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Mechanism-Based Inactivation of Benzoylformate Decarboxylase, A Thiamin Diphosphate-Dependent Enzyme

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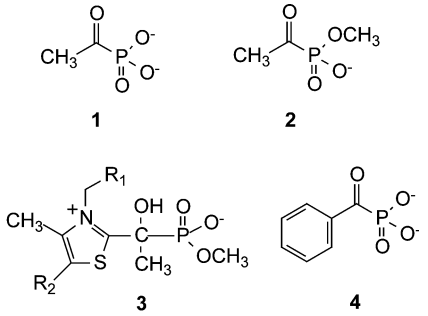
Benzoylformate decarboxylase from *Pseudomonas putida* (BFD; EC 4.1.1.7) belongs to a family of thiamin diphosphate (ThDP, vitamin B1)-dependent enzymes that catalyze the non-oxidative decarboxylation of 2-keto acids (Scheme 1).[1] Mechanism-based inhibitors of such thiamin-dependent decarboxylases are rare.[2] In this communication, we report the discovery of a mechanism-based inhibitor of benzoylformate decarboxylase that covalently modifies the enzyme via active site phosphorylation, a type of mechanism-based inactivation that is virtually unprecedented.

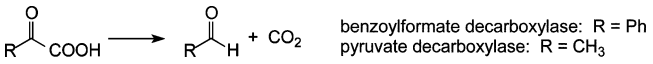
There are several instances where phosphonate analogues of 2-keto acids have proved to be effective inhibitors of non-oxidative decarboxylation.[3] In particular, acetylphosphonate (**1**) and its monoester, methyl acetylphosphonate (**2**), have been shown to inhibit enzymes catalyzing the decarboxylation of pyruvate via the reversible addition of the cofactor to these inhibitors to form structures such as **3**.[3a,b]



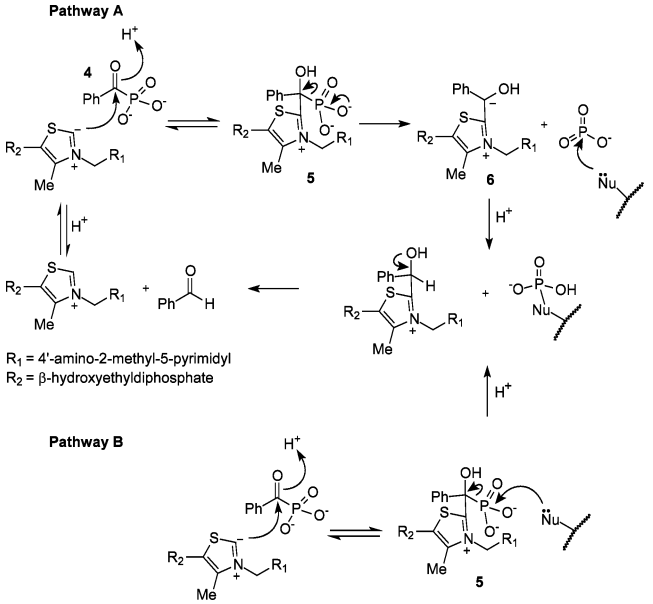
We hypothesized that benzoylphosphonate (**4**), the phenyl analogue of **1**, might function as a mechanism-based, irreversible inactivator of BFD. It was thought that, as shown in Scheme 2, **4** would undergo nucleophilic attack by the ylid of ThDP to provide the adduct, **5**. However, unlike **3**, **5** has an anion-stabilizing phenyl group. This gives **5** the potential for a more facile elimination of metaphosphate which might lead to enzyme phosphorylation and, concomitantly, irreversible inhibition. Two possible pathways for this process are shown in Scheme 2.

To explore this possibility, **4** was prepared using a modification of the method of Karaman et al.[4] and was found to act as a competitive inhibitor of BFD. The K_i value of 0.36 ± 0.03 mM indicated that it was likely to be acting as a ground-state analogue of benzoylformate, which has a K_m value around 0.4 mM.[1e] More

Scheme 1



Scheme 2



importantly, it was also observed that prolonged incubation of BFD with **4** resulted in permanent inactivation of the enzyme. Further investigation showed that the inactivation of BFD was both time- and concentration-dependent (Figure 1A). The data could most simply be described by the scheme in which **4** initially binds reversibly to the benzoylformate binding site, and in a subsequent slower reaction, BFD is inactivated:

$$E + I \overset{K_i}{\rightleftharpoons} EI \xrightarrow{k_{inact}} E - I$$

Kitz and Wilson[5] (Figure 1B) analysis of the inactivation data, using eq 1, provided an inactivation rate constant (k_{inact}) of 0.0185 min^{-1}, that is, a half-life of ca. 38 min at 30 °C.

