General base catalyzed deprotonation of a thiamin-derived intermediate: Evidence for sequential proton transfer in pyridine catalyzed decarboxylation

by

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Abstract

General base catalyzed deprotonation of a thiamin-derived intermediate: Evidence for sequential proton transfer in pyridine catalyzed decarboxylation

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The conjugate acid of pyridine had been found to catalyze decarboxylation of α-mandelylthiamin (MTh). It was proposed this occurs by association between the substrate and pyridinium ion in a π-stacked complex prior to cleavage of the C-C bond. Despite the evidence for selective acid catalyzed decarboxylation of MTh with pyridine and its derivatives, the nature of proton transfer occurring after the C-C bond breaks and before the final products form had not been investigated. General base catalyzed deprotonation of hydroxybenzylthiamin (HBnTh) has been applied as a model for the reverse reaction of acid-catalyzed decarboxylation. Kinetic analysis of this process suggests the acceleration by a preassociated pyridinium ion and the product-determining step in the decarboxylation of MTh are facilitated by independent sequential proton transfers.
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<tr>
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<td>Phenylthiazole ketone</td>
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<tr>
<td>DMAP</td>
<td>Dimethyamino pyrimidine</td>
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1 Introduction

1.1 Thiamin diphosphate dependent decarboxylation

The decarboxylation of \( \alpha \)-ketoacids in metabolism requires covalent participation of coenzymes within their respective enzymes.\(^1\), \(^2\) The high-energy acyl carbanion generated from the loss of \( \text{CO}_2 \) must be stabilized. Thus, the protein acts in conjunction with the coenzyme thiamin diphosphate (ThDP) (figure 1.1) to delocalize the reactive intermediate as an acyl carbanion equivalent in the form of a covalent complex between the substrate and enzyme bound ThDP with the carbanion stabilized through electrostatic and resonance interactions.\(^1\) The diphosphate moiety of the coenzyme, along with a divalent metal ion such as \( \text{Mg}^{2+} \) or \( \text{Ca}^{2+} \), anchors ThDP within the active site.\(^3\) The C2 position of the thiazolium ring is the reactive site of the cofactor.\(^4\) Covalent intermediates formed from nucleophilic addition of the C2 carbanion to \( \alpha \)-keto acids act in an analogous manner to cyanide in the benzoin condensation by stabilizing the incipient acyl carbanion ylide through resonance delocalization into the heterocycle. The covalent conjugate can subsequently react with other electrophiles as in acetoacetate synthase or undergo protonation and elimination as in pyruvate (PDC) (scheme 1.1a) and benzoylformate decarboxylase (BFD) (scheme 1.1b).\(^2\)
Figure 1.1 Numbered structure of thiamin diphosphate.

Scheme 1.1 Decarboxylation of an α-keto acid by enzyme bound ThDP (a. PDC, b. BFD).

1.2 Mechanism of thiamin diphosphate decarboxylation

Site-directed mutagenesis of ThDP dependent holoenzymes has emphasized the importance of the cofactor in catalysis. Despite evidence from X-ray crystal structures of BFD that suggest the importance of certain residues in the catalytic mechanism, mutations at any site fail to inactivate the enzyme completely.\textsuperscript{5-12} Residual activity of the enzyme with non-conserved active site mutations illustrates the robust role of ThDP. Although the cofactor can function independently of the active site, the protein provides a $10^6$-fold acceleration over and above the non-enzymic rate when the intermediate is
produced synthetically.\textsuperscript{13} The rate enhancement is obtained from a number of sequential and consecutive processes provided by multiple active residues and conformational changes. In particular, the mechanism for the ThDP-dependent enzyme, BFD, begins when the cofactor is activated by deprotonation of C2 and added to the substrate, benzoylformate. The covalent intermediate undergoes decarboxylation, leaving the C2α carbanion/enamine intermediate, which is protonated and subsequently eliminated following deprotonation of the C2α hydroxyl (scheme 1.1).\textsuperscript{1}

The efficiency of the holoenzyme is achieved by meeting a number of challenges. First, the weakly acidic C2 (pK\textsubscript{a} 17-18)\textsuperscript{3} thiazolium carbon must be deprotonated to react with substrate to form α-mandelylthiamin diphosphate (MThDP). Second, proper orientation of the substrate to both enforce nucleophilic attack by the ylide and enable stabilization of the nascent carbanion through delocalization into the thiazolium ring must be achieved (figure 1.2). Finally, the C2α carbon must be protonated following the loss of CO\textsubscript{2}. Depending on the enzyme, the intermediate following decarboxylation exists predominantly as either a highly reactive zwitterionic C2α carbanion (pKa 15-16) or a less reactive, but still unstable, enamine (figure 1.1).\textsuperscript{14-17} These entities can also be considered as resonance contributors of a single common structure with rehybridization. The reactivity of both species necessitates a mechanism that irreversibly quenches the nucleophilic C2α position by protonation, producing 2-(1-hydroxybenzylthiamin) diphosphate (HBnThDP). This will serve to both increase the commitment by preventing internal return of CO\textsubscript{2} (k\textsubscript{p}) and suppress the destruction of the carbanion/enamine cofactor conjugate by fragmentation (k\textsubscript{f}) to a phenyl thiazole ketone (PTK) and dimethylamino pyrimidine (DMAP) (scheme 1.2).\textsuperscript{18}
Figure 1.2 Orientation of the C2α carboxylate prior to decarboxylation of MThDP to ensure maximum overlap of the nascent carbanion into the adjacent thiazolium ring.

Scheme 1.2 Fragmentation of the carbanion/enamine into PTK and DMAP following decarboxylation of MThDP.

