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Diverting Thiamin from Catalysis to Destruction. Mechanism of Fragmentation of *N*(1′)-Methyl-2-(1-hydroxybenzyl)thiamin†

Ronald Kluger,* John Francis Lam, John Paul Pezacki, and Chi-Ming Yang

Contribution from the Lash Miller Laboratories, Department of Chemistry, University of Toronto, Toronto, Ontario M5S 1A1, Canada

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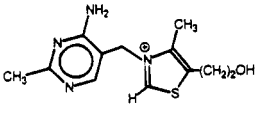
Abstract: Thiamin (**1**) promotes reactions involving acyl carbanion equivalents derived from benzaldehyde through a covalent intermediate, 2-(1-hydroxybenzyl)thiamin (HBzT, **2**). HBzT reverts to benzaldehyde and thiamin in alkaline solution. However, in neutral solution thiamin is not released as HBzT fragments irreversibly into thiazole and pyrimidine derivatives. Since thiamin is produced from HBzT only at high pH, protonation of the pyrimidine of HBzT may lead to preference for fragmentation. To test the effect of charge on the reaction pathway, *N*(1′)-methyl-2-(1-hydroxybenzyl)thiamin (**5**, MBzT) was prepared. Unlike HBzT, MBzT fragments in acidic, neutral, and alkaline solutions to give ketone **3** and a substituted pyrimidinium ion. Exchange of the C(2α) hydrogen for deuterium is faster than fragmentation with proton removal from C(2α) a necessary step preliminary to fragmentation. A mechanism that is consistent with these data converts the C(2α) conjugate base of HBzT to the tautomer in which a proton is removed from the C(2α) and another is added to N(3). This zwitterion apparently fragments by C−N cleavage along with a 1−2 proton shift from nitrogen to carbon. The cleavage step involves an incipient carbanion in the transition state which is stabilized by the positively charged pyrimidine. The path leading to elimination of benzaldehyde is favored only when the neutral pyrimidine is present in much higher concentration than its conjugate acid. The C(2α) conjugate base of 2-(1-hydroxybenzyl)thiamin diphosphate is an intermediate in the reaction catalyzed by benzoylformate decarboxylase where fragmentation of the thiamin derivative would block the normal catalytic process. In that case, forces in the active site of the enzyme must direct reaction away from fragmentation towards release of benzaldehyde.

Thiamin pyrophosphate serves as a coenzyme for reactions that involve acyl carbanion equivalents.[1] These reactions include decarboxylation of 2-ketocarboxylic acids and α condensation reactions of aldehydes.[2] Thiamin (**1**, vitamin B1)-is itself a catalyst for reactions that proceed via acyl carbanion equivalents.[1] On the basis of studies of catalysis and exchange reactions in thiazolium compounds related to thiamin, Breslow deduced the established mechanism involving base-catalyzed ionization of the C−H bond at the 2′ position of the thiazolium



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moiety of thiamin which adds to the carbonyl group of the reactant, leading to a series of stabilized carbanion equivalents.[3]

A notable example of catalysis by thiamin is the formation of benzoin from benzaldehyde in weakly alkaline solutions.[4-6]

† Dedicated to the memory of Professor John W. Bunting (1943−1995).
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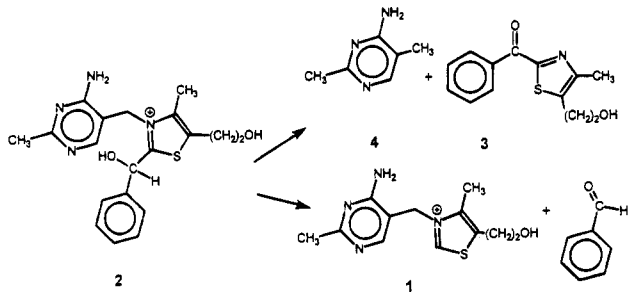
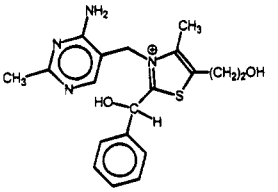
Scheme 1. Fragmentation (Upper) and Elimination (Lower) Pathways



Thiamin functions by an extension of the Breslow mechanism in which it serves to stabilize carbanions as cyanide does in the classical benzoin condensation.[7] Thiamin undergoes base-catalyzed addition to benzaldehyde to give 2-(1-hydroxybenzyl)-thiamin (**2**, HBzT). Reaction of the C(2α) conjugate base of

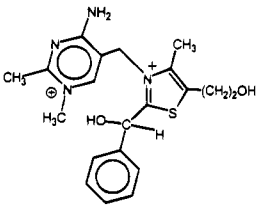


HBzT with benzaldehyde produces the thiamin adduct of benzoin. Consistent with this pathway, HBzT in the presence of base (but in the absence of added benzaldehdye) establishes an equilibrium with thiamin and benzaldehyde.[8,9]

While the thiamin-catalyzed formation of benzoin from benzaldehyde is explained by this mechanism, we recently showed that HBzT does not form thiamin and benzaldehyde in neutral and weakly acidic solutions.[9] This provides an explanation for what had been considered to be unusual catalysis of elimination.[8] In neutral and acidic solutions, HBzT undergoes an unexpected irreversible fragmentation, incorporating the elements of benzaldehyde into a derivative of the thiazolium moiety to give a phenyl thiazolyl ketone (**3**) and the substituted pyrimidine **4** (Scheme 1).

