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Reactivity of Intermediates in Benzoylformate Decarboxylase: Avoiding the Path to Destruction

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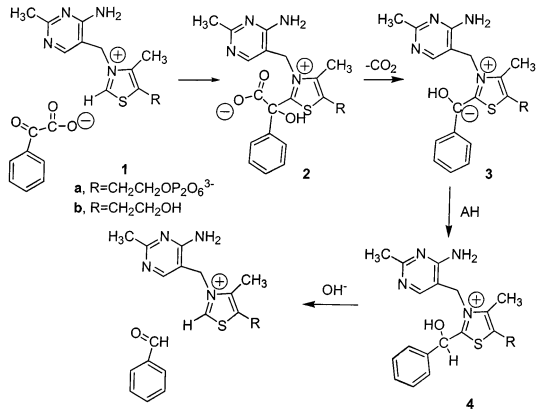
Enzymes that promote the decarboxylation of 2-oxocarboxylic acids utilize thiamin diphosphate (TDP, **1a** in Scheme 1) as a cofactor, following the general pathway that was deduced by Breslow.[1−3] Benzoylformate decarboxylase (BFD) is a bacterial TDP-dependent decarboxylase that has been the subject of structural and mechanistic studies.[4−9] On the basis of the pattern of reactions of α-ketoacid decarboxylases, we assume that α-mandelylTDP (MTDP, **2a**) forms on the enzyme from TDP and benzoylformate, permitting the release of carbon dioxide, to produce a carbanion-enamine (**3a**).[9] This is protonated to give 2-(1-hydroxybenzyl)TDP, HBzTDP (**4a**), the precursor of benzaldehyde.

To study the reactivity of intermediates in the benzoylformate decarboxylation process, we developed a synthesis of α-mandelylthiamin (MT, **2b**). Direct analogy to producing α-lactylthiamin from ethyl pyruvate and thiamin[11] did not produce MT. This was overcome by addition of magnesium chloride. Thus, thiamin hydrochloride (5.0 g, 15 mmol) was suspended in 80 mL of absolute ethanol, and 50 mL of anhydrous ethanol containing 2 equiv of sodium ethoxide was added. Ethyl benzoylformate solution, containing 8.2 mL of ethyl benzoylformate and 0.6 g of anhydrous magnesium chloride in 50 mL of absolute ethanol, was deoxygenated and immediately added under nitrogen to the basic thiamin solution. After 30 min of stirring at 0 °C, gaseous hydrogen chloride was added to acidify the solution, and the precipitate, consisting of sodium chloride and thiamin, was removed by filtration. The filtrate was concentrated, and the yellow liquid was dissolved in 25 mL of water and extracted with three 50 mL portions of dichloromethane to remove excess ethyl benzoylformate. The aqueous layer was stirred with Chelex for 1 h at pH 6 to remove magnesium chloride. After being filtered, the aqueous solution was lyophilized to dryness. The yellow solid was dissolved in methanol and passed through a 3 × 30 cm cellulose column and dried to give the product. ^{1}H NMR (400 MHz DCl in D_2O relative to internal DSS): δ 1.30 (3H, t, J = 7.2 Hz, CH_3CH$_2$OCO), 2.41 (3H, s, CH_3-pyrimidine), 2.49 (3H, s, CH_3-thiazole), 3.25 (2H, t, J = 5.8 Hz, CH_2CH$_2$OH), 3.94 (2H, t, J = 5.8 Hz, CH$_2$CH_2OH), 4.43 (2H, q, J = 7.2 Hz, CH$_3$CH_2OCO), 5.36 (1H, d, J = 18 Hz, H_aH$_b$CN), 5.86 (1H, d, J = 18 Hz, H$_a$$H_b$CN), 6.81 (1H, s, *H*-pyrimidine), 7.32 (3H, m, aromatic), 7.54 (2H, m, aromatic). The ethyl ester was hydrolyzed by dissolving in 12 M HCl and left for 3 days at room temperature. After concentration to remove excess hydrogen chloride and lyophilization, the MT chloride hydrochloride was obtained (stored dry at −20 °C). ^{1}H NMR (400 MHz, DCl in D_2O relative to internal DSS): δ 2.39 (3H, s, CH_3-pyrimidine), 2.49 (3H, s, CH_3-thiazole), 3.23 (2H, t, J = 5.8 Hz, CH_2CH$_2$OH), 3.94 (2H, t, J = 5.8 Hz, CH$_2$CH_2OH), 5.41 (1H, d, J = 18 Hz, H_aH$_b$-CN), 5.90 (1H, d, J = 18 Hz, H$_a$$H_b$CN), 6.82 (1H, s, *H*-pyrimidine), 7.30 (3H, m, aromatic), 7.55 (2H, m, aromatic).