Deprotonation of C2 is accomplished by cooperation of the enzyme active site and the aminopyrimidine moiety of the cofactor. The pyrimidine is in equilibrium with its tautomer, 1’,4’-iminopyrimidine, which is stabilized by three active site-derived...
hydrogen bonds. A proton transfer network extends from a glutamate residue (in PDC), through deprotonation of the 1’ nitrogen, and finally deprotonation of C2 is provided by the 4’ imino group. The chromophoric nature of the imino tautomer has aided in showing that it persists throughout the remainder of the catalytic cycle until it reprotonates the C2 carbanion after elimination of the product (scheme 1.3). In addition to the bonding interactions that stabilize the imino tautomer, an interaction with a large hydrophobic residue enforces a V-conformation of the cofactor that has been observed in crystal structures of most ThDP dependent enzymes. This conformation is not a potential energy minimum of the cofactor and may contribute ground state destabilization. Furthermore, structural analysis suggests that the conformation may aid in deprotonation of C2 by providing close proximity between C2 and the 4’ imino nitrogen base.

Scheme 1.3 Enzymic deprotonation at C2 of thiazolium to generate the nucleophilic zwitterions that can react with the substrate α-keto acid.

Tittmann and coworkers have used both X-ray crystal structures and $^1$H NMR analysis of quenched enzyme-bound intermediates on BFD and PDC. They have proposed a ‘least-motion’ mechanism for substrate attack and decarboxylation. The enzyme accomplishes this by orienting the carboxylate of the α-keto acid substrate perpendicular to the thiazolium ring prior to nucleophilic attack of the C2 conjugate base.
The orientation provides maximum π-overlap to stabilize the C2α carbanion following decarboxylation. This is an extension of Dunathan’s hypothesis regarding the mechanism of pyridoxal dependent enzymes. Site-directed mutageneis has implicated H70 as a residue that may hydrogen bond with the carbonyl of the α-keto acid substrate for the purpose of both activating it as an electrophile and to orient it perpendicular to the thiazolium ring.

The intermediate following decarboxylation must be protonated rapidly to allow the forward commitment of the process. With a high local concentration of CO2 adjacent to the reactive C2α conjugate base, internal return may proceed with little or no enthalpic barrier. Furthermore, rapid protonation of the C2α carbanion/enamine is necessary to prevent fragmentation of the cofactor, which occurs in solution at a rate 100 times that of enzymatic decarboxylation. The reactivity of the C2α carbanion as a nucleophile towards CO2 and as a precursor of fragmentation emphasizes the importance of proton transfer that occurs at a rate that suppresses these processes and contributes to the overall efficiency of the enzyme. X-ray and mutagenesis studies have shown that active site histidine residues could serve this purpose. This is consistent with the ~100 fold decrease in $k_{cat}$ from mutation of H281 of BFD to alanine. Although the role for H281 has been called into question based on its distance of >5 Å from C2α, the residue is part of a flexible loop on the enzyme that is likely to undergo a conformational change when the substrate binds. This phenomenon has been observed in other ThDP dependent enzymes. Based on the previous discussion, rapid protonation from histidine will increase the commitment of the enzymatic reaction by inhibiting reversion by internal return of CO2. Rapid protonation to produce the HBnThDP (in BFD) also serves to
suppress fragmentation of the carbanion/enamine since protonation will occur faster in
the preassociated complex than the cofactor conjugate can fragment.

Although internal return is irreversibly inhibited by rapid protonation,
fragmentation is only suppressed to the extent that the equilibrium between the active site
residue, H281, and the C2α conjugate acid favours HBnThDP over the
carbanion/enamine. Since fragmentation of ThDP conjugates on BFD is not observed on
the enzyme at all, an additional component to catalysis must be operative that impedes or
eliminates the back proton transfer from the C2α conjugate acid to H281 (figure 1.4).
This aspect of ThDP-dependent catalysis had not been addressed prior to my work.
Figure 1.4 Protonation of a preassociated Brønsted acid in the active site prevents internal return of CO₂ (k⁻¹) and suppresses fragmentation (k₉). The protonated intermediate can then undergo elimination to yield benzaldehyde as the product and regenerate the cofactor.

1.3 Dynamic proton transfer in enzymatic catalysis

As previously discussed, the decrease in \( k_{cat} \) of the H281A by two orders of magnitude indicates this residue is important in catalysis; however, the residual \( k_{cat} \) of 1-2 s⁻¹ is still 10 000 times that of the non-enzymic counterpart.⁶, ¹⁰ In order to understand how enzyme catalysis still proceeds in the absence of the proposed proton donor adjacent to the incipient carbanion intermediate; the notion of proton donation can be extended beyond discreet hydrogen bonding donor-acceptor interactions. In this extension, enzyme active sites are composed of electrostatic networks where bimolecular equilibria between donor and acceptor are part of a larger flux of protons moving from regions of low to high potential along the lowest energy paths. This type of dynamic process is implicated in the mechanism of OMP decarboxylase (ODCase), and provides an illustration of the interdependence of multiple residues in the process of protonating a carbanion following decarboxylation. Composed of alternating lysine and aspartate residues, this network is important for catalysis as indicated by decreases in \( k_{cat} \) of up to five orders of magnitude upon mutation of one amino acid in the chain. Considering that ODCase accelerates decarboxylation of OMP ~10¹⁷ times above the spontaneous reaction, a decrease in the rate by 10⁵ is considerable. However, it indicates that the enzyme is still capable of significantly lowering transition state energy in its altered form.¹⁷, ²² In BFD, despite the
loss of an important catalytic group, the dynamic interactions between enzyme and
substrate compensate by providing a proton from an alternative site. Although an
analogous network is not as evident in the active site of BFD, the retention of ~10 000
fold acceleration following mutation of H281 indicates that the enzyme benefits from a
similar network of proton sources.