Zoltewicz and Uray describe this as a hydride transfer. The hydroxyl group of the hydroxybenzyl side moiety becomes a carbonyl while the methylene bridge between the thiazolium and pyrimidine groups becomes a methyl as a consequence of fragmentation. C(2α) loses a hydride equivalent which the methylene bridge gains.[10] However, the observed base catalysis is not consistent with such a mechanism.[10] In a related observation, Oka and co-workers noted the formation of **3** and **4** as the result of combining thiamin, benzaldehyde, and triethylamine in refluxing methanol.[11] Zoltewicz and Baugh[12] proposed that **3** and **4** result from addition of methoxide to the protonated pyrimidine, expulsion of the thiazole, and bimolecular hydride transfer. Zoltewicz and Uray[10] concur that such a mechanism cannot apply to the aqueous reaction. Since fragmentation appears to be a fundamental route by which thiamin can be destroyed (in competition with a catalytic process), we sought to find the basis for fragmentation.

Because fragmentation of HBzT is favored over elimination of benzaldeyhde in neutral and acidic solutions, it is possible that the ionization status of HBzT and resulting intermediates affects the reaction pathway. The pyrimidine ring of thiamin is a Brønsted base (The pK_a' of the conjugate acid is 5.3 at N(1′).[19]) Thus, the predominance of fragmentation in neutral and acidic solutions would be associated with conditions in which the pyrimidine is present as the conjugate acid. In order to assess the reactivity of the species in which the pyrimidine contains a positive charge, we prepared the *N*(1′)-methyl derivative of HBzT (**5**, MBzT) and have analyzed its reaction



pathway. Unlike (protonated) HBzT, which has a dissociable proton on its pyrimidinium ring, the *N*(1′)-methyl derivative MBzT retains a localized positive charge on the pyrimidine in neutral and alkaline solutions. We find that this change is sufficient to direct reaction completely to fragmentation, even at high pH. This permits a more direct mechanistic analysis of the generalized fragmentation process.

Experimental Section

Methods. Kinetics of Reactions in Water. Reaction mixtures were kept at 40.0 °C in a water bath or in the jacketed cell holder of a UV/vis spectrophotometer. The ionic strength of all buffer solutions was maintained at 0.10 by the addition of sodium chloride or potassium chloride. The extent of completion of reactions was followed by monitoring the increase in absorbance at 328 nm (ϵ = 10 000), characteristic of the ketone product. Data were collected with an interfaced computer. First-order rate constants were calculated from nonlinear regression fits of the data to the integrated first-order rate expression. Where products and kinetic order were established independently, slower reactions were analyzed by the method of initial rates, as indicated.

Kinetics in Deuterium Oxide. For reactions in deuterium oxide, the pK_a' of each of the buffers in deuterium oxide and of ionic strength 1.0 was determined. Equivalent amounts of the acidic and basic buffer components (0.0250 mmol) were placed 25.0 mL volumetric flasks and diluted with 1.0 M potassium chloride in deuterium oxide. pH meter readings were noted and converted to pD.[13]

Rates of exchange of the proton at C(2α) of MBzT were measured by monitoring the decrease of the ^{1}H NMR singlet at δ 6.4 of the sample in buffered solutions. The fragmentation of MBzT was measured by monitoring the decrease of the ^{1}H NMR peak at δ 6.5 (due to the C(6′) H of the pyrimidinium moiety which is shifted in the fragmented product) or by UV/vis measurement of the increase in absorbance at 328 nm due to the formation of ketone **3** as described for the reaction of HBzT. In the case of NMR analysis of reactions in phosphate buffers, integrated areas for the signals of the C(2α) and C(6′) protons were measured three times independently and compared to those of the internal standard. The resulting peak areas were then compared to the area of the same peaks in the initial spectrum.

For reactions in phosphate buffer, first-order rate constants were determined from the slopes of linear regression fits of the data to the integrated rate law, with the reaction followed for three half-lives. For exchange reactions in acetate and formate buffers, half-lives were more than 12 h, so initial rate measurements were used. Samples in NMR

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Table 1. Observed First-Order Rate Constants (k_0) for Fragmentation of MBzT at Various Acidities[a]

