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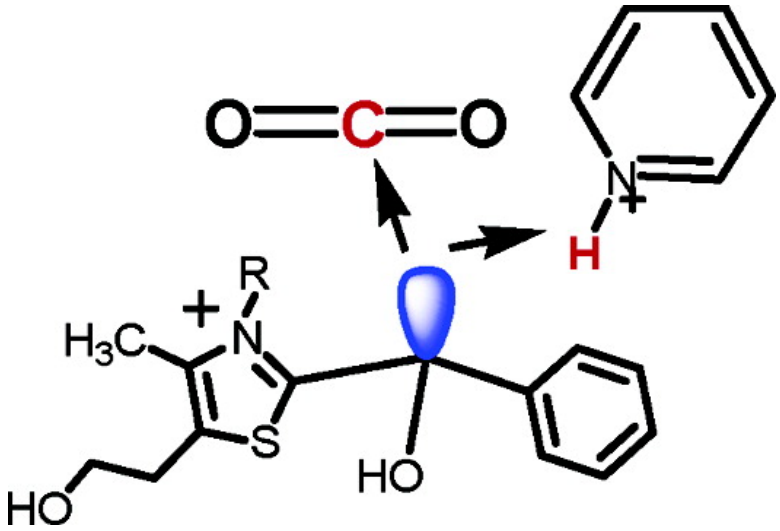

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Making Thiamin Work Faster: Acid-Promoted Separation of Carbon Dioxide

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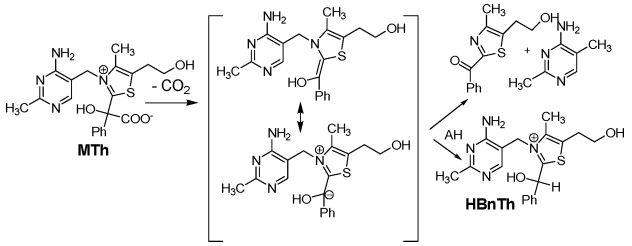
Almost 50 years ago, Breslow deduced that thiamin diphosphate (ThDP)-dependent 2-ketoacid decarboxylases form C-2 thiazolium ThDP conjugates with substrates to yield resonance-stabilized carbanions upon loss of CO_2.[1,2] The route has been experimentally confirmed,[3] but the intermediates themselves are not sufficiently reactive to account for the enzymic process.[4−7]

Mandelylthiamin (MTh) is the conjugate of thiamin and benzoylformate,[8] an analogue of the conjugate in benzoylformate decarboxylase (BFD).[9] Decarboxylation of MTh generates the C2α conjugate base of 2-(1-hydroxybenzyl)thiamin (HBnTh) with a first-order rate constant that is about 10^6 times smaller than k_{cat} for BFD.[8] Thus, delocalization of the carbanion does not account for the catalytic power of the enzyme. Furthermore, while BFD efficiently releases benzaldehyde and regenerates ThDP, the loss of CO_2 from MTh leads to a fragmentation reaction that destroys thiamin (in competition with protonation to form HBnTh).[10−15] The processes are summarized in Scheme 1. Since the rate constant for nonenzymatic fragmentation is considerably *larger* than k_{cat} for BFD,[12] the enzyme's mechanism for accelerating decarboxylation must coincidentally suppress fragmentation.

Scheme 1. Fragmentation Competes with Protonation after Loss of Carbon Dioxide



In the course of examining the reactivity patterns of MTh, we discovered that its decarboxylation is accelerated by certain buffers, although there is no apparent way that the C−C bond breaking might be stabilized by an acid or base. The data in Figure 1 show that the decarboxylation of MTh is accelerated by buffers containing pyridine or 4-picoline. We used solutions at pH = pK_a (pH 4.6−6.5), where the rate of decarboxylation of MTh is pH-independent ($k_{obs} = 3.2 \times 10^{-4}$ s^{-1}).