1.4 Synthetic thiamin derived intermediates

Studying the mechanism of ThDP dependent enzymes by subjecting the
holoenzyme to experiments such as structure determination by X-ray diffraction in the
presence of substrate analogues, kinetic analysis of mutant enzymes, and \(^1\)HNMR
analysis of covalent intermediates bound to quenched enzymes has provided important
mechanistic information. Included in this data is the identity of enzyme bound thiamin
conjugates that indicate the stable intermediates along the catalytic path. Both MThDP
and HBnThDP have been observed as intermediates in the catalytic cycle of BFD.\(^5\) In
order to complement the understanding gained from observing the reactivity of the native
substrates and intermediates within the active site of an enzyme, analysis of synthetic
analogues of enzyme-bound intermediates have provided the means for observing the
catalytic function of thiamin in a controlled environment. Both \(\alpha\)-mandelylthiamin
(MTh) and 2-(1-hydroxybenzylthiamin) (HBnTh) have been synthesized for this purpose
(figure 1.5).\(^{23, 24}\) These intermediate analogues differ from the natural enzymic
intermediates only by the absence of the diphosphate moiety, a group that is not
implicated in catalysis.
Figure 1.5 Structures of ThDP intermediate analogues synthesized to mimic the reactivity of enzyme bound MThDP and HBnThDP.

1.5 **Fragmentation of HBnTh**

Kinetic analysis of HBnTh has provided insights on the mechanism of the fragmentation of HBnTh to PTK and DMAP, a reaction that was first observed by Oka under different conditions (scheme 1.2). Fragmentation is a general-base-catalyzed process that is a consequence of protonation of N1’ on the pyrimidine ring. The N-methylated derivative of HBnTh behaves similarly and has been utilized to avoid a divergence between fragmentation and elimination. Fragmentation proceeds with the same mechanism across all buffer concentrations; however, a change in rate-limiting step from deprotonation of C2α (kB) to fragmentation (kf) occurs as buffer concentration increases (scheme 1.4). The reaction proceeds by an E1CB mechanism as deduced from the large kinetic isotope effect of kH/kD = 5.8 for buffer catalyzed deprotonation of HBnTh and HBnTh-C2α-d and the inverse solvent isotope effect (kH/kD = 0.33) from rates in water and deuterium oxide. Furthermore, a reaction constant (ρ = 1.6) was
found for the fragmentation under conditions where proton transfer is rate-limiting, indicating the formation of negative charge at C2α.28 Despite the kinetic data available for this process, the transition state for fragmentation is not established. Based on the negligible reaction constant for fragmentation a concerted electrocyclic mechanism that resembles a [1,5]-sigmatropic is plausible but unprecedented.28 If this were the case, then it is possible that the enzyme can suppresses this process in a stereoelectronic manner by misaligning the carbanion/enamine relative to the orbitals of the putative pericyclic transition state.28 However, the lack of rotational freedom would provide a severe entropic restraint on the reaction.

Scheme 1.4 General base catalyzed deprotonation of HBnTh


1.6 Pyridine catalyzed decarboxylation of MTh

Kinetic analysis of the reactions of MTh has provided valuable information regarding key steps in the catalytic mechanism of BFD. This has helped to consolidate our understanding of the mechanism in terms of X-ray structures with substrate analogues and mutational analysis of the holoenzyme. Based on the rate of decarboxylation of MTh, the enhancement provided by BFD is on the order of $10^6$. This enhancement is very similar to that for PDC, which was determined using the rate of decarboxylation of $\alpha$-lactylthiamin (LTh). Buffer catalysis of the decarboxylation of MTh was not expected since a role for a Brønsted acid is not apparent in the mechanism; however, rapid protonation of the incipient carbanion following decarboxylation suppresses fragmentation. This led to the hypothesis that an active site Brønsted acid could serve to suppress fragmentation in BFD. More in-depth examination of this phenomenon in the presence of several buffers led to the discovery that pyridine and its alkylated derivatives, in protonated form, selectively catalyze decarboxylation. Furthermore, they are also the most efficient acids that suppress fragmentation. Since there is no conventional location on MTh for a Brønsted or Lewis acid to enhance the rate of carbon-carbon bond breaking, the mechanism of pyridine as a catalyst of decarboxylation requires that it is a spectator of carbon bond breaking. Since there is no site for H-bonding and the system contains a benzene ring, it was proposed that this involves a $\pi$-stacked preassociated complex between the C2α phenyl moiety and the aryl component of pyridinium (scheme 1.5). Preassociation allows for the barrier of transporting a Brønsted acid to the C2α carbanion following decarboxylation to be overcome. This provides a mechanism of protonation that competes effectively with internal return of CO$_2$ to the highly reactive
carbanion (pKa 15-16). Overall, this should lead to an increase in the commitment towards loss of CO₂ and a larger rate. This is an important observation that correlates with the previously mentioned mutational analysis of BFD where H281 has been identified to be in the correct proximity and orientation to fulfill the role of a preassociated Brønsted acid poised for rapid protonation of the C2α carbanion.

Scheme 1.5 Pyridine catalyzed decarboxylation of MTh.