buffer	pK_a	pH	[buffer], M	k_0, s^{-1}	k_b, M^{-1} s^{-1}
acetate	4.76	4.30	0.1, 0.05, 0.01	$(2.7 \pm 0.4) \times 10^{-7}$	$(1.4 \pm 0.2) \times 10^{-6}$
		5.00	0.1, 0.05, 0.01	$(1.9 \pm 0.02) \times 10^{-7}$	$(9.2 \pm 0.4) \times 10^{-6}$
succinate	5.64	5.30	0.6, 0.3, 0.08	$(4.0 \pm 0.3) \times 10^{-6}$	$(5.4 \pm 0.6) \times 10^{-5}$
		5.90	0.04, 0.02, 0.004	$(1.1 \pm 0.2) \times 10^{-5}$	$(3.2 \pm 0.7) \times 10^{-4}$
MES	6.15	5.70	0.1, 0.05, 0.01	$(9.0 \pm 0.4) \times 10^{-6}$	
		6.20	0.1, 0.05, 0.01	$(2.5 \pm 0.2) \times 10^{-5}$	
PIPES	6.82	6.40	0.03, 0.017, 0.0034	$(4.4 \pm 0.2) \times 10^{-5}$	
		6.90	0.03, 0.017, 0.0034	$(1.3 \pm 0.02) \times 10^{-4}$	
phosphate	7.20	6.90	0.06, 0.03, 0.006	$(1.2 \pm 0.08) \times 10^{-4}$	$(2.3 \pm 0.2) \times 10^{-3}$
		7.40	0.04, 0.02, 0.004	$(3.01 \pm 0.06) \times 10^{-4}$	$(4.7 \pm 0.2) \times 10^{-3}$
HEPES	7.55	7.00	0.1, 0.5, 0.001	$(1.63 \pm 0.02) \times 10^{-4}$	
		7.60	0.1, 0.5, 0.001	$(5.0 \pm 0.3) \times 10^{-4}$	
POPSO	7.85	7.80	0.04, 0.02, 0.004	$(1.1 \pm 0.1) \times 10^{-3}$	$(4.2 \pm 0.6) \times 10^{-3}$
		8.10	0.04, 0.02, 0.004	$(2.65 \pm 0.03) \times 10^{-3}$	$(1.6 \pm 0.2) \times 10^{-2}$
hydroxide	14				1×10^{-3}

[a] Where buffer catalysis was observed, second-order rate constants (k_b) are listed.

tubes were placed in a 40.0 °C water bath. At 4−12 h intervals integrations of the C(2α) and C(6′) proton signals were measured.

Synthesis of *N*(1′)-Methyl-2-(1-hydroxybenzyl)thiamin. 2-(1-Hydroxybenzyl)thiamin chloride hydrochloride (HBzT·Cl·HCl) was prepared by condensation of benzaldehyde and thiamin.[8,11,14,15] HBzT·Cl·HCl (1.0 g, 2.3 mmol) was dissolved in distilled water (2.8 mL). Sodium bicarbonate (0.25 g, 3 mmol) was added in portions. The reaction mixture was warmed until it became clear. Calcium carbonate (0.15 g, 1.5 mmol) was added to the reaction mixture, and the pH was adjusted to 6.5 by the addition of hydrochloric acid. Dimethyl sulfate (0.70 mL, 7.4 mmol) was added by syringe in three portions during 1.5 h, with the solution acidity maintained at pH 6.6 (±0.1) by addition of sodium bicarbonate. The solution was clarified by filtration and the collected solid washed with water (1 mL). Sodium perchlorate (1.5 g in 2 mL of water) was added to the filtrate. The resulting suspension was stirred for 2 h, filtered, and washed with 1% perchloric acid (2 × 1 mL). The white powder was recrystallized from 5 mL of 1% perchloric acid, producing 0.93 g of MBzT (74% from HBzT): white crystals; mp 122−123 °C; UV (1% $HClO_4$) λ_{max} = 262.4 (ϵ = 15 500); FABMS for $M^{2+}(ClO_4)_2$, *m/z* 487 ($M^{2+}(^{37}ClO_4)$, 12.9), 485 ($M^{2+}(^{35}ClO_4)$, 42); high-resolution FABMS for MH^+ peak ($C_{20}H_{24}N_4O_2S$), calcd 385.1698, obsd 385.1691; ^1H NMR (200 MHz, D_2O) δ 7.30−7.46 (m, 5H), 6.51 (s, 1H), 6.41 (s, 1H), 5.33 (dd, *J* = 18.2, 29.6 Hz), 3.96 (t, 2H, *J* = 5.8 Hz), 3.52 (s, 3H), 3.22 (t, 2H, *J* = 5.8 Hz), 2.53 (s, 3H), 2.38 (s, 3H); ^{13}C APT NMR (50 MHz, D_2O) δ 162.4 (−), 144.0 (−), 141.8 (+), 134.1 (−), 136.0 (−), 129.4 (+), 129.3 (+), 127.5 (+), 107.5 (−), 81.0 (+), 75.7 (−), 71.3 (+), 59.7 (−), 46.1 (−), 41.5 (+), 28.9 (−), 20.5 (+), 10.5 (+).

Preparative-Scale Fragmentation of MBzT. In order to isolate products of the reaction of MBzT under conditions related to the kinetic studies, a larger scale reaction was conducted. The bisperchlorate of MBzT (0.17 g) was dissolved in 5 mL of water, and the pH adjusted to 8.0. The pH was maintained by addition of 0.5 M potassium hydroxide from an automatic buret in a pH-stat. After 2 h, 1 M hydrochloric acid was added to produce a final acidity of 0.01 M. The product was then extracted into two 50 mL portions of ethyl acetate and dried over magnesium sulfate, and the solvent was removed. The residue was passed through a column of silica gel (eluted with 1:1 ethyl acetate/hexane) to give 0.050 g of a yellow oil, identified as the previously reported phenyl thiazolyl ketone **3**:[9,14] ^1H NMR (200 MHz, $CDCl_3$) δ 2.44 (s, 3H), 3.03 (t, 2H, *J* = 6.3 Hz), 3.84 (t, 2H, *J* = 6.3 Hz), 7.43−7.62 (m, 3H), 8.35−8.40 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 15.4, 30.2, 62.6, 128.2, 131.0 133,2, 135.3, 137.3, 152.1, 163.4, 184.0. The aqueous phase was freeze-dried, leaving 0.12 g of a powder, which was a mixture of 1,2,5-trimethylpyrimidinium perchlorate (the *N*(1′)-methyl analogue of **4**) and potassium chloride: ^1H NMR (200 MHz, D_2O) δ 7.90 (s, 1H), 3.79 (s, 3H), 2.59 (s, 3H), 2.11 (s, 3H); ^{13}C NMR (100 MHz, D_2O) δ 13.6, 21.9, 42.7, 63.3, 115.0, 146.6, 162.5, 164.4.

