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Active-Site Engineering of Benzaldehyde Lyase Shows That a Point Mutation Can Confer Both New Reactivity and Susceptibility to Mechanism-Based Inhibition

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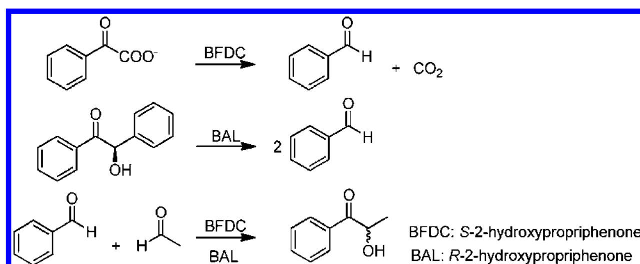
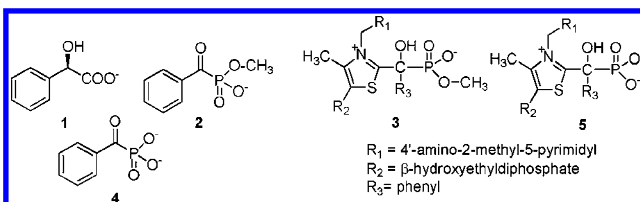
Benzoylformate decarboxylase (BFDC, EC 4.1.1.7) and benzaldehyde lyase (BAL, EC 4.1.2.38) are thiamin diphosphate (ThDP, vitamin B1)-dependent enzymes that share 24% sequence identity. Both act on aromatic substrates (Scheme 1). BFDC from *Pseudomonas putida* catalyzes the decarboxylation of benzoylformate, producing benzaldehyde and carbon dioxide,[1] whereas BAL from *Pseudomonas fluorescens* Biovar I catalyzes the decomposition of (*R*)-benzoin to 2 equiv of benzaldehyde.[2] The two enzymes are of interest to the synthesis community because, as shown in Scheme 1, they can also carry out stereospecific carboligation reactions, often providing products with the opposite stereochemistry.[3]

Scheme 1



Analogues of benzoylformate have proved to be useful in identifying residues involved in substrate binding and catalysis. For example, (*R*)-mandelate (**1**, Scheme 2) binds reversibly in the BFDC active site, and the X-ray structure of the BFDC−**1** complex, as well as mutagenesis studies, suggest that Ser26 provides a hydrogen bond to the carboxylate of the substrate.[4]

Scheme 2



2-Ketophosphonates have been shown to be effective inhibitors of enzymes that catalyze nonoxidative phosphorylation.[5] Methyl benzoylphosphonate (MBP, **2**) was found to be converted by BFDC to the stable predecarboxylation intermediate analogue **3**, thus inactivating the enzyme.[6,7]

Benzoylphosphonate (BP, **4**), the dianionic analogue of benzoylformate, was also found to inactivate BFDC. However, in an unprecedented result, the inactivation was found to result from the phosphorylation of Ser26, possibly via reaction of Ser26 with metaphosphate eliminated from the intermediate analogue **5**.[8] Methyl metaphosphate is more difficult to generate than metaphosphate,[9] which explains the relative stability of **3** in comparison with **5**. The phosphorylation of Ser26 is made more intriguing by a recent suggestion that this residue may act as the nucleophile in a carbon dioxide trapping mechanism.[10] Such a mechanism, originally proposed by Sauers, Jencks, and Groh for the biotin-dependent decarboxylases,[11] would prevent back-reaction of carbon dioxide, thereby promoting separation of CO_2 from the BFDC active site.[10]

Circular dichroism analysis indicates that wild-type (wt) BAL can also decarboxylate benzoylformate,[12] but the activity level is too low to be measured by the standard coupled assay.[13] In accord with this low level of decarboxylase activity, wt BAL was also found to react with **2** to form **3**.[14] However, it showed no reaction with **4** (data not shown). Examination of their active sites showed that Ser26 and Ala28 occupied similar positions in BFDC and BAL, respectively. Replacement of Ala28 by serine provided a BAL variant whose K_m value for (*R*)-benzoin was virtually identical to that of the wt enzyme but whose k_{cat} value was reduced ~10-fold.[13] More importantly, the A28S variant was readily able to decarboxylate benzoylformate (K_m = 20 mM, k_{cat} = 1.2 s^{-1})[13] and was found to have a substrate spectrum comparable to that of BFDC, albeit with reduced activity.[15]

On the basis of the X-ray structure of the BFDC−**1** complex,[4] it is likely that in BAL A28S, the newly introduced serine forms a hydrogen bond to the carboxylate of benzoylformate. Potentially, an enhanced ability to bind benzoylformate could be the sole reason for the increased decarboxylase activity observed with BAL A28S. Of course, if Ser28 also acts as the nucleophile in a carbon dioxide trapping mechanism, it is possible that, like Ser26 of BFDC, it will be phosphorylated by **4**.

To explore this possibility, BAL A28S was prepared[1] and reacted, in turn, with **2** and **4**. Steady-state analysis showed that the 2-ketophosphonates acted as competitive inhibitors of BAL A28S, with K_i values of ~0.13 and 1.2 mM, respectively. In comparison, the K_i values for BFDC were 0.38 and 0.36 mM, respectively.[6,8] At higher inhibitor concentrations, some deviation from linearity in the progress curves was observed, suggestive of time-dependent inactivation. Dilution studies showed that the inactivation of BAL A28S by **2** and **4** (Figure 1A,B, respectively) was both time- and concentration-dependent but gave no evidence of saturation.