We tested whether the acceleration from pyridine and 4-picoline arises from their effect on solvent polarity, as desolvation should destabilize MTh.[6,7] We used ethanol as an additive with a dielectric constant similar to that of pyridine. The addition of ethanol (0.1−0.8 M) to 0.1 M phosphate buffer does not change significantly the observed rate coefficient for decarboxylation. *N*-ethyl pyridinium chloride was used to assess the magnitude of the nonspecific medium effect of pyridinium ions, and this is also minimal.

A plot of the observed rate constant for decarboxylation of MTh as a function of the mole fraction of the acid component indicates that this component is catalytic (Figure 2). We assume that curvature is the result of saturation in formation of the pyridinium complex.

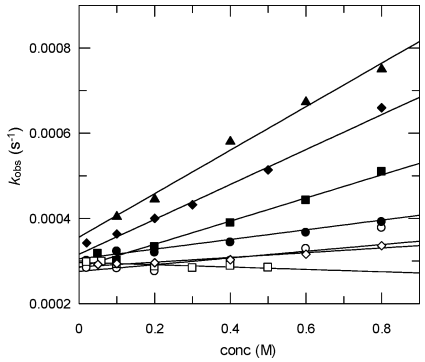


Figure 1. MTh decarboxylation in acetate (○), bis-tris (●), phosphate (□), pyridine (▲), and 4-picoline (◆) buffers, pH = pK_a of each buffer (acetate at pH 4.6, bis-tris at pH 6.7, phosphate at pH 6.5, 4-picoline at pH 6.0, and pyridine at pH 5.4). In the case of ethanol (◇), 0.1 M phosphate at pH 6.5 was used as buffer, and ethanol was added as a solvent component. In the case of ethyl pyridinium (■), 0.1 M phosphate at pH 6.5 was used as buffer, and ethyl pyridinium chloride was added as a component for maintenance of ionic strength.

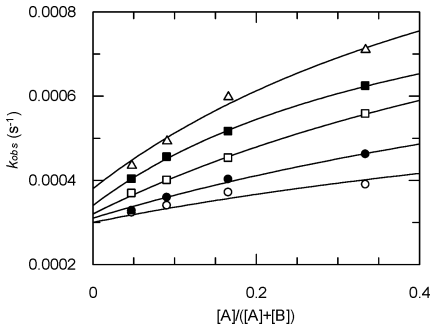


Figure 2. MTh decarboxylation rate constant in pyridine buffer versus [pyridinium ion]/[total pyridine]. The total pyridine concentration varied from 0.1 to 0.8 M (○ for 0.1 M, ● for 0.2 M, □ for 0.4 M, ■ for 0.6 M, and △ for 0.8 M). [A] refers to the acid component, pyridinium ion, and [B] refers to the base component, pyridine.

The derived second-order rate constant for catalysis by pyridine is 0.001 M^{-1} s^{-1}, and that for 4-picoline is 8×10^{-4} M^{-1} s^{-1}. A very small amount of complexation would give these apparent values, despite a much larger actual value in the complex.

The thiazolium ring of MTh has no site where a proton donor might reasonably associate to promote the loss of CO_2. However, if the back reaction is prevented by protonation prior to complete separation of CO_2, the overall reaction will be faster; diffusion of CO_2 is rate-limiting.[16] (Guthrie's analysis of decarboxylation by the no barrier theory provides a very clear picture of the detailed processes to consider, including changes in hybridization.[17]) Addition of CO_2 to the HBnTh carbanion must compete with separation of these species. This is reminiscent of the mechanism (in reverse) proposed for biotin-enzyme, ATP-driven carboxylation reactions.[18] Where "low entropy CO_2" is generated in situ, an adjacent nucleophile will react most efficiently. The mechanistic issues of