Results

HBzT is conveniently prepared by the base-catalyzed addition of thiamin to benzaldehyde.[16] In order to prepare the *N*(1′)-methyl derivative **5**, we attempted condensation of benzaldehyde with *N*(1′)-methylthiamin.[17,18] The desired product was not detected, probably because of its tendency to fragment. However, *N*(1′)-methyl-2(1-hydroxybenzyl)thiamin (MBzT, **5**) was accessible by methylation of HBzT.

N(1′)-Methylthiamin was originally studied by Jordan and Mariam who prepared the derivative by reaction of thiamin with methyl iodide.[17] Later, Zoltewicz and Uray showed that dimethyl sulfate is a more effective and reproducible reagent for this process.[18] We prepared MBzT by following the Zoltewicz−Uray procedure for thiamin with HBzT. We could find no previous report of the synthesis or physical properties of MBzT. Crane and Washabaugh mention in passing a study of buffer catalysis of formation of benzaldehyde from MBzT. No details of the preparation or reaction are presented.[19]

Rates and Products of Decomposition of MBzT. The pH−rate profile for the disappearance of MBzT, whether followed by NMR or UV, is a straight line with unit slope across the entire pH range (4.3−9, Figure 1). The only products we observe are those that result from fragmentation, in analogy to what we observe for HBzT in neutral solution: the phenyl thiazolyl ketone, **3**, and the trimethylaminopyrimidine **6**. This contrasts with the passing mention by Crane and Washabaugh of the production of benzaldehyde from MBzT.[19] The methylation of the pyrimidine has a dramatic effect on the reaction pathway: HBzT eliminates benzaldehyde at pH 8 and higher and also has a complex pH−rate profile for fragmentation.[9,20]

Our data for the pH−rate profile for MBzT were measured in the presence of buffers, and these results were extrapolated to rate coefficients at zero buffer concentration. The slopes of plots of observed rate coefficients versus buffer concentration give the second-order rate constants for buffer catalysis (Table 1). Since the rates increase with the concentration of the basic component of the buffer, they are associated with general base catalysis. Plots of the data for general base catalysis vs pK_a of

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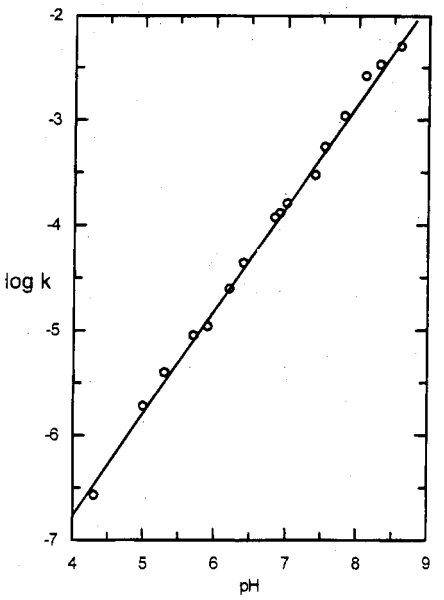

Figure 1. pH−rate profile for fragmentation of MBzT.

the acid component of the buffer following the Brønsted procedure gives β for the reaction (~1.0). The kinetic term for general base catalysis of the fragmentation of MBzT is observed only if the base is anionic; uncharged bases have no observable effect on the rate of the reaction (Table 1). The point for the rate constant due to hydroxide falls below the line of the Brønsted plot, permitting the observation of the rates due to other catalysts.

Rate of Exchange of the C(2α) Proton of MBzT. Finding the source of the observed base catalysis should provide an important insight into the mechanism of the overall reaction. The magnitude of the Brønsted coefficient suggests that the transition state associated with the observed general base catalysis involves a proton transfer that is not concerted with heavy atom bond formation or cleavage.[21] The rate law for general base catalysis is consistent with the action of a Brønsted base upon the substrate or its tautomer, or of a Brønsted acid upon the conjugate base of the substrate or its tautomer. The relationship of the structures of the reactants and products helps to define possible modes for catalysis.

The fragmentation of MBzT requires the loss of its C(2α) proton since the product is the corresponding ketone. If the step in which this proton is removed is rate-determining, then the hydroxide and buffer catalysis we observe would be associated with removal of that proton. The loss of a proton from the C(2α) position is a common occurrence in a number of reactions involving HBzT and related model systems[1,15,22,23] and should also occur in MBzT.

