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Review

Structure and mechanism of enzymes involved in biosynthesis and breakdown of the phosphonates fosfomycin, dehydrophos, and phosphinothricin

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ABSTRACT

Recent years have seen a rapid increase in the mechanistic and structural information on enzymes that are involved in the biosynthesis and breakdown of naturally occurring phosphonates. This review focuses on these recent developments with an emphasis on those enzymes that have been characterized crystallographically in the past five years, including proteins involved in the biosynthesis of phosphinothricin, fosfomycin, and dehydrophos and proteins involved in resistance mechanisms.

Introduction

The recent surge in available genomic information for a wide range of bacteria has coincided with, and at the same time stimulated, a strong renewed interest in natural product biosynthesis. Many recent genome-aided studies have focused on the products of non-ribosomal peptide synthesis, polyketides, isoprenoids, and ribosomally synthesized natural products [1–3]. By comparison, relatively little was known until recently about the biosynthetic pathways leading to phosphonate natural products. Seminal pioneering studies by Seto and coworkers established that the C–P bond in most phosphonates is formed by the enzyme phosphoenolpyruvate (PEP) mutase [4], which converts PEP to phosphonopyruvate (PnPy, Fig. 1), and that the biosynthetic pathways of this class of compounds contain many unusual chemical transformations [5,6]. Recent years have seen the characterization of the first biosynthetic clusters for phosphonates, including phosphinothricin [7,8], fosfomycin [9], FR900098 [10], dehydrophos [11], and rhizocticin [12] (Fig. 1). The genetic information in turn has allowed the elucidation of the biosynthetic pathways and study of individual enzymes. In parallel, the use of phosphonates in the clinic has fueled studies of emerging resistance mechanisms. This review will focus on recent developments in phosphonate biosynthesis and resistance with emphasis on those enzymes that have been investigated mechanistically and characterized crystallographically.

Phosphinothricin biosynthesis

Metcalf and coworkers recently revised the early steps in the biosynthesis of phosphinothricin [13], an unusual phosphinate containing two C–P bonds (Fig. 1). Phosphinothricin is the active ingredient of commercial herbicides as Herbiace and BASTA, and is used in combination with LibertyLink genetically modified crops including corn, canola, soybean, and cotton. Phosphinothricin mimics the tetrahedral intermediate generated in Gln synthase [14,15] by using a monoanionic phosphinate group containing two C–P bonds. One of the revised biosynthetic steps features the conversion of 2-hydroxyethyl phosphonate (2-HEP)[1] to hydroxymethylphosphonate (HMP, Fig. 2A). *In vitro* reconstitution of this

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[1] *Abbreviations used:* 2-HEP, 2-hydroxyethylphosphonate; 2-HPP, (2*S*)-hydroxypropylphosphonate; SAM, *S*-adenosylmethionine; HEPD, 2-hydroxyethylphosphonate dioxygenase; HppE, (2*S*)-hydroxypropylphosphonate epoxidase; DhpI, phosphonate methyltransferase; FosA/B, fosfomycin thiol transferases; FosX, fosfomycin hydrolase; HMP, hydroxymethylphosphonate; SAH, *S*-adenosylhomocysteine.

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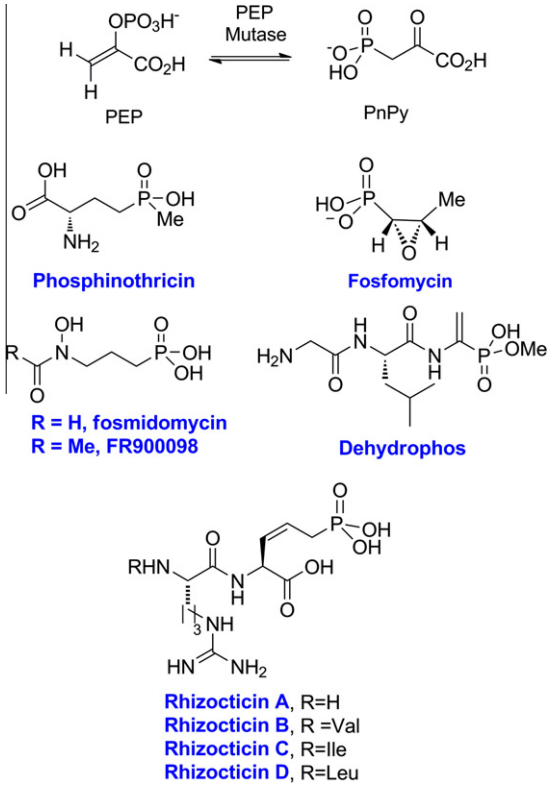

Fig. 1. Phosphorus–carbon bond formation by PEP mutase and structures of the phosphonate natural products for which the gene clusters have been determined.

transformation catalyzed by the enzyme encoded by the *phpD* gene demonstrated that the protein only requires ferrous ion and oxygen for activity, and that 2-HEP is converted into HMP and formate [16]. Experiments with ^{18}O-labeled molecular oxygen showed that the label is incorporated into both HMP and formate, demonstrating the enzyme is a 2-hydroxyethylphosphonate dioxygenase (HEPD). However, about 40% of the hydroxyl oxygen in HMP was derived from solvent, requiring a solvent exchangeable intermediate during catalysis. Use of deuterium labeled substrates showed that the enzyme abstracts a hydrogen atom from C2, and that the hydrogens on C1 are retained in the HMP product.