Kitz and Wilson (double-reciprocal) plots[16] of the data in Figure 1 provided straight lines with *y*-intercepts approaching zero (data not shown). This suggests that only weak complexes are formed between BAL A28S and the two inhibitors and that inactivation is fast relative to the rate of complex formation.[16] Plots of k_{inact} versus

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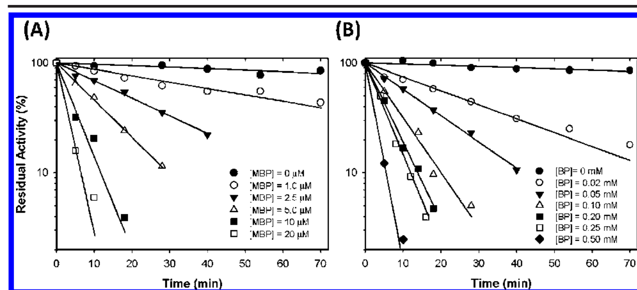

Figure 1. Inactivation of BAL A28S with increasing concentrations of (A) methyl benzoylphosphonate (**2**) and (B) benzoylphosphonate (**4**).

inhibitor concentration provided second-order rate constants of 1.8 × 10^4 and 7.9 × 10^2 M^{-1} s^{-1} for **2** and **4**, respectively (see the Supporting Information). The former result is consistent with that obtained earlier for the reaction of **2** with wt BAL,[14] which had a rate constant of 8.9 × 10^2 M^{-1} s^{-1}.

The inactivation of wt BAL and BFDC by **2** was attributed to formation of the stable adduct **3**,[6,7,14] whereas inactivation of BFDC by **4** was attributed to phosphorylation of the active-site serine.[8] To compare the modes of inactivation of BAL A28S by **2** and **4**, we turned to X-ray crystallography. Cocrystallization of BAL A28S with the two inhibitors was undertaken as previously described for BAL.[14] The coordinates and structure factors for **2**- and **4**-modified BAL A28S have been deposited as Protein Data Bank entries 3IAE and 3IAF, respectively.

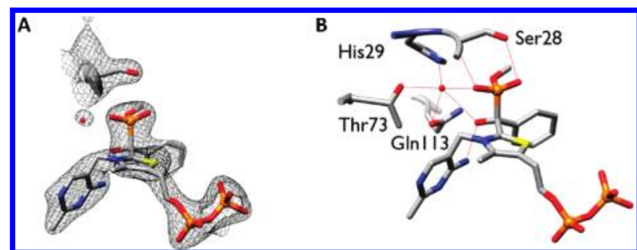

Figure 2. Structure of **2**-modified BAL A28S. (A) Electron densities of Ser 28 and the ThDP−**2** adduct. The mesh represents a $2F_o - F_c$ simulated annealing omit map contoured to 1.5σ. (B) Likely hydrogen-bonding interactions.

The X-ray structure of BAL A28S treated with **2** (Figure 2A) revealed the covalent adduct **3**, which is similar to that observed for wt BFDC,[6,7] BAL,[14] and other ThDP-dependent enzymes.[17,18] The newly introduced serine is positioned appropriately to hydrogen bond with the phosphonate oxygen of the inhibitor (Figure 2B). Comparison of this structure to that of the wt BAL−**3**[14] complex provides an overall active-site root-mean-square deviation of 0.20 Å. The exceptional degree of spatial conservation of other active-site residues between the wt and A28S variant is strong evidence that Ser28 is solely responsible for the added reactivity toward benzoylformate.

The structure of BAL A28S treated with **4** (Figure 3) shows two striking differences from that of the enzyme treated with **2**. First, there is no evidence for covalent modification of the cofactor at C2 of the ThDP thiazolium ring. Second, a simulated annealing omit map, using wt BAL as the model, shows considerable electron density contiguous with the side chain of Ala28. As with BFDC treated with **4**,[8] the additional electron density is best explained by phosphorylation of the adjacent serine residue, here Ser28. It is notable that in the two structures the active-site water occupies an almost identical position and bonding network.

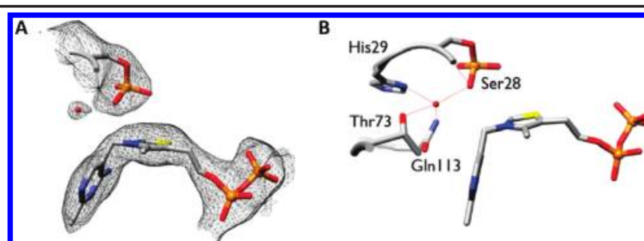

Figure 3. Structure of **4**-modified BAL A28S. (A) Electron density of the phosphorylated Ser28. The mesh represents a $2F_o - F_c$ simulated annealing omit map contoured to 1.5σ. (B) Likely hydrogen-bonding interactions.

Taken together, these data demonstrate that a point mutation can convert BAL into a true BFDC. Not only does BAL A28S decarboxylate benzoylformate, but also, in a highly unusual reaction previously observed only with BFDC, the newly introduced serine residue is phosphorylated by benzoyl phosphonate (**4**). This confirms that **4** is a novel mechanism-based inactivator of ThDP-dependent decarboxylases and lends further credence to the suggestion that Ser26 may play a role in removing CO_2 from the active site of BFDC.[4,10] It is notable that the majority of ThDP-dependent decarboxylases contain an active-site nucleophile positioned appropriately to facilitate the expulsion of carbon dioxide. Consequently, the trapping mechanism may hold true for the broad spectrum of ThDP-dependent decarboxylases, certainly for those that give rise to an aldehyde as a product.

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

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