The rate of base-catalyzed exchange of a weakly acidic proton for a deuteron in deuterium oxide normally involves rate-determining removal of the proton.[1,24] Thus, a model for the removal of the proton in the fragmentation process is the exchange reaction. As in the fragmentation reaction, only buffers whose basic component is anionic accelerate the exchange above the background rate (Table 2). Removal of the C(2α) proton by the basic component of the buffer is rate-determining, so the observed general base catalysis kinetics is a result of the step involving the Brønsted base. A Brønsted correlation treatment of the resulting observed rate constants for general base catalysis in Table 2 gives $\beta \geq 0.9$ for the

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Table 2. Rate Constants for Buffer-Catalyzed Exchange of the C(2α) Proton of MBzT in Deuterium Oxide Buffer Solutions at 40 °C[a]

buffer	pK_a'[b]	concentration, M	k_b, M^{-1} s^{-1}
formate	4.2	1.0, 0.75, 0.5	2.7×10^{-6}
acetate	5.0	1.0, 0.75, 0.5	2.6×10^{-5}
phosphate	6.8	0.28, 0.20, 0.15	7.0×10^{-4}

[a] Ionic strength 1.0 (KCl). Reactions were carried out at pD = pK_a. [b] pK_a of the conjugate acid in D_2O (I = 1.0 M) determined as described in the Experimental Section.

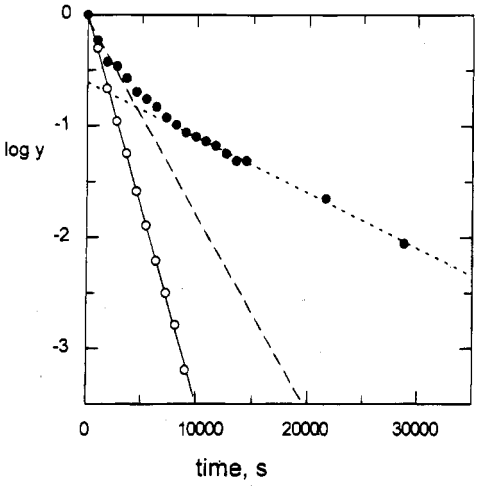


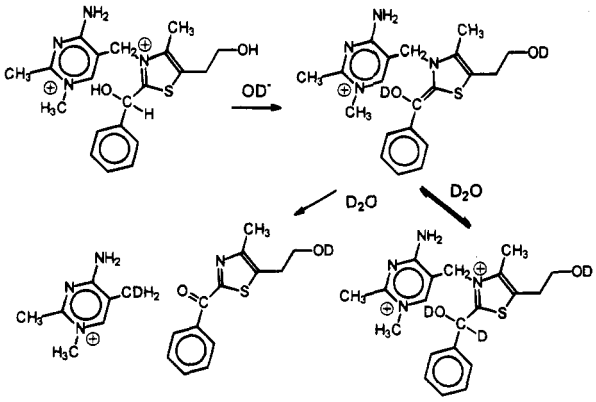
Figure 2. Extent (y) of fragmentation of MBzT followed by the proton NMR signal of the C(6′) proton (●) and exchange of C(2α), from the decreased proton signal (○) in 0.2 M phosphate in deuterium oxide. The regression line for C(2α) H−D exchange is shown by a solid line. The fit for fragmentation before a significant amount of C(2α) H−D exchange has occurred is shown by a dashed line while fragmentation of the C(2α)-deuterated reactant is shown by a dotted line.

removal of the C(2α) proton, a subject analyzed in detail by Washabaugh for a related process.[25]

If proton removal is rate-determining, then the exchange reaction should be no faster than the overall fragmentation process. The rate coefficients for exchange of the C(2α) proton of MBzT are larger than those for fragmentation under the same conditions, as can be seen directly by following the reaction of MBzT in deuterium oxide−phosphate. The signal for the C(2α) proton decreases more rapidly due to exchange than do the peaks associated with fragmentation. Exchange is nearly complete prior to the observation of a significant extent of fragmentation. This is based on the specific behavior of the peak due to C(2α) which decreases as the proton is exchanged for a deuteron and that of the peak of the proton on N(6′) which is shifted during fragmentation. These results indicate that a proton transfer, other than removal of the proton from C(2α), is rate-determining.

Biphasic Rate of Fragmentation of MBzT in Deuterium Oxide. The plot of fragmentation of MBzT in deuterium oxide according to the first-order rate equation is biphasic, with a somewhat slower second phase (Figure 2). This observation is consistent with a step subsequent to proton removal being rate-determining. Early in the reaction, a proton is removed from the C(2α) position of MBzT to give the enamine intermediate (Scheme 2). This intermediate can either acquire a deuteron from the solvent, to give the C(2α)-deuterated analogue of MBzT, or go on to fragmentation. A simple scheme is shown below. As the reactant becomes converted to its deuterated analogue, the rate decreases since the rate of removal of the C(2α) proton is subject to a primary kinetic isotope effect. The mechanism associated with the rate constants for the process in water is discussed later in this paper.