Scheme 1. Thiamin and TDP Pathways for Benzoylformate



The observed first-order rate coefficient for decarboxylation of MT at pH 5, 6, and 7 (25 °C) is 3.1 (±0.3) × 10^{-4} s^{-1}. For comparison, the enzymic k_{cat} is 8.1 × 10^1 s^{-1}. Thus, the decarboxylation step is at least 200 000 times faster on the enzyme. This is similar to the acceleration in pyruvate decarboxylase.[11] We assume that the acceleration is achieved by desolvation of the intermediate as it is produced from thiamin diphosphate and the substrate.[10,11]

The decarboxylation of MT tests an important mechanistic issue. The carbanion that results from the loss of carbon dioxide from MT should be the same as that which arises from transfer of a proton from the C2α position of HBzT to a Brønsted base. It has been surmised that the species generated from HBzT fragments directly into pyrimidine and thiazole moieties[12−14] (Scheme 2, k_f): phenyl thiazole ketone, PTK, 5-(2-hydroxyethyl-4-methyl-thiazol-2-yl)-phenyl-methanone, and 2,5-dimethyl-pyrimidin-4-ylamine, DPA. This requires that loss of CO_2 from MT generates the carbanion at C2α,[15] whose protonation by Brønsted acids to give HBzT will compete with the uncatalyzed fragmentation.[14] Furthermore, because the decarboxylation step is rate determining, added acids should not affect the observed first-order rate coefficient for decomposition of MT. They will divert the resulting carbanion toward HBzT and away from PTK and DPA (Scheme 2).

Consistent with these predictions, significant amounts of PTK and DPA form from MT along with HBzT (pH 6.2, 0.03 M KP_i), appearing with the same rate coefficient (k_1). Furthermore, increasing the concentration of buffer increases the proportion of HBzT without affecting the observed rate constant (Figure 1).

From measurements of the reactivity of derivatives of HBzT,[14] we know that fragmentation of the C2α carbanion of pyrimidine-protonated HBzT (HBzT(H$^+$)) is very rapid: k_f is ∼10^5 s^{-1} at 40 °C (we assume $k_f \approx 10^3-10^4$ s^{-1} at 25 °C). Because we are working

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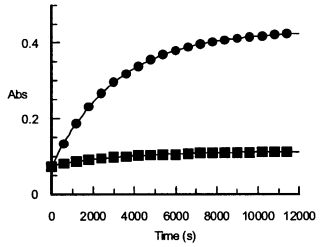


Figure 1. Absorbance at 328 nm (due to formation of the fragmentation product, PTK) from solutions of MT at 25 °C in 0.03 M pH 6.2 potassium phosphate buffer (●) and 0.4 M pH 6.2 potassium phosphate buffer (■). Initial concentrations of MT are identical, and ionic strength is 1.0. Both curves are fit to the first-order rate law and give $k_{obs} = 3.1 \pm 0.2 \times 10^{-4}$ s^{-1}.

