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The reactivity of lactyl-oxythiamin implies the role of the amino-pyrimidine in thiamin catalyzed decarboxylation

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ABSTRACT

It has previously been established that the deprotonated amino substituent of the pyrimidine of thiamin diphosphate (ThDP) acts as an internal base to accept the C2H of the thiazolium in ThDP-dependent enzymes. The amino group has also been implicated in assisting the departure of the aldehydic product formed after loss of CO_2 from ketoacid substrates. However, the potential role for the pyrimidine amino group in the key decarboxylation step has not been assessed. Oxythiamin contains a hydroxyl group in place of the pyrimidine amino group in thiamin, providing a basis for comparison of reactivity. Lactyl-oxythiamin (LOTh), the conjugate of pyruvic acid and oxythiamin was prepared by condensation of ethyl pyruvate and hydroxyl-protected oxythiamin followed by deprotection and acidic hydrolysis of the ethyl ester. The rate constants observed for the decarboxylation of LOTh in neutral and acidic solutions are about four times smaller than those for the corresponding compound that contains the amino group, lactylthiamin. The difference in reactivity is consistent with the amino group's participation in facilitating the decarboxylation step by allowing a competitive addition pathway that produces bicarbonate and has implications for the corresponding enzymic reaction.

1. Introduction

Enzymes that catalyze the formation of acyl carbanion equivalents utilize ThDP as a cofactor. The diphosphate portion of the cofactor is associated with binding to a metal ion and the protein in forming the holoenzyme. The non-phosphate portion of the cofactor is thiamin, which consists of linked thiazolium and pyrimidine derivatives. The catalytic functions of the coenzyme are associated with formation of the nucleophilic carbene derived from the thiazolium portion of thiamin. The covalent pre-decarboxylation intermediates resulting from addition of that carbene to carbonyl groups of α-ketoacids undergo decarboxylation to produce the functional equivalent of an acyl carbanion as the immediate product, which is stabilized by protonation.

A major catalytic role of the aminopyrimidine portion of ThDP has been associated with the deprotonated 1,4-iminopyrimidine tautomer acting as an intramolecular base in the critical transfer of the C-2 proton from the thiazolium ring to form the nucleophilic carbene that adds to the substrate's carbonyl [1]. It has also been suggested that this imino tautomer is associated with elimination from the precursor to the aldehydic products that results from the loss of CO_2 in the addition intermediate from pyruvate [2,3]. However, there is no specific information that has been reported on the effects of the amino group derived from thiamin on the critical decarboxylation step of the intermediate resulting from addition of the carbene from ThDP to the ketoacid substrate. We have now developed a synthesis of the conjugate of pyruvate and oxythiamin, lactyl-oxythiamin, which differs from the native thiamin-derived intermediate with a single alteration: a hydroxyl group replaces the amino group of the pyruvate-thiamin conjugate, lactyl-thiamin. The distinctions in the intrinsic reactivity of lactyl-thiamin and lactyl-oxythiamin should serve as accurate models for the corresponding diphosphates as the diphosphate group is remote from the reaction site on enzymes and it is involved only in binding the coenzyme permanently to a metal ion and the protein.

2. Experimental section

2.1. Materials

Commercial materials were purchased and used without further purification.

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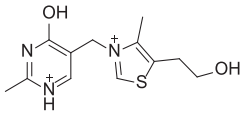
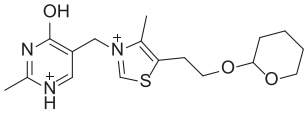
2.2. Syntheses



2.2.1. Oxythiamin

Oxythiamin was prepared from thiamin according to the published procedure [4]. As oxythiamin and thiamin would have very similar ^{1}H NMR spectra, thiamin was added to a solution of the product of the oxythiamin preparation. The appearance of a second set of non-coincident peaks confirmed that the product had formed successfully



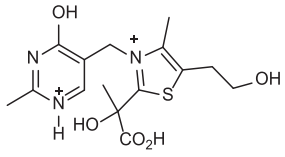
2.2.2. O-THP oxythiamin

A suspension of oxythiamin (5 g, 14.8 mmol) and *p*-toluene sulfonic acid monohydrate (0.75 g, 4 mmol) was prepared in dichloromethane (150 mL). 3,4-dihydropyran (12.5 mL, 137 mmol) was added to this suspension. After stirring at room temperature for 8 h, an additional portion of 3,4-dihydropyran (5 mL) was added. The mixture was stirred overnight. The progress of the reaction was monitored by thin layer chromatography (TLC). After the starting material was consumed, the reaction was quenched by adding 2.5 mL of trimethylamine. A yellow solid was obtained after removing solvents. The crude material was purified on a sodium bromide-loaded silica gel column chromatography [5] (eluent: 10–20% MeOH/dichloromethane). The fractions containing the product were combined and the solvent was removed by rotary evaporation. The residual material was dissolved in small amount of isopropanol to remove sodium bromide and *p*-toluene sulfonate by vacuum filtration. Finally, the solvent was evaporated under reduced pressure to obtain the product

^{1}H NMR (400 MHz, D_2O): δ 9.97 (s, 1H), 8.26 (s, 1H), 5.48 (s, 2H), 4.64 (t, 1H, J = 4.0 Hz), 3.99–3.91 (m, 1H), 3.74–3.60 (m, 2H), 3.50–3.43 (m, 1H), 3.30–3.27 (m, 1H), 3.25–3.18 (m, 2H), 2.61 (s, 3H), 2.42 (s, 3H), 1.85–1.65 (m, 2H), 1.61–1.42 (m, 3H).