Scheme 2



Discussion

MBzT does not release benzaldehyde over the entire range of this study since the rate of fragmentation increases in proportion to hydroxide concentration. The formation of benzaldehyde would require that the fragmentation cease to increase with hydroxide so that a competing hydroxide-dependent process could occur.[9] Thus, fragmentation is clearly the only overall reaction at all acidities. In contrast, HBzT produces thiamin and benzaldehyde in more alkaline solutions.[9] This supports the proposal that the fragmentation is accelerated by a positive charge on the pyrimidine, while elimination occurs only from the neutral pyrimidine version of HBzT. The uniform increase in observed first-order rate constants for fragmentation of MBzT with increasing hydroxide concentration (with no change in product distribution) indicates that a common specific base catalyzed mechanism for fragmentation of MBzT is operative for the entire pH range studied. The decomposition of MBzT is also subject to general base catalysis by negatively charged bases. The rate law is thus

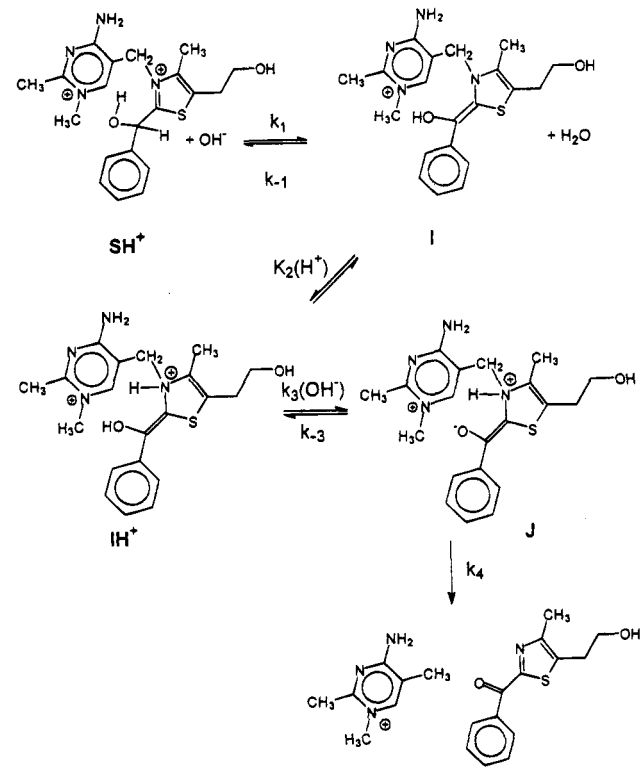
$$v = k_{obs}[S] = (k_{OH}[OH^-] + k_b[B^-])[S] \qquad (1)$$

The Brønsted plot of k_b has a slope >0.9 (with the point for hydroxide considerably below the line), indicating that the rate-determining step in the mechanism is proton removal or addition independent of any cleavage or formation of bonds between heavy atoms.[21]

The observation that MBzT undergoes fragmentation in alkaline as well as in neutral and acidic solutions supports the hypothesis that a positive charge at N(1′) of the pyrimidine promotes fragmentation. In the case of HBzT, the changeover from fragmentation to elimination occurs between pH 7 and pH 8, 2 pH units above the value equal to the pK_a of the protonated pyrimidine. If fragmentation occurs only from the N-protonated species, then this process is about 10^2 times faster than elimination from the unprotonated species, indicative of a rate-determining transition state with high-energy carbanionic character within the range of stabilization by the inductive effect of the cationic substituent. Determination of the rate of the fragmentation reaction of the unprotonated species will require additional studies.

The detailed mechanism of the fragmentation of MBzT involves initial loss of the C(2α) proton to give the enamine which is also the intermediate in the proton exchange reaction.

Scheme 3



However, since exchange is faster than fragmentation, this step is not rate-determining. The structure of the products from fragmentation requires loss of the proton from the hydroxyl group at C(2α) and protonation of the methylene bridge along with cleavage of the bond connecting the bridge to the thiazole nitrogen. The structure of the intermediate makes direct transfer of a proton from the hydroxyl group to carbon unlikely. The mechanism in Scheme 3 proposes a route to the products from the enamine consistent with our results.

The overall reaction is base-catalyzed. The enamine intermediate **I** is formed from MBzT (**SH**$^+$) by removal of a proton (k_1), but this step is not rate-determining. Removal of the hydroxyl proton from **I** (not shown) cannot be rate-determining because the overall process would then be second-order in hydroxide. Addition of a proton to this second conjugate base at the methylene carbon in the rate-determining step would involve a protonation level for the transition state which would be equivalent to a neutral reaction of the first conjugate base of HBzT with hydroxide. This implicates a process whereby a proton is transferred to the developing negative charge at carbon in the transition state of the rate-determining step. This is equivalent to protonation of a C−N σ bond by an external Brønsted acid, accounting for the observed general base catalysis (reaction of a Brønsted acid with a conjugate base) as well as the acceleration provided by positive character on the pyrimidine. It is unlikely that such a transfer could occur efficiently given the structures involved and the lack of an opportunity for preformation of a hydrogen bond. The large magnitude of the Brønsted coefficient is also inconsistent with such a mechanism.

