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Fragmentation of the Conjugate Base of 2-(1-Hydroxybenzyl)thiamin: Does Benzoylformate Decarboxylase Prevent Orbital Overlap To Avoid It?

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Thiamin (vitamin B1) catalyzes the formation of benzoin[1,2] through the initial formation of 2-(1-hydroxybenzyl)thiamin (HBnT, **1**).[3−5] The enzyme benzoylformate decarboxylase (BFD)[6−8] releases benzaldehyde from a similar intermediate, regenerating the thiamin coenzyme. In contrast, in neutral solutions, HBnT undergoes irreversible fragmentation into a pyrimidine (**2**) and a phenyl thiazole ketone (**3**), products that were originally reported by Oka for other reaction conditions.[9,10] The release of benzaldehyde from HBnT occurs after ionization of the hydroxyl at C2α. The fragmentation reaction, which is inherently faster, proceeds by initial removal of the proton from the carbon at C2α.[11,12] The rate of the fragmentation step from the carbon conjugate base is competitive with protonation at C2α.[13] Empirically, fragmentation of HBnT competes with formation of benzaldehyde in proportion to the extent that HBnT is present as a dication (from protonation or alkylation of N1′ of the pyrimidine).[11,14] The positive charge on the pyrimidine might promote cleavage of the methylene bridge of HBnT, which must acquire carbanionic character in a polar mechanism. However, we now find that when we generate the conjugate base of HBnT at C2α by decarboxylation,[15] we observe that the fragmentation step is independent of the state of N1′. Because BFD generates the analogous intermediate by decarboxylation, its avoidance of fragmentation cannot be due to its control of the protonation state of the pyrimidine.

As shown in Scheme 1, the addition product of thiamin and benzoylformate is mandelylthiamin (MT, **4**; 2-(2-hydroxy-2-phenylacetoxy)thiamin).[15] Loss of carbon dioxide from MT produces the C2α conjugate base of HBnT (**5**). When **5** is generated in this way, it partitions between formation of the fragmentation products (**2** and **3**) and its conjugate acid, HBnT. The formation of the latter is promoted by Brønsted acids.[15]

We prepared the N1′-methyl derivative of MT (NMMT, **6**) by reaction of the ethyl ester of MT with dimethyl sulfate in pH 6.6 phosphate buffer, following the general method of Zoltewicz.[16] The ester was isolated and characterized after lyophilization, extraction into ethanol, filtration, and evaporation. The ester was converted to the acid in 12 M hydrochloric acid (3 days, room temperature) − decarboxylation of thiamin conjugates is very slow in strongly acidic solutions.[17] After concentration to remove excess hydrogen chloride, and lyophilization, NMMT chloride hydrochloride was obtained as a pale yellow solid (stored dry at −20° C). ^{1}H NMR (300 MHz DCl in D$_2$O): δ 2.23 (3H, s, CH$_3$-pyrimidine), 2.33 (3H, s, CH$_3$-thiazole), 3.07 (2H, t, $J = 5.8$ Hz, CH$_2$CH$_2$OH), 3.56 (3H, s, N1′-CH$_3$), 3.79 (2H, t, $J = 5.8$ Hz, CH$_2$CH$_2$OH), 5.28 (1H, d, $J = 18$ Hz, H$_a$H$_b$CN), 5.62 (1H, d, $J = 18$ Hz, H$_a$H$_b$CN), 6.64 (1H, s, H-pyrimidine), 7.14 (3H, m, H-aromatic), 7.39 (2H, m, H-aromatic). ^{13}C NMR (75 MHz, D$_2$O): δ 169.6, 161.5, 160.8, 145.3, 143.7, 137.6, 136.1, 135.1, 129.9, 129.7, 125.9, 107.4, 79.0, 65.3, 10.0, 47.1, 29.3, 20.4, 16.8, 13.0, 11.2. ESIMS: (C$_{21}$H$_{25}$N$_4$O$_4$S)$^+$, calcd 429.1591, found 429.1585. The site of alkylation was

Scheme 1. Mandelylthiamin (**4**) Decarboxylates and Gives the Carbanion, Which Either Fragments (Fast) or Is Protonated (Slow) To Give HBnT (**1**)^a
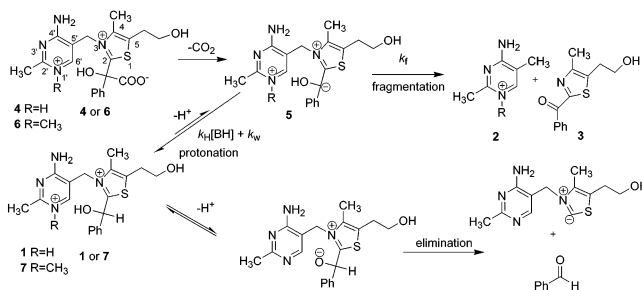
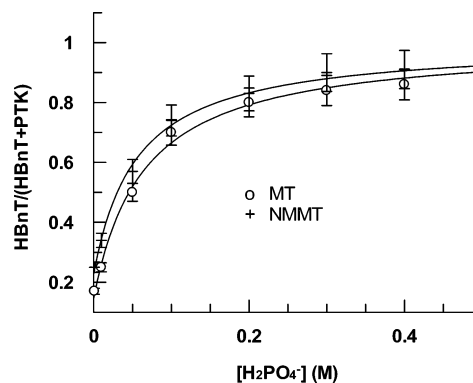^{a} Loss of a proton from HBnT forms a carbanion (with a positive charge on the pyrimidine ring) or an alkoxide.