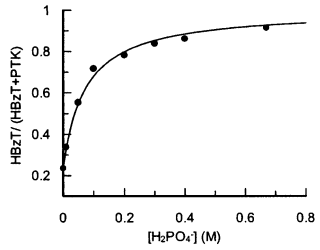
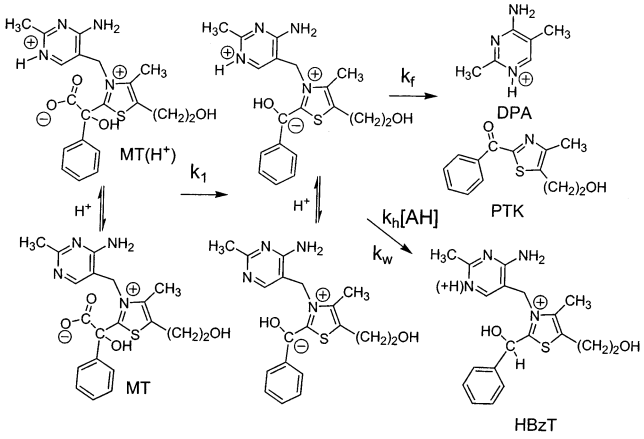


Figure 2. Relative initial yield of HBzT (as a fraction of the sum of the concentrations of PTK and HBzT) as a function of concentration at pH 6.16 (at which $[HPO_4^{2-}]/[H_2PO_4^-] = 0.5$) phosphate buffer following eq 1.

Scheme 2. Competitive Routes from Decarboxylation of MT



at pH > pK_a of the protonated pyrimidine of MT, the apparent magnitude of k_f is reduced by the proportion that is not protonated.[13]

Measuring the effects of a range of buffer concentrations on product distributions gives data that yield the apparent rate constant ratio for the competing pathways. The amount of PTK that is formed from decarboxylation is proportional to the absorbance at 328 nm, while the corresponding amount of HBzT can be measured by the final absorbance at 328 nm which includes the slow secondary reaction to give PTK and DPA from HBzT. (We heated the sample containing the products of the initial reaction at 80 °C for 8 h to obtain complete conversion.) The complete spectrum is that of the combination of PTK and DPA.

The data in Figure 2 give the relative yield of HBzT as a function of buffer concentration. We fit the data to eq 1, which is based on Scheme 2.

$$[\mathrm{HBzT}]/([\mathrm{HBzT}] + [\mathrm{PTK}]) = (k_w + k_h[\mathrm{AH}])/(k_f + k_w + k_h[\mathrm{AH}]) \quad (1)$$

From the fit of eq 1 we obtain

$$k_w/k_f = 0.18 \pm 0.02 \quad (2)$$

and

$$k_h/k_f = 15.0 \pm 1.5\ \mathrm{M}^{-1} \quad (3)$$

This indicates that in the absence of buffer, fragmentation is highly favored. High concentrations of Brønsted acids are needed to produce HBzT in reasonable yield.

Jordan and co-workers report that the 4-nitro analogue of the carbanion intermediate derived from MTDP is formed on the enzyme and can be observed spectroscopically.[9] It has a half-life that is several orders of magnitude longer than that which we observe for fragmenting the intermediate,[9] so its potential fragmentation is suppressed. Barletta et al. report that protonation of the conjugate base of a model of HBzT is very slow.[16] On the enzyme, an acidic residue may be poised to accomplish a proton transfer with great efficiency (after loss of carbon dioxide).[16,17]

In conclusion, we have shown that α-mandelylthiamin (MT), the conjugate of benzoylformate and thiamin, can be prepared and isolated. Its spontaneous decarboxylation is very slow as compared to the comparable step in catalysis in the enzymic reaction. The fragmentation of the intermediate that follows the loss of carbon dioxide from MT is as fast as the normal enzymic reaction. However, the enzyme avoids this route and instead produces benzaldehyde, regenerating TDP. The structural basis of BFD's productive diversion is an important feature that remains to be discovered.

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