Alternatively, the nitrogen of the enamine could acquire the proton to produce **IH**$^+$ (shown as an equilibrium, K_2, among steady state intermediates). Removal of the hydroxyl proton (k_3) will generate enolate **J** (which is also locally cationic at two places). The proton, which is already present on nitrogen, only needs to shift to the adjacent carbon in the transition state of the C−N bond-breaking step (k_4). Again, the positively

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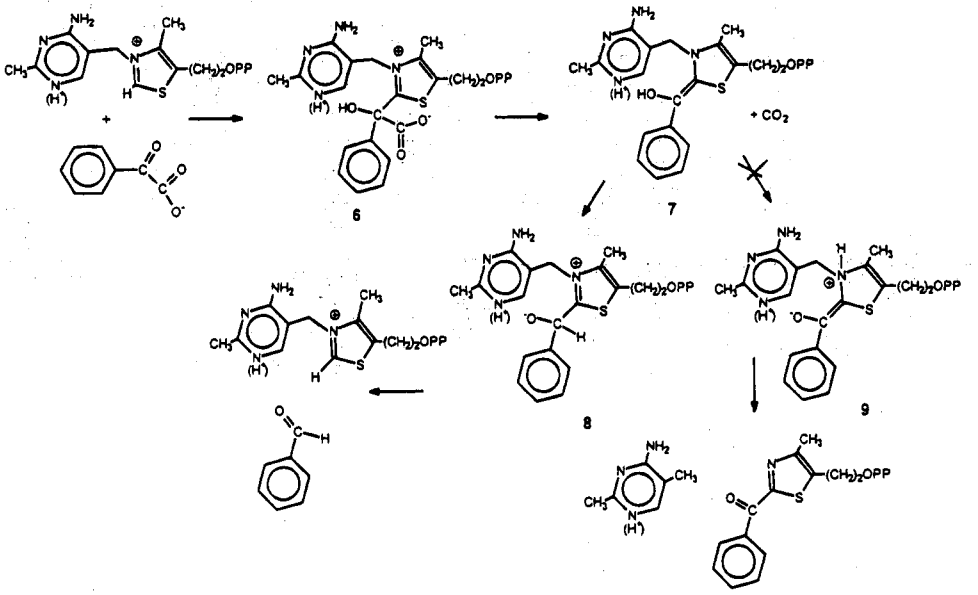
Scheme 4



charged pyrimidine can promote the breaking of the C−N bond by its inductive effect in stabilizing the carbanionic state.

The electronic structures of intermediate **J** and the transition state associated with its fragmentation as proposed in the mechanism above are subjects of further study. The structure shown for **J** is one resonance contributor for the enolate. The resonance contribution to the enolate where negative charge resides on the C(2) carbon is reminiscent of the ylide derived from thiamin.[10,26]

Fragmentation vs Elimination. On the basis of the reaction pattern for MBzT, the branching between pathways with pH for HBzT is clearly a function of the state of protonation of the pyrimidine ring, with the fragmentation from the protonated material favored by $\sim 10^2$ over elimination. Our studies do not reveal the rates of the lesser reactions in competition: fragmentation of (unprotonated) HBzT, formation of benzaldehyde and thiamin from the conjugate acid of HBzT, formation of *N*(1′)-methylthiamin and benzaldehyde from MBzT.

The mechanism in Scheme 3 is consistent with the observed base catalysis for fragmentation of MBzT, with removal of the C(2α) proton being part of the mechanism but not rate-determining. In addition, conversion of intermediate **J** to the indicated products is faster than protonation to give $\mathbf{IH}^+$ (in order to account for the observed general base catalysis, a proton transfer must be part of the rate-determining step). Intermediates **I**, $\mathbf{IH}^+$, and **J** are present at low steady state levels. The rate expression is

$$v = -\mathrm{d}[\mathrm{MBzT}]/\mathrm{d}t = k_{\mathrm{obs}}[\mathbf{SH}^+][\mathrm{OH}^-] = k_4[\mathbf{J}] \quad (2)$$

with formation of **J** being rate-determining and the concentration of **J** being given by the steady state assumption:

$$k_{-3}[\mathrm{OH}^-][\mathbf{IH}^+] = (k_{-3} + k_4)[\mathbf{J}] \quad (3)$$

and

$$[\mathbf{J}] = k_3[\mathrm{OH}^-][\mathbf{IH}^+]/(k_{-3} + k_4) \quad (4)$$

$$v = (k_3k_4[\mathrm{OH}^-]/(k_{-3} + k_4))[\mathbf{IH}^+] \quad (5)$$

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We can treat prior steps as equilibria,

$$k_1/k_{-1} = K_1 = [\mathbf{I}]/([\mathbf{SH}^+][\mathrm{OH}^-]) \quad (6)$$

$$k_2/k_{-2} = K_2 = [\mathbf{IH}^+]/[\mathbf{I}][\mathrm{H}^+] \quad (7)$$

so that

$$[\mathbf{IH}^+] = K_2[\mathbf{I}][\mathrm{H}^+] \quad (8)$$

and

$$[\mathbf{IH}^+] = (K_1K_2K_w)[\mathbf{SH}^+] \quad (9)$$

$$v = (K_1K_2K_wk_3k_4[\mathrm{OH}^-]/(k_{-3} + k_4))[\mathbf{SH}^+] = k_{\mathrm{obs}}[\mathrm{OH}^-][\mathbf{SH}^+] \quad (10)$$