Figure 1. Fraction of decarboxylation of MT (○) and NMMT (+) producing proton-transfer products HBnT (**1**) and NMHBnT (**7**, N1′ methylated HBnT) determined from the concentration of **3**.

confirmed by NOE analysis. The 6′ proton and the C2′ methyl are adjacent to N1′-methyl.

Decarboxylation of NMMT leads to an increase in absorbance at 328 nm at pH 6.2 (in phosphate buffer) from formation of **3**. We quantified the outcome as described for the reaction of MT by completing the fragmentation of HBnT.[15] Figure 1 yields the apparent rate constant ratio for the competing pathways (fragmentation vs protonation) for NMMT and MT. For MT, k_H/k_f is 17.0 ± 2.0 M^{-1}, and k_w/k_f is 0.18 ± 0.02. For NMMT, k_H/k_f is 23.3 ± 1.3 M^{-1}, and k_w/k_f is 0.21 ± 0.02. The rate constants for decarboxylation are also similar in these conditions, and the yields of fragmentation products **3** and **2** (or the N1′-methyl analogue) relative to HBnT (or its N1′-methyl analogue **7**) are similar over a wide range of phosphate buffer concentrations. This clearly establishes that the positive charge on the pyrimidine does not promote the fragmentation step and suggests that the transition state occurs before significant charge develops at the carbon. Because the intermediate that leads to fragmentation is generated from HBnT

**Scheme 2.** Fragmentation of the C2α Conjugate Base[a]
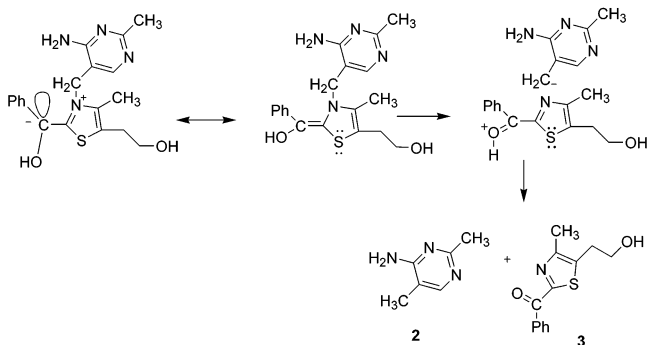
[a] Delocalization of the carbanion triggers fragmentation.

by the removal of the C2α proton, this must be the step that is affected by the state of N1′.

N1′-methylated 2-(1-hydroxybenzyl)thiamin, NMHBnT (**7**), contains two quaternary nitrogens. Removal of the C2α proton generates a formal carbanion whose primary resonance structure is an uncharged enamine (overall charge +1, with only one ionic center). As shown in Scheme 2, the slow proton removal from C2α results in annihilation of both the nascent negative charge and the existing thiazolium charge. In contrast, loss of the proton from the OH, which is required for formation of benzaldehyde, creates a localized oxyanion. In neutral or alkaline solution, loss of the hydroxyl proton from HBnT leads to a simple zwitterion; elimination competes with fragmentation (and C2α-proton exchange) in sufficiently alkaline solution.

We note that while the rate constant for fragmentation from the intermediate is competitive with the measured rate constant for the enzymic process, the enzyme avoids fragmenting its cofactor.[13,18] Our results establish that suppression of protonation of the pyrimidine in the enzymic reaction would not prevent fragmentation: the C−N cleavage process itself must be avoided. We suggest that an extension of Dunathan's hypothesis for the maintenance of specificity in PLP enzymes[19] can be adapted for the present case. Loss of carbon dioxide from MT generates a carbanion that can be delocalized as an enamine. If the fragmentation step requires that the enamine's orbital overlap be present in its transition state, then preventing this overlap will make fragmentation inaccessible and would also make protonation orders of magnitude faster, as in the protonation of the localized thiamin ylide.[20] A hydrogen bond from the enzyme to the C2α hydroxyl through the active site's H70[21] and other binding interactions could provide such a restriction. The transition state for decarboxylation could then be accelerated by specific interactions in the enzymic environment,[22] with H70 serving as an acceptor in the elimination step (which would be aligned for formation of benzaldehyde),[23−25] as would specific binding of other groups (Scheme 3).

The BFD H70A mutant is essentially inactive.[21] This suggests that without the orienting effect of H70, thiamin diphosphate and benzoylformate may not form MT-diphosphate. If orientation in

Scheme 3. Avoidance of Orbital Overlap To Prevent Fragmentation and Accelerate Protonation in an Enzyme
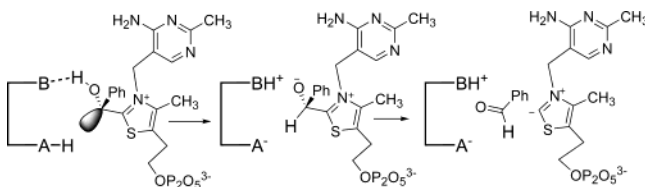
addition and elimination are achieved in the same way, achieving a least-motion pathway, then fragmentation is blocked and protonation is enhanced as a direct consequence of the formation of MT-diphosphate. Thus, we propose that thiamin diphosphate readily adds to small molecules to enable multiple binding interactions. This provides energy so that the protein can stabilize and control transition states in a defined yet flexible environment.[26]

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JA0390505