R)-[2H1]-HMP was incubated with HEPD, the generated formate contained no deuterium, whereas when (S)-[2H1]-HMP was used, the formate produced contained 1 equiv of deuterium. After it had been established that oxidation of HMP is stereospecific, (R)- and (S)-2-[1-2H1]-HEP were incubated with HEPD until full conversion of both 2-HEP and the initial HMP product to phosphate and 2 equiv of formate was achieved (Scheme 3b). If the conversion of 2-HEP to HMP were stereospecific at C1, the total formate produced from (R)- and (S)-2-[1-2H1]-HEP should contain either 50% deuterium or no deuterium. However, we observed experimentally that both enantiomers of 2-[1-2H1]-HEP resulted in 25% deuterium incorporation in the formate. This finding is consistent with racemization at C1 during the conversion of 2-HEP to HMP (Scheme 3b).

A large kinetic isotope effect (KIE) on H/D abstraction from HMP could potentially be a complicating factor in these experiments. Therefore, we measured the kinetics of oxidation of (R)-[2H1]-HMP in comparison with unlabeled HMP using an oxygen electrode. As shown in Figure 2, this reaction exhibited a substantial primary KIE of 7.6 (0.4 at saturating HMP concentrations. This observed isotope effect provided the opportunity to confirm racemization at C1 with one more experiment. (R)- and (S)-2-[1-2H1]-HEP were separately incubated with HEPD until all of the 2-HEP was consumed, as determined by 31P NMR spectroscopy. The resulting deuterium-labeled HMP was then used as substrate for a kinetics experiment, and
the rate of oxidation was measured at saturating HMP concentrations. Comparison with the observed rate of oxidation of unlabeled HMP under identical conditions showed that the HMP produced from (S)-2-[1-2H1]-HEP displayed an observed KIE of 2.4 ± 0.2 and the HMP produced from (R)-2-[1-2H1]-HEP displayed an observed KIE of 2.5 ± 0.3. Importantly, a 1:1 mixture of authentic (R)– and (S)-[2H1]-HMP displayed an observed KIE of 2.2 ± 0.2 under the same conditions, agreeing well with the expected value of 2.4 for a racemic mixture (see the Supporting Information). Thus, all of the experiments point to the same conclusion: the stereochemo-

cal integrity at C1 of 2-HEP is lost during the transformation to HMP.

The loss of stereochemistry can be rationalized in several ways. First, the hydroxylation mechanism in Scheme 2 may still be operational, but the carbocation formed upon the retro-Claisen-like step may have a lower barrier for rotation than an enolate, resulting in loss of stereochemical information. Alternatively, the electron-rich carbocation in intermediate II may be oxidized by proton-coupled electron transfer to provide ferric hydroxide and product radical IV, which could recombine in much the same way as a rebound-type mechanism often invoked for other iron-dependent enzymes (Scheme 4A).20,21 The radical would be expected to have a lower barrier for rotation than the carbanion, potentially explaining the observed racemization.

An alternative explanation for racemization is shown in Scheme 4B. Here the initial radical at C2 could undergo electron transfer to the iron center to generate an aldehyde. Subsequent nucleophilic attack by the peroxide at the carbonyl would then produce bridged alkyperoxide V. Similar mechanisms have been proposed for isopenicillin N synthase-catalyzed oxidation of a substrate analogue,22 for the reaction catalyzed by Clostridium propionicum myo-inositol oxidase (MIOX),25 and very recently for HEPD in a computational study.26 In the last of these reports, the bridged peroxide was proposed to cleave homolytically,26 resulting in a geminal diolate radical that could generate the same intermediate IV depicted in Scheme 4A, in which the stereochemical information at C1 may be lost.

In summary, this investigation has provided new insights into the novel chemistry catalyzed by HEPD. Abstraction of a hydrogen atom from C2 has been shown to proceed stereo-specifically. Interestingly, however, all of the stereochemical information at C1 of 2-HEP is lost in HMP. Thus, the hydroperoxylation mechanism, previously proposed on the basis of the direct observation of the product of a Criegee rearrangement with the substrate analogue 1-HEP,10 cannot be operational in the conversion of 2-HEP to HMP.

ASSOCIATED CONTENT

Supporting Information. Synthetic procedures, spectral characterization of compounds, and procedures for enzymatic assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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