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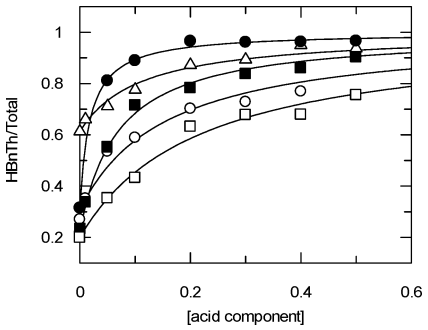
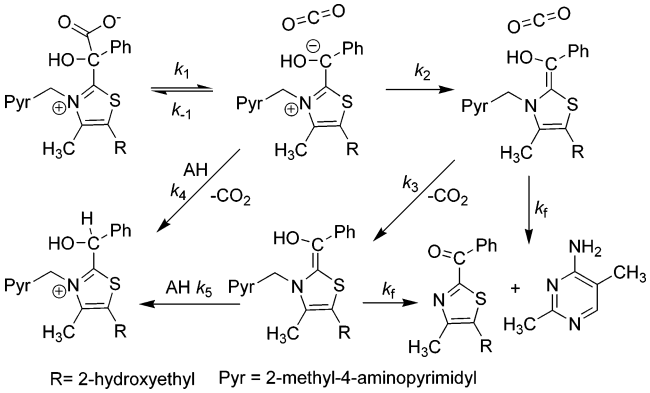


Figure 3. Increased yield of HBnTh by increasing acid concentration of the buffer components. The data were collected at pH = pK_a (○ bis-tris pH 6.7, ● pyridine pH 5.4, □ (2,2,2)-trifluoroethylamine pH 5.7, ■ phosphate pH 6.5, and △ acetate pH 4.6).

separation and recombination are also similar to those in stepwise nucleophilic substitution, in which ion pair separation is kinetically significant.[19−21] Thus, a mechanism that accounts for our results involves the acid catalyst as a spectator to the carbon−carbon bond-breaking event[22] (Scheme 2).

Scheme 2. A Brønsted Acid Intercepts the Carbanion in Competition with Nascent CO_2: Rehybridization[17] and Partial Separation are Schematically Incorporated into k_2



This mechanism predicts that the catalytic Brønsted acids will be uniquely effective in blocking fragmentation.[10] They can transfer a proton prior to dissociation of CO_2 and also after diffusion has occurred. The yields of fragmented products versus formation of HBnTh are summarized in Figure 3. As predicted, the conjugate acid of pyridine is especially effective at blocking fragmentation, requiring a considerably lower concentration to have a maximal effect.

On the basis of these results, we expect that enzymes can promote decarboxylation of a ThDP conjugate through early protonation of the carbanion, preventing recombination with nascent CO_2. Gao's calculations of OMP decarboxylase conclude that separation can be the major rate-liming process.[23] Since recombination should have a lower barrier than diffusion, this could account for the additional acceleration in BFD compared to MTh and BFD's avoidance of fragmentation. A conformation of the conjugate base that would lead to overlap with the thiazolium (along with rehybridization[17]) would be less efficient as it would increase the barrier to protonation on an enzyme.

The general role of thiamin in promoting decarboxylation has often been summarized by showing the thiazolium serving as a "sink" for the electron density generated in conjunction with formation of CO_2 in a concerted transition state. However, our results show that the transition state for breaking the C−C bond need not involve such delocalization to be concerted with formation of CO_2.

The many aspects of ThDP as a catalyst should be emphasized. Its ability to ionize to the C2 ylide, which can readily add to a substrate's carbonyl group, is central in positioning the substrate for reaction, providing the benefits of forming a covalent intermediate.[24] The adjacent positive charge of the thiazolium nitrogen provides very significant electrostatic stabilization of anions and of anionic transition states.[25] After a bound substrate is converted to a protein-associated ThDP conjugate in a decarboxylase,[7] it can be oriented to be destabilized by electrostatic stress[26] and desolvation,[6,7] while the bond breaking can be facilitated by protonation of the product carbanion to facilitate the departure of CO_2, a process that our results suggest can be rate-determining. Finally, the need for the quick arrival of a proton in the proper location can be facilitated by the proton tunnel found in related enzymes.[27]

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