1.7 **Sequence of proton transfer in pyridine catalyzed decarboxylation**

The importance of proton transfer for both catalysis and preservation of the cofactor in the mechanism of BFD has been established from analysis of both HBnTh and MTh. Protonation by pre-associated pyridine-derived acids suppresses fragmentation of the conjugate base of HBnTh following departure of CO₂. However, unlike decarboxylation, this depends on both the acidity and degree of alkyl substitution of the pyridine-derived acid and other Brønsted acids. Although decarboxylation and suppression of fragmentation both involve a proton transfer, it is apparent that the structural requirements for promoting decarboxylation and preventing fragmentation are
different. Since removal of a proton from HBnTh is the microscopic reverse of
protonation after separation of CO₂, the kinetics of formation of the C₂α carbanion with a
wide range of Brønsted bases has been investigated. From the Brønsted catalysis law, the
transition state for general base catalyzed deprotonation of HBnTh with both pyridine and
non-pyridine buffers is deduced in the current study and compared to our understanding
of the transition state for selective acid catalysis of decarboxylation via preassociation.
Identifying how these transition states intersect in the mechanism of decarboxylation of
MTh provides important insight into the sequence of proton transfers in ThDP-dependent
decarboxylation.
2 Experimental

2.1 Materials

Commercial reagents were used as purchased without further purification. The pH of buffer solutions was monitored in titrations by a glass Ag/AgCl electrode in KCl calibrated with IUPAC standards. Kinetic measurements were performed on a GBC Cintra 40 UV/Vis with a peltier thermostat.

2.2 Synthesis of N1'-methyl-2-(1-hydroxybenzylthiamin) (MHBnTh)

2.2.1 Condensation of thiamin hydrochloride with benzaldehyde

HBnTh was synthesized by condensing thiamin hydrochloride with benzaldehyde according to the procedure of Doughty. Under an argon atmosphere, two equivalents of sodium ethoxide (80.1 mmol of sodium in 135 mL) was added slowly to 40 mmol (10.62g) thiamin. Benzaldehyde (80.1 mmol, 8.1 mL) was dissolved in 135 mL of ethanol and added in one portion to the thiamin/ethoxide solution and stirred for 10 minutes. The reaction was quenched with two equivalents of concentrated hydrochloric acid (80.1 mmol, 6.7 mL), filtered, and dried under vacuum. The solid was washed with dichloromethane and the aqueous fraction was frozen and lyophilized. The resulting solid was recrystallized from ddH2O. 2-(1-hydroxybenzylthiamin) (HBnTh) was obtained in 15% yield (2.21 g).
\textsuperscript{1}HNMR (400MHz, DMSO-\textit{d}_6) \delta: 7.90 (1H, s), 7.50-7.47 (m, 2H), 7.29-7.26 (m, 3H), 7.15 (s, 1H), 6.45 (s, 1H), 5.74 (d, 1H, $^2J = 17.8$ Hz), 5.47 (d, 1H, $^2J = 17.9$ Hz), 3.73-3.64 (m, 2H), 3.06-3.03 (t, 2H), 2.50 (s, 3H), 2.30 (s, 3H).

\subsection*{2.2.2 N1’ methylation of 2-(1-hydroxybenzyl) thiamin}

The N1’ position on the pyrimidine of HBnTh was methylated according to a procedure adapted from that of Zoltewicz\textsuperscript{29}. HBnTh (2.21 g, 6.0 mmol) was dissolved in ddH\textsubscript{2}O (9.2 mL), and both one equivalent (6.0 mmol) of HCO\textsubscript{3} and a half equivalent of CaCO\textsubscript{3} (3.0 mmol) were added slowly to the aqueous solution. The pH was adjusted to 6.5 with 1M HCl and solid NaHCO\textsubscript{3} and 2.5 equivalents of dimethylsulfate (DMS) (14.9 mmol, 1.4 mL) was added in three portions. Thirty minutes of reaction time separated the addition of the first two quarter equivalents (3.7 mmol, 0.35 mL). The solution was adjusted to pH 6.5 before the addition of the second quarter equivalent and again after another 30 minutes of reaction. The remaining half equivalent (7.4 mmol, 0.7 mL) was added and the reaction was allowed to proceed for an additional 1.5 hours. The reaction was filtered, chilled on ice, and 6.4 equivalents (38.1 mmol) of NaClO\textsubscript{4} as a 6M solution was added slowly. The crude product was isolated by filtration after crystallizing overnight at 4 °C. The crude product, N1’-methyl 2-(1-hydroxybenzylthiamin) (MHBnTh), was recrystallized from 1% HClO\textsubscript{4} and obtained in a yield of 81% (1.86 g).
\[^{1}\text{HNMR} (400\text{MHz, DMSO-}d_6) \delta: 9.26 (s, 1H), 8.42 (s, 1H), 7.72 (s, 1H), 7.40-7.38 (m, 2H), 7.30-7.23 (m, 3H), 6.72 (s, 1H), 6.30 (s, 1H), 5.31 (s, 2H), 3.80-3.67 (m, 2H), 3.52 (s, 3H), 3.14-3.00 (m, 2H), 2.50 (s, 3H), 2.29 (s, 3H).\]

\[^{13}\text{CNMR} (100\text{MHz, DMSO-}d_6) \delta: 178.5, 162.2, 160.6, 143.9, 143.2, 138.5, 135.0, 129.8, 129.5, 128.5, 108.5, 71.0, 60.1, 46.8, 42.1, 30.3, 21.9, 11.9.\]

ESIMS $[^{\text{C}_{20}\text{H}_{25}\text{N}_{4}\text{O}_{2}\text{S}}]^+$, calculated: 385.1692, observed: 385.2.