The X-ray crystal structure of HEPD, determined to a resolution of 1.8 Å (Fig. 2B), demonstrates that the polypeptide is composed of two repeats of a cupin domain [16]. This structural homology is unexpected given that the enzyme does not show sequence similarities to other proteins that share the cupin architecture. Members of the cupin superfamily are non-heme mononuclear iron-dependent proteins that adopt a characteristic antiparallel β-barrel fold. The two cupin domains in HEPD are decorated with a helical region consisting of roughly five α-helices and this topology of a cupin domain fused to a helical region is unique to HEPD and HppE, an iron-dependent enzyme involved in the biosynthesis of fosfomycin (see below).

Although the structure of HEPD consists of two structurally intertwined repeats of this fold, only one of the two repeats contains a functional metal-binding site made up of the characteristic 2-His-1-Glu facial triad composed of residues His129, Glu176, and His182. As crystallization was conducted in the presence of a cadmium salt, a Cd(II) ion is positioned at the metal-binding site with three solvent molecules completing the six-coordinate metal center. The corresponding metal-binding site in the second repeat lacks this facial triad and is further occluded by residues Tyr359 and Lys404. Mutational studies document that residues within this vestigial metal site are not essential for catalytic activity. The crystal structure of Cd(II)-HEPD in complex with 2-HEP was solved to 1.9 Å resolution and reveals bi-dentate coordination of the substrate to the metal. Although substrate binding does not induce any major structural reorganization of the polypeptide, the side chain of Tyr98 undergoes a torsional rotation to form a hydrogen bond with one of the phosphonate oxygen atoms of the substrate, with Asn126 providing an additional hydrogen bond with the substrate.

On the basis of the mechanistic studies and crystallographic information, two mechanisms were proposed for the conversion of 2-HEP to HMP and formate [16] (Fig. 3A). For both, the ferrous enzyme reacts with oxygen to form a formally ferric-superoxo species **I** that abstracts a hydrogen atom from the substrate. The resulting substrate radical can then either be hydroxylated or hydroperoxylated. In the former case, an acetal results that can undergo a retro-Claisen type reaction to provide formate and the stabilized anion in intermediate **II**, which may attack the electrophilic ferryl species resulting in HMP. In the case of hydroperoxylation, a Criegee rearrangement would afford ester **III**, which upon hydrolysis would also provide HMP and formate. For both mechanisms exchange of oxygen from O_2 with solvent can be explained but by different mechanisms. For the hydroxylation pathway, the ferryl intermediate in **II** can undergo exchange as reported previously for other systems [17–19]. For the hydroperoxylation mechanism, exchange requires an unusual hydrolysis of the ester **III** via attack of hydroxide (which can exchange with solvent) on C1 rather than at the carbonyl carbon. Experiments with a series of substrate analogs provide direct support for hydroperoxylation. For instance, with 1-hydroxyethyl phosphonate, the direct product of the Criegee rearrangement step, 1-acetylphosphate, was produced (Fig. 3B) [20].

Fosfomycin biosynthesis

Fosfomycin is a clinically used antibiotic that inhibits UDP-GlcNAc enolpyruvoyl transferase (MurZ), an essential enzyme in cell wall biosynthesis, via a covalent mechanism [21]. The biosynthetic gene cluster in *Streptomyces wedmorensis* has been characterized [22,23], as has its biosynthetic pathway. The most interesting of the biosynthetic enzymes that have been studied thus far is an unusual epoxidase that converts (2*S*)-hydroxypropyl phosphonate (2-HPP) to fosfomycin (Fig. 2A). During this transformation, the hydroxyl oxygen of 2-HPP ends up in the epoxide [24]. Liu and co-workers have extensively studied this process [25–30] and Drennan and co-workers solved the structure of the enzyme [31].

The crystal structure of the oxidative cyclase consists of a single β-barrel containing cupin domain augmented with a novel helical region composed of five α-helices (Fig. 2C). The facial triad of HppE consists of His138, Glu142 and His180 and crystallographic studies of Fe(II)-HppE revealed a hexacoordinate metal with three solvent molecules completing the coordination sphere. Structural studies of catalytically inert aerobic Co(II)-HppE and active Fe(II)-HppE, obtained anaerobically, showed two modes of binding for the substrate 2-HPP suggestive of a precatalytic two-step binding process. Within the two copies of the polypeptide in the crystallographic asymmetric unit, one molecule shows a monodentate coordination of a phosphonate oxygen to the metal and the second shows a bidentate interaction. Notably, bidentate coordination of the substrate is accompanied by a reorganization of an active site loop that results in the reorganization of active site residue Tyr105 to form a hydrogen bond with one of the oxygen atoms in the 2-HPP substrate. Interestingly, substrate binding in HEPD induced a torsional rotation in the side chain of the equivalent Tyr98 to similarly form a hydrogen bond interaction with an oxygen atom in the substrate 2-HEP.