$$k_{obs} = \frac{k_{inact}[I]}{K_i + [I]} \quad (1)$$

To identify the residue(s) affected by reaction with **4**, a sample of the inactivated BFD was crystallized at pH 7.0 using the hanging drop vapor diffusion method. The inactivated enzyme crystallized in space group *I*222, the same form as that of the WT BFD crystal used to determine the original BFD structure (PDB entry 1BFD).[6]

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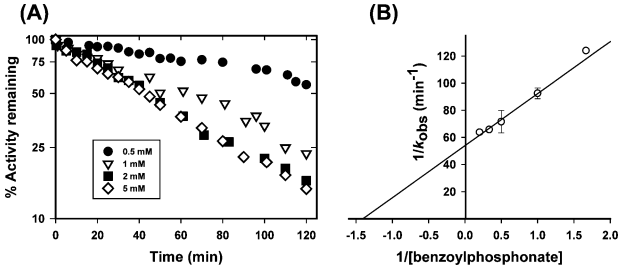

Figure 1. (A) Inactivation of BFD showing saturation at increasing concentrations of benzoylphosphonate (**4**). (B) Reciprocal (Kitz and Wilson[5]) plot of 1/observed k_{inact} versus 1/[benzoylphosphonate]. The solid line represents the fit of the data to eq 1 and provides a K_i of 0.71 mM and a k_{inact} of 0.0185 min^{-1}.

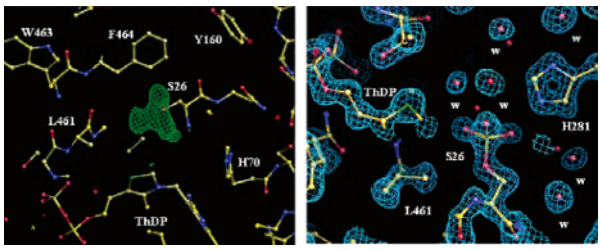

Figure 2. (A) View of the active site region near Ser26 in **4**-modified BFD. The unbiased $F_o - F_c$ (green, 3.5 σ) electron density at 1.39 Å resolution is based on phases from the BFD model before addition of the phosphate group. (B) $2F_o - F_c$ (blue) electron density map contoured at 1.5 σ.

The crystals of the modified enzyme diffracted to 1.39 Å. Significant electron density was found near Ser26 (Figure 2A) with the $2F_o - F_c$ electron density map clearly showing that Ser26 had been phosphorylated, with the phosphorus atom being covalently attached to the γ-oxygen of Ser26 (Figure 2B). The cofactor was found to be intact, and there was no evidence for benzaldehyde remaining in the active site. Coordinates and structure factors for the modified enzyme are deposited as Protein Data Bank entry 2FWN.

Both site-directed mutagenesis and X-ray crystallography have shown that Ser26 is important in the catalytic mechanism of BFD.[1e] Based on the structure of BFD in complex with the substrate analogue, R-mandelate, the Ser26 side chain was predicted to interact with the carboxyl group of the substrate benzoylformate. Further, the BFD S26A variant showed more than 20-fold increase in K_m value for benzoylformate as well as a 50-fold decrease in the value of k_{cat}.[1e] Presumably, phosphorylation of Ser26 would render the active site unavailable for productive binding of benzoylformate, resulting in irreversible inhibition. This suggestion would also lead to the prediction, subsequently proven to be correct, that the BFD S26A variant would not be irreversibly inactivated by **4** (data not shown).

Phosphonates have been used extensively as inhibitors of various enzyme types, including serine proteases,[7] protein kinases,[8] and phosphatases.[9] Generally, the inhibitory properties of the phosphonates are based on their ability to mimic the tetrahedral intermediates of the enzymatic reactions while maintaining chemical stability.[10] By contrast, it has been shown that **1** reversibly inhibits the decarboxylase activity of both pyruvate oxidase and pyruvate dehydrogenase simply by acting as an analogue of pyruvate. Given the mechanistic similarities between PDC and BFD, it is notable that the phenyl analogue of **1**, benzoylphosphonate (**4**), displays a distinctly different pattern of inhibition with BFD. It seems likely that two important factors govern this difference in reactivity. First, the conjugate base of hydroxybenzyl thiamin diphosphate (HBThDP, **6**) is more stable than that of its pyruvate-derived analogue (the pK_a of HBThDP is ca. 2 units lower than that of HEThDP, F. Jordan, personal communication). Therefore, **6** could serve as a sufficiently good leaving group to enable direct phosphoryl transfer to an active site nucleophile (Scheme 2). Second, the presence of a nucleophilic serine prepositioned to attack the phosphoryl group may be a requirement for the phosphoryl transfer process. Such nucleophilic precoordination to phosphorus is known to be a requirement for the mechanistically similar Conant−Swan fragmentation.[11]

In summary, we suggest that **4** acts as a mechanism-based inhibitor of BFD, with inactivation resulting from the phosphorylation of Ser26.

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Supporting Information Available: Experimental details and crystallographic statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

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