2.3 $pK_A$ determination

Acid dissociation constants for all pyridine derivatives were measured at 40 °C and ionic strength (I) of 1.0 using the procedure of Andon and Cox.\textsuperscript{36} Stock solutions of the pyridine derivative (0.05 M) in 3 mL quartz cuvettes were prepared with both 0.1 M KOH and 0.015 M HCl to a final concentration of 1.67 x 10\textsuperscript{-4} M. Three samples of each acid and base were prepared at 40 °C and scanned from 230 to 290 nm. A third solution with the same concentration of base was prepared at 40 °C in various ratios of 0.015 M potassium acetate/0.005 M acetic acid. The buffer ratio was adjusted until three wavelength scans of the base were approximately halfway between the scans in acid and base resulting in a $pK_A$ with an error equal to or less than 0.05. The $pK_A$ was calculated by first identifying $\lambda_{\text{max}}$ for each base and subtracting $A_{\lambda_{\text{max}}}$ from the baseline at $A_{290}$ for each of the acid ($\Delta A_{\text{Acid}}$), base ($\Delta A_{\text{base}}$), and buffer solutions ($\Delta A_{\text{Buffer}}$). The pH of each buffer solution was measured at 40 °C and the $pK_A$ was calculated according
to equation 1 and 2 for each of three independent scans of the pyridine base in acid, base, and buffer.

Equation 2.1 Activity coefficient of the acid component for each buffer at 40 °C and \( I = 1 \).

\[
\log \gamma^*_{BH} = -0.5262 \left( \frac{\sqrt{I}}{1 + \sqrt{I}} \right)
\]

Equation 2.2 Calculation of the pKa using the activity coefficient and the pH of the buffer at 40 °C.

\[
pK_a = pH - \log \left( \frac{1}{\left( \frac{\Delta A_{Buffer} - \Delta A_{Base}}{\Delta A_{Acid} - \Delta A_{Buffer}} \right)} \right) + \log \gamma^*_{BH}
\]

The pKa for imidazole at 40 °C and \( I = 1 \) was calculated using the temperature coefficient \((dpK_a/dT)\) of -0.022^\circ C and the Debye-Hückel relationship (equation 2.3).^{37, 38}

Equation 2.3 Debye-Huckel relationship used to determine the pKa at variable ionic strength. \( A = \) Temperature constant \((A = 0.5262 \) at 40 oC\), \( z = \) charge of conjugate acid.

\[
pK_{a(I=1)} = pK_a + (2z - 1) \left( \frac{A\sqrt{I}}{(1 + \sqrt{I})} - 0.1 \right)
\]
2.4 Kinetics

2.4.1 Buffer preparation

Different concentrations of buffer were measured by weight or volume. According to calculations based on equation 4, KCl was added to each buffer as a 2.35 M solution to adjust the ionic strength to $I = 1.0$. Each solution was made to ~20 mL and titrated in a thermoregulated jacketed beaker at 40 °C with KOH and HCl to the desired pH. Titrated buffers were made up to 25.0 mL with ddH$_2$O in volumetric flasks. Concentrations of buffers ranged from 0.1 M to 0.9 M.

Equation 2.4 Calculation of the ionic strength in solution as the sum of the product between the molar concentration ($c_i$) and charge of each species ($z_i$).

$$I = \sum_{i=1}^{n} c_i z_i^2$$

2.4.2 Ultraviolet spectroscopy

Reactions were monitored in 3 mL quartz cuvettes in the thermoregulated UV spectrophotometer. Reactions were conducted under pseudo-first-order conditions by adding 50 μL of a 6.6 mM stock solution of HBNTh to 2.9 mL of buffer at 40 °C. The final substrate concentration for each reaction was approximately 10 μM. The formation of PTK from the fragmentation of the conjugate base of HBNTh was monitored at 328 nm for 9 hours. The increase in absorbance at 328 nm resulting from fragmentation is illustrated in figure 2.1 by wavelength scans of a solution containing HBNTh buffered
with 2,4-lutidine. Following the initial 9 hours, each reaction solution was transferred into vials and incubated for 4-5 days at 60 °C. The final UV absorbance of each reaction after incubation was recorded.

Figure 2.1 Wavelength scans of HBnTh buffered with 2,4-lutidine over a 24 hour period. Solid lines represent wavelength scans at 1 hour intervals and the dotted line corresponds to a scan performed after 24 hours.

2.4.3 Kinetic data analysis

Observed first order rate constants were obtained using the method of initial rates. The change in absorbance with respect to time was measured for the first ~2 % of each reaction and then divided by the final absorbance in order to accurately account for the initial concentration of HBnTh. The observed first order rate constants (k_{obs}) were plotted relative to the concentration of the base component in each buffer. Using the assumption
that $k_f \ (8 \times 10^3 \ \text{s}^{-1}) >> k_{-1}([\text{BH}] + k_{\text{H}_2\text{O}})$ and $k_1[\text{B}] > k_{\text{OH}^-}[\text{OH}]$, equation 2.5 was simplified to equation 2.6. This allowed for the second order rate constants ($k_b$) to be calculated from the slope of the linear regression for the relationship between $k_{\text{obs}}$ and base concentration. The second order rate constants were used along with the measured pK$_A$'s to construct a Brønsted plot.

Equation 2.5 Equation for the observed first order rate constant for deprotonation of HBnTh.

$$k_{\text{obs}} = \frac{(k_b[B] + k_{\text{OH}^-}[\text{OH}^-])k_f}{k_{-b}[\text{BH}^+] + k_{\text{H}_2\text{O}} + k_f}$$

Equation 2.6 Approximated equation for the observed first order rate constant for deprotonation of HBnTh.

$$k_{\text{obs}} = k_b[B]$$
3 Results

3.1 $pK_A$ determination

Spectrophotometric analysis of each buffer was performed in triplicate. A sample wavelength scan of pyridine in the presence of acid, base, and buffer is shown in figure 3.1.