ESI-MS $[C_{17}H_{24}N_3O_3S]^+$, calc: 350.15; found: 350.2.

2.2.3. Synthesis of lactyl-oxythiamin



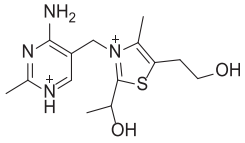
O-THP-oxythiamin (4 g) and ethyl pyruvate (7 mL) were suspended in 30 mL of dry dichloromethane and cooled to -20 °C in a 35% ethanol: water bath with dry ice under dry nitrogen. LiHMDS (3.0 eq) was added dropwise to the stirred mixture. After 30 min at −20 °C, the reaction mixture was transferred to vigorously stirred trifluroacetic acid (50 mL) through a cannula. The acid and solvent were removed by rotary evaporation at 25 °C. The crude material was purified using a sodium bromide-treated silica gel column (eluent: 5–15% methanol/dichloromethane with 0.1% TFA). The isolated yield was 30%.

^{1}H NMR: (400 MHz, D_2O): δ 7.40 (s, 1H), 5.6 (d, 1H, J = 12.0 Hz), 5.35 (d, 1H, J = 12.0 Hz), 3.96–3.9 (m, 1H), 3.8–3.73 (m, 3H), 3.06 (t, 2H, J = 4.0 Hz), 2.38 (s, 3H), 2.33 (s, 3H), 1.97 (s, 3H), 1.04 (t, 3H, J = 4.0 Hz)

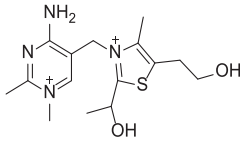
ESI-MS $[C_{17}H_{24}N_3O_5S]^+$, calc: 382.14; found: 382.14.

The ethyl ester (ethyl lactyl-oxythiamin) was converted to the free acid (lactyl-oxythiamin) in concentrated hydrochloric acid during 3 days with stirring at room temperature. Excess hydrogen chloride was then removed by rotary evaporation. The product was obtained as an off-white solid and stored with a very small amount of concentrated hydrochloric acid (to prevent decarboxylation) at −20 °C. The solid was dissolved in 20% DCl in deuterium oxide and characterized by ^{1}H NMR.

^{1}H NMR: (400 MHz, D_2O): δ 7.40 (s, 1H), 5.38 (d, 1H, J = 16.0 Hz), 5.17(d, 1H, J = 16.0 Hz), 3.62 (t, 2H, J = 8.0 Hz), 2.89 (t, 2H, J = 8.0 Hz), 2.51 (s, 3H), 2.14 (s, 3H), 1.83 (t, 3H, J = 4.0 Hz).



2-(1-Hydroxyethyl)thiamin was prepared from thiamin and acetaldehyde according to the literature procedure [6].

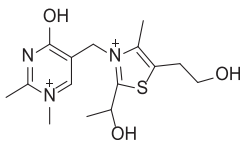


N1′-Methyl-2-(1-hydroxyethyl)thiamin (MHETh) was prepared by alkylation of 2-(1-hydroxyethyl)thiamin. This material (2 g, 5.2 mmol) and 2,6-di-tert-butyl-4-methylpyridine (1.28 g, 6.24 mmol) were suspended in 70 mL acetonitrile and the solution was subjected sonication. Methyl triflate (0.85 mL, 7.8 mmol) was added to the resulting mixture and stirred. After the starting material was consumed, the reaction was quenched with concentrated hydrochloric acid and the solvent was removed by rotary evaporation. The crude material was purified through a sodium bromide-loaded silica gel column (eluent: 5–15% MeOH/dichloromethane). This material was partially dissolved in isopropanol. Sodium bromide was removed by vacuum filtration. After evaporating the solvent, the product was obtained as a white powder with an isolated yield of 50%.

^{1}H NMR (400 MHz, D_2O): δ 7.37 (s, 1H), 5.59 (s, 2H), 5.48 (q, 1H, J = 8.0 Hz), 3.91 (t, 2H, J = 4.0 Hz), 3.78 (s, 3H), 3.19 (t, 2H, J = 4.0 Hz), 2.67 (s, 3H), 2.41 (s, 3H), 1.72 (d, 3H, J = 8.0 Hz).

ESI-MS $[C_{15}H_{24}N_4O_2S]^+$, calc: 324.16; found: 324.14.

2.2.4. Synthesis of N-methyl hydroxyethyloxythiamin



N1′-methyl-2-(1-hydroxyethyl)-oxythiamin (MHETh) was prepared from MHETh and was converted to N1′-methyl-hydroxye thyl-thiamin (MHEOTH) using the procedure of Rydon [4].

^{1}H NMR (400 MHz, D_2O): δ 7.71 (s, 1H), 5.66 (q, 1H, J = 8.0 Hz), 5.41 (s, 2H), 3.86 (t, 2H, J = 4.0 Hz), 3.71 (s, 3H), 3.10 (t, 2H, J = 4.0 Hz), 2.52 (s, 3H), 2.41 (s, 3H), 1.68 (d, 3H, J = 8.0 Hz).

ESI-MS $[C_{15}H_{23}N_3O_3S]^+$, calc: 325.14; found: 325.14.