$$k_{\mathrm{obs}} = K_1K_2K_wk_3k_4/(k_{-3} + k_4) \quad (11)$$

Since $k_4 > k_{-3}$, we can approximate

$$k_{\mathrm{obs}} \approx K_1K_2K_wk_3 \quad (12)$$

The rate-determining step associated with k_3 is base catalyzed removal of the hydroxyl proton from **J**. The enol is expected to be weakly acidic so that transfer to hydroxide should have the rate constant of a diffusion-controlled process. The relatively low concentration of hydroxide across the range of our study (pH 4−9) makes the effective rate of the step much slower than for reactions at standard state. Since k_3 is rate-determining, $k_{-3} < k_4$, where k_{-3} is protonation of the enolate by water and k_4 is the C−N bond-breaking step. Transfer of a proton from water (p$K \approx 15$) to the enolate (pK of the substituted enol ~7) is a thermodynamically unfavorable process, and $k = 10^{10-\Delta pK}$ and $k_{-3} \approx 10^2\ \mathrm{s}^{-1}$ with water at unit activity. Thus, the rate constant for C−N bond breaking can be estimated as $k_4 \geq 10^2\ \mathrm{s}^{-1}$. This corresponds to an activation free energy for breaking the bond of only 1 kcal/mol, which is well below the strength of an unactivated C−N bond.

Control of Competing Pathways. The acidity of the proton at C(2α) of HBzT is not known. However, related values have been reported. Washabaugh estimates p$K_a \approx 15$ for that position in the analogue of HBzT in which a hydroxyl group replaces

the pyrimidinyl amino group.[25] Bordwell and Jordan calculate that the pK_a at the same position of 2-(1-hydroxyethyl)thiamin (HET) is 14.1 from measurements in DMSO.[27] Stivers and Washabaugh deduce the pK_a of HET from indirect kinetic measurements as ~19.[28] On the basis rates of exchange and extrapolation of pK_a values of carbon acids, Lienhard proposed that the pK_a of HET is 17.[29] The loss of a proton from HBzT can also occur from the hydroxyl at C(2α), and this pK_a is estimated to be ~10.7 by Crane and Washabaugh.[8] Thus, the precursor to fragmentation (loss of a proton from carbon) is the less favored tautomer in competition with the precursor to the elimination reaction (loss of a proton from the adjacent oxygen). This does not determine the product distribution since it is only the relative free energies of the transition states of the competing rate-determining steps that control the outcome. The transition state for fragmentation of the N-protonated form of HBzT is lower in energy than that for formation of benzaldehyde and thiamin from this reactant. The energetics are reversed for HBzT with a neutral pyrimidine. In the case of MBzT, since a charge remains on the pyrimidine throughout the pH range, fragmentation remains the favored route under conditions where HBzT produces benzaldehyde and thiamin.

Relation to Enzymic Mechanisms. Benzoylformate decarboxylase is a thiamin diphosphate-dependent enzyme, converting benzoylformate to benzaldehyde and carbon dioxide. The sequence of events in Scheme 4 indicates the likely pathway for the interaction of the substrate and coenzyme.

The mechanism involves a covalent intermediate, **6**, that releases carbon dioxide to form the enamine conjugate base of 2-(1-hydroxybenzyl)thiamin diphosphate, **7**. This is the coenzymic analogue of the conjugate base of HBzT. Since the enzyme functions in neutral solution, the inherently favored pathway would lead to fragmentation of the intermediate rather than the observed elimination of benzaldehdye.[30] The branching between the pathways from **7** is shown in Scheme 4. The path to benzaldehyde with regeneration of thiamin diphosphate requires protonation of C(2α) and loss of the adjacent hydroxyl proton to give **8**, prior to loss of benzaldehyde.

From our present results we formulate fragmentation occurring by protonation of the thiazole nitrogen and deprotonation of the C(2α) hydroxyl proton to give **9**. The free energy difference between **8** and **9** is the difference in energy for loss of the proton from carbon versus nitrogen. The C(2α) carbon acid is likely to have pK_a > 14 on the basis of measurements of related materials.[25,27] A protonated enamine such as **9** will be at least 7 pK units more acidic, so **8** is at least 5 kcal/mol lower in free energy than **9**. Our results indicate that in the absence of enzyme the transition state for fragmentation of the pyrimidine-protonated thiamin derivative analogous to **9** is about 2 kcal/mol lower in energy than that for elimination of benzaldehyde from the analogue of **8** in which the pyrimidine is not protonated.

The decarboxylation of **6** is promoted by a medium of low polarity, on the basis of observations of acceleration of the decarboxylation of the related compound derived from thiamin and pyruvate.[31] We have proposed that thiamin diphosphate-dependent decarboxylases utilize the energy gained in forming the covalent intermediate to desolvate it within the active site.[32] After decarboxylation, the covalent intermediate becomes accessible to the external medium.[32] On the basis of Oka's observation of fragmentation of HBzT in a low dielectric medium,[11] the proposed change to higher polarity after decarboxylation reduces the possibilities for fragmentation. A resonance contributor for the structure shown as **9** has the charges on adjacent atoms, an arrangement favored in a low-polarity medium that is not possible for **8**. Protonation of the pyrmidine can be suppressed by adjacent positive charges of the protein side chains, and this could all but eliminate the possibilities for fragmentation.

A substrate analogue, *p*-[(bromomethyl)benzoyl]formate, reacts with benzoylformate decarboxylase to lose carbon dioxide and bromide, giving toluoylthiamin diphosphate, a material that rapidly hydrolyzes.[33] This requires that the shift of the hydroxyl proton to carbon be circumvented. Since fragmentation was not observed, loss of bromide must also be faster than transfer of the proton from oxygen to nitrogen and C–N cleavage.

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