![Wavelength scan of pyridine in acid (solid line), base (dashed line), and buffered solution (dot-dash line).](image)

Figure 3.1 Wavelength scan of pyridine in acid (solid line), base (dashed line), and buffered solution (dot-dash line).

Applying equations 2.1 and 2.2 to the data in each wavelength scan provided three $pK_A$ measurements for each base that were averaged. The resulting constants for seven pyridine derivatives used in the current study are reported in table 3.1.
Table 3.1 Acid dissociation constants and their margin of error reported as standard deviations of triplicate spectrophotometric measurements at 40 °C and I = 1. Also listed are pKₐ values measured at 25 °C by Andon.³⁶

<table>
<thead>
<tr>
<th>Base</th>
<th>pKₐ</th>
<th>Std. Dev.</th>
<th>pKₐ³⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>4.92</td>
<td>0.03</td>
<td>5.22</td>
</tr>
<tr>
<td>2-Picoline</td>
<td>5.54</td>
<td>0.02</td>
<td>5.96</td>
</tr>
<tr>
<td>3-Picoline</td>
<td>5.35</td>
<td>0.04</td>
<td>5.63</td>
</tr>
<tr>
<td>4-Picoline</td>
<td>5.94</td>
<td>0.05</td>
<td>5.98</td>
</tr>
<tr>
<td>2,4-Lutidine</td>
<td>6.42</td>
<td>0.03</td>
<td>6.63</td>
</tr>
<tr>
<td>2,6-Lutidine</td>
<td>6.26</td>
<td>0.05</td>
<td>6.72</td>
</tr>
<tr>
<td>2,4,6-Collidine</td>
<td>6.94</td>
<td>0.01</td>
<td>7.45</td>
</tr>
</tbody>
</table>

3.2 Kinetics

A linear increase in rate was observed over all concentrations of each buffer. A sample first-order rate plot for pyridine-catalyzed deprotonation of HBnTh is shown in figure 3.2. According to equation 2.6 the slope from linear regression analysis of each first order plot provided the second order base-dependent rate constants reported in table 3.2.
Figure 3.2 First order rate plot for general base catalyzed deprotonation of HBnTh by pyridine. The concentration dependence on both base (circles) and buffer (triangles) is shown.

Table 3.2 Second order base-dependent rate constants for general base catalyzed deprotonation of HBnTh

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>pK_a</th>
<th>$k_b$ (M$^{-1}$s$^{-1}$)</th>
<th>log$k_b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridine</td>
<td>5.22</td>
<td>4.92</td>
<td>$4.96 \times 10^{-6}$</td>
<td>-5.30</td>
</tr>
<tr>
<td>2-Picoline</td>
<td>5.24</td>
<td>5.54</td>
<td>$8.79 \times 10^{-6}$</td>
<td>-5.06</td>
</tr>
<tr>
<td>3-Picoline</td>
<td>5.05</td>
<td>5.35</td>
<td>$1.01 \times 10^{-5}$</td>
<td>-5.00</td>
</tr>
<tr>
<td>4-Picoline</td>
<td>5.64</td>
<td>5.94</td>
<td>$3.57 \times 10^{-5}$</td>
<td>-4.45</td>
</tr>
<tr>
<td>2,4-Lutidine</td>
<td>5.82</td>
<td>6.42</td>
<td>$7.03 \times 10^{-5}$</td>
<td>-4.15</td>
</tr>
<tr>
<td>2,6-Lutidine</td>
<td>5.66</td>
<td>6.26</td>
<td>$2.26 \times 10^{-5}$</td>
<td>-4.65</td>
</tr>
<tr>
<td>2,4,6-Collidine</td>
<td>6.24</td>
<td>6.94</td>
<td>$1.23 \times 10^{-4}$</td>
<td>-3.91</td>
</tr>
<tr>
<td>Imidazole</td>
<td>6.48</td>
<td>6.7</td>
<td>$1.74 \times 10^{-4}$</td>
<td>-3.76</td>
</tr>
</tbody>
</table>
3.3 Brønsted linear free energy relationship

Plotting the base-dependent second order rate constants for each base relative to its pKₐ produced the Brønsted plot shown in figure 3.3. The resulting plot for imidazole and non-hindered pyridine bases, which include pyridine and methyl pyridines substituted at 3, 4, and 5, gives $\beta = 0.85$. The rate constants for pyridines substituted with a methyl group at the 2-position fall below the expected value and those for pyridines with substituents at both the 2- and 6-positions are farther below the normal line.

Figure 3.3 Brønsted plot with a slope of $\beta = 0.85$ for the general base catalyzed deprotonation of HBnTh with unhindered pyrdines (black circles), hindered (white circles) pyridines, and imidazole (grey circle). Unhindered bases (pKa): Pyridine (4.92), 3-picoline (5.35), 3,5-lutidine (5.82), 4-picoline (5.94), imidazole (6.70). Hindered bases (pKa): 2-Picoline (5.54), 2,6-lutidine (6.26), 2,4-lutidine (6.42), 2,4,6-collidine (6.94).
4 Discussion

4.1 General base catalyzed deprotonation of HBnTh

4.1.1 No change in rate-limiting step with tertiary amines

In the general base catalyzed deprotonation of HBnTh by anionic buffers such as phosphate, saturation of the rate is observed with increasing concentration, indicating a change in rate-limiting step is occurring. Tertiary amine buffers did not indicate a concentration dependent change in rate-limiting step. First order rate plots of phosphate catalyzed deprotonation saturate at high buffer concentrations. This observation is attributed to a change from rate-limiting deprotonation at low buffer concentrations to rate-limiting fragmentation at high buffer concentrations. Under all conditions of this study, first order kinetic plots were linear as illustrated for pyridine catalyzed deprotonation in figure 3.2. Considering that this is a one step reaction, there must be a base dependent change in the transition state for deprotonation when the catalyst is changed from an oxyanion to a tertiary amine. This is also evident from comparison of the Brønsted coefficients of $\beta = 0.85$ for tertiary amine derivatives and $\beta = 0.5$ for acetate derivates in the deprotonation of HBnTh.\textsuperscript{18} The magnitudes of the Brønsted coefficients suggest that proton transfer is later in the case of tertiary amines relative to acetates, which indicates that the former case is thermodynamically less favourable. This can be justified by considering electrostatic interactions in the transition state for the two cases. Anionic oxygen sites acquire a proton from the C2$\alpha$ carbon acid producing a neutral
carboxylic acid and a carbanion. Alternatively, a neutral pyridine (or imidazole) nitrogen will acquire a proton from the C2α carbon acid to become a positively charged pyridinium (or imidazolium) ion that is adjacent to a negatively charged carbanion. The electrostatic interaction of the carbanion paired with a pyridinium ion provides a lower barrier for reprotonation over reaction of a neutral acid, justifying the proposal of a late transition state in tertiary amine-catalyzed deprotonation of HBnTh. The slower rate of deprotonation with tertiary amines explains the absence of saturation in the first order rate plots since that rate does not exceed that of fragmentation under these conditions.

4.1.2 Steric effect in general base catalyzed deprotonation of HBnTh with pyridine derivatives: A stereoelectronic phenomenon

Negative deviations in the linear correlation by hindered pyridine derivatives in the Brønsted plot shown in figure 3.3 are in accord with the observations of Gold and Hine.\textsuperscript{39, 40} Their observation of similar deviations for the deprotonation of acetone and its derivatives by pyridine buffers was accounted for by a steric effect imposed by methyl substituents in the 2 and 6 positions on the pyridine ring. Considering the rotational freedom about the C-C bonds of acetone, it would be expected that a steric effect in general base catalyzed deprotonation could be avoided. Although steric effects destabilize the transition state, the stability gained by orienting the incipient carbanion to enforce delocalization into an adjacent π-system outweighs the destabilization from steric interactions imposed by the stereoelectronic constraint (figure 4.1). Considering that the C2α carbon of HBnTh is flanked by a phenyl group and a substituted thiazolium ring, a
steric effect in deprotonation by substituted pyridines is a reasonable expectation. This is due to both the steric bulk and extended $\pi$-systems of these groups that will readily collide with alkyl substituents on pyridine derivatives and enforce a perpendicular orientation between the incipient carbanion $p$-orbital and their conjugated systems, respectively.

Figure 4.1 Stereoelectronic constraints in the deprotonation of acetone resulting in the observed steric effect in general base catalysis by substituted pyridines.

4.2 Sequential proton transfer in decarboxylation of MTh

4.2.1 Selective acid catalysis in the decarboxylation of MTh

It was pointed out earlier that MTh does not contain a location for a Brønsted acid to act as a catalyst. Alternatively, decarboxylation can be catalyzed by preassociation of a spectator Brønsted acid in a $\pi$-stacked complex (figure 4.2). The spectator Brønsted acid in this case is pyridinium and is alkyl derivatives. The positive deviation from the pH-rate profile in figure 4.3 by both hindered and non-hindered pyridine derivatives illustrates
that their catalytic ability is independent of substitution. The absence of a similar
deviation in the presence of imidazole shows that it is not catalytic.41

Figure 4.2 \( \pi \)-stacked pyridinium acting as a spectator catalyst in the decarboxylation of
MTh by protonating the incipient carbanion following C-C bond breaking.

Figure 4.3 pH-rate profile for the decarboxylation of MTh in catalytic and non-catalytic
buffers (1 M). The ionic strength was maintained at 1 in all cases. All kinetic runs at pH 4
were buffered with acetate/acetic acid.
The observed first order rate coefficient for the decarboxylation of MTh in acetate buffer at pH 4 is $6.4 \times 10^{-4}$ s$^{-1}$. With 1.0 M imidazolium in acetate buffer, the rate is unchanged but with 1.0 M pyridinium the observed rate coefficient increases to $8.8 \times 10^{-4}$ s$^{-1}$, consistent with a preassociation mechanism that permits protonation of the carbanion by pyridinium without diffusion, preventing reaction of the carbanion with CO$_2$. As a result, the sequence of departure of CO$_2$ and transfer of the proton from a pre-associated acid is important for our understanding of how the process is both enforced to proceed in the forward direction and how fragmentation is inhibited in the presence of catalyst.

### 4.2.2 Consistency in selective acid-catalyzed decarboxylation of MTh and general base-catalyzed deprotonation of HBnTh

We note that in the transfer of a proton from the product, HBnTh, there is a steric effect from $\alpha$-methyl groups on the catalyst, while the rates for unhindered pyridines conform to the Brønsted plot. These results require that the proton transfer that accelerates decarboxylation is completed prior to that which leads to the final protonation. The latter competes with fragmentation rather than with carboxylation.$^{13, 23}$ In other words, upon loss of CO$_2$, the HBnTh is associated with a Brønsted base that can accept the C2$\alpha$ proton in the absence of CO$_2$ (and lead to fragmentation), or which can separate and lead to formation of HBnTh. Analysis of proton transfer from HBnTh provides information only about the process in the absence of carbanion-associated CO$_2$ while catalysis of the decarboxylation of MTh provides information about proton transfer in the presence and then absence of associated CO$_2$. 
Irreversible loss of CO₂ connects the sequential proton transfer steps. Nominally, the C₂α conjugate base can be accessed by loss of a proton from HBnTh or loss of CO₂ from MTh. However, in reality there is a significant difference. Starting from MTh, an electrophilic substitution occurs through an unstable C₂α carbanion that is either quenched by a proton or by internal return of CO₂. A preassociated acid is required to accelerate irreversible loss of CO₂, presumably by enforcing the progress towards the protonated product. However, if the C₂α conjugate base is generated from HBnTh, no pre-association occurs and there are conventional steric effects in the direct transfer of a proton to a Brønsted base. This process is the microscopic reverse of the subsequent proton transfer after departure of CO₂ in the decarboxylation of MTh. Scheme 4.1 illustrates the difference between coincident proton transfer and sequential proton transfer steps in decarboxylation. Only the latter is consistent with my results.

Scheme 4.1 Coincident (upper) and sequential (lower) proton transfer routes in acid catalyzed decarboxylation.
4.2.3 Mechanism for acid catalyzed decarboxylation of MTh

We can consolidate our understanding of the mechanism for formation of the C2α conjugate base from both MTh and HBnTh into a mechanism for decarboxylation of MTh (Scheme 4.2). This involves C-C bond breaking, with or without catalytic protonation. The resulting complex in the absence of CO₂ equilibrates a proton in the step where formation of HBnTh competes with fragmentation. The role of pyridine is evident in the mechanism as a preassociated acid that accelerates the electrophilic substitution of CO₂ by enforcing the evolution of CO₂ (kₚ) in competition with its internal return (k⁻¹).

The highly exergonic process is independent of the acidity and steric character of the pre-associated catalyst. Following the loss of CO₂, the conjugate base of the catalyst is present in a high local concentration relative to the C2α carbon acid. The basicity and steric constraints of the catalyst determine the final products that are the result of a partition between diffusion of the base (k_D) and removal of a proton from the carbon acid (k_b).
4.3 Implications of sequential proton transfer in enzymatic decarboxylation

4.3.1 Suppressing fragmentation of enzyme-bound ThDP

Catalysis by BFD accelerates decarboxylation by a factor of $10^6$ and avoids fragmentation of its cofactor conjugate, HBnThDP.\cite{23} If a mechanism similar to that for preassociated acids with MTh applies, we can understand a likely role for the protein in combination with the cofactor. This is consistent with the enzyme functioning via the inherently pre-associated catalytic groups in its active site. In order for the catalytic groups to suppress fragmentation effectively, once the proton transfer has occurred, a conformational change could increase the distance from the potential proton acceptor, preventing formation of the conjugate base and facilitating the departure of CO$_2$. Consistent with comparisons between solution and enzymatic reactions,\cite{42} if this conformational change were coupled to the channeling of CO$_2$ out of the active site, it would be responsible for increasing the net flux of enzymatic decarboxylation.
5 Conclusions and future work

Covalent thiamin conjugates provide a realistic model to investigate the mechanism of ThDP dependent enzymes. HBnTh, in this case, provided the means to study the reverse of acid-catalyzed deprotonation. By comparing the structure-reactivity characteristics of both processes it is apparent that the transition state that accelerates decarboxylation of MTh is not the microscopic reverse of deprotonation of HBnTh. The selectivity for pyridine buffers and the absence of steric effects in pyridine-catalyzed decarboxylation is what precludes this process from being directly related to general base-catalyzed deprotonation of HBnTh. Unlike decarboxylation, the rates of deprotonation for planar tertiary amines are subject to steric effects and produce a linear Brønsted plot. These differences indicate that two independent sequential proton transfers are responsible for accelerating decarboxylation and then for providing a partition in the product determining step between fragmentation and protonation. Since fragmentation only results from the second proton transfer following the irreversible loss of CO₂, the absence of fragmentation on an enzyme is simply avoided by the protonation that is part of catalysis. A conformational change resulting in displacement of the conjugate base of H281 to inhibit an equilibrium with the C2α carbon acid will prevent formation of the carbanion leading to fragmentation.

Further studies with different classes of Brønsted bases is of interest for the purpose of understanding the extent of delocalization of the C2α carbanion in the transition state for deprotonation of HBnTh. This information would provide a better understanding of the energetics for internal return of CO₂ in the decarboxylation of MTh.
A more localized carbanion will result in a lower barrier for internal return, which would emphasize the importance of preassociated acid-catalysis both on and off the enzyme.

Further study with the holoenzyme enzyme, BFD, is necessary to apply the understanding gained from current and previous work with covalent thiamin intermediates. Previous mechanistic studies with BFD have focused on rates of catalysis and substrate affinity, which has been important for our understanding of catalysis. However, in consideration of the current work, it would be valuable to focus on the enzyme’s ability to suppress destruction of thiamin by fragmentation. Site-directed mutagenesis of residues flanking the catalytic H281 residue may inhibit a necessary conformation change resulting in deprotonation of enzyme-bound HBnTh leading to fragmentation. Since the C2α carbanion intermediate is instrumental for catalysis of many ThDP dependent enzymes, it is important that the mechanism by which these enzymes avoid this destructive path is understood to clarify their catalytic mechanisms.
6 References


41. Data submitted for publication.