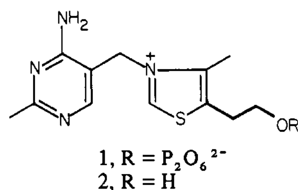
Thiamin-Catalyzed Decarboxylation of Pyruvate. Synthesis and Reactivity Analysis of the Central, Elusive Intermediate, α-Lactylthiamin

Ronald Kluger,* Jik Chin, and Timothy Smyth

Contribution from the Department of Chemistry, University of Toronto, Toronto, Ontario, Canada, M5S 1A1. *Received April 7, 1980*

Abstract: The thiamin-catalyzed decarboxylation of pyruvate has been proposed to proceed via a compound formed from the addition of thiamin to pyruvate, α-lactylthiamin (2-(1-carboxy-1-hydroxyethyl)thiamin). However, this adduct had eluded attempts at its synthesis or isolation. A straightforward synthesis has now been developed involving ethoxide-catalyzed reaction of ethyl pyruvate and thiamin forming ethyl 2-(1-carboxy-1-hydroxyethyl)thiamin. In concentrated hydrochloric acid the ester is converted to α-lactylthiamin. Kinetic methods were developed which permit all rate and equilibrium constants for the reaction system to be determined by UV or NMR spectroscopy. In some cases, consecutive reactions were analyzed by a two wavelength analysis which is explained in the Appendix to the paper. Measured rate constants (25 °C) include k_1, formation of α-lactylthiamin from pyruvate and thiamin (specific base catalyzed), 1.3 M^{-2} s^{-1}; k_{-1}, reverse of that process, 1.3 M^{-1} s^{-1}; k_2, decarboxylation of α-lactylthiamin at pH 7, 4.0×10^{-5} s^{-1}; k_2 at pH 4, 1.1×10^{-4} s^{-1}. Reaction of thiamin with 2 equiv of hydroxide to give the ring-opened product occurs with a rate constant of 1.3×10^{1} M^{-1} s^{-1} (since the rate-determining step occurs when the first hydroxide reacts). Ring closing to give thiamin is acid catalyzed, $k_3 = 5.2 \times 10^{5}$ M^{-1} s^{-1}. Application of these values, known and estimated pK_a's, and data in the literature for enzymic processes reveals that decarboxylation of α-lactylthiamin diphosphate on an enzyme is subject to less acceleration by the enzyme than is its reversion to pyruvate and thiamin by about a factor of 10^2. It also appears that the inherent stability of α-lactylthiamin diphosphate on an enzyme may require it to be destabilized. The gain in energy by the enzyme in such a process could be used for catalysis.

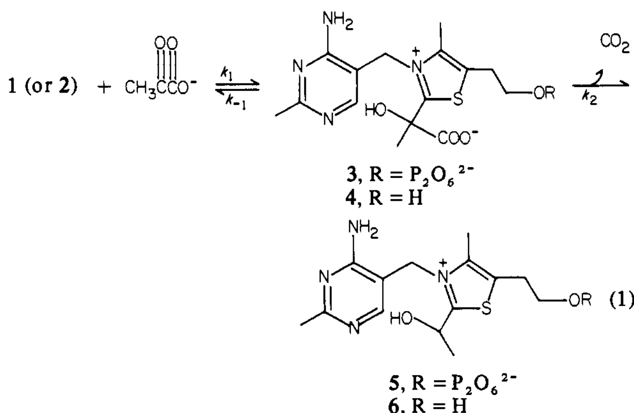
The decarboxylation of pyruvate in enzymic reactions, which formally requires generation of the acetyl C_1 carbanion, involves thiamin diphosphate (1) as a required cofactor.[1] The nonenzymic



1, R = $P_2O_6^{2-}$
2, R = H

decarboxylation is also catalyzed by **1** or its nonphosphorylated parent, thiamin (**2**), which is vitamin B_1.[2]

The catalytic reaction in the absence of enzyme is much less efficient than its enzymic counterpart, but the existence of the parallel led to studies of both systems in which common features were sought. After considerable effort by many groups, Breslow obtained evidence which suggested the now accepted pathway[3,4] which is given in eq 1.



3, R = $P_2O_6^{2-}$
4, R = H

5, R = $P_2O_6^{2-}$
6, R = H

The two key intermediates in the Breslow mechanism are α-lactylthiamin (**4**) and hydroxyethylthiamin (**6**). Soon after the route was proposed, hydroxyethylthiamin diphosphate (**5**) was isolated from the enzymic system.[5] Hydroxyethylthiamin was synthesized and its reactions were studied.[1,6] However, quantitative studies of both systems required the availability of the precursors that lose carbon dioxide, α-lactylthiamin (**4**) and α-lactylthiamin disphosphate (**3**). Reactions leading to and from (**3**) must involve transition states in need of stabilization by an enzyme[7] which should be compared with the nonenzymic reactions of **4**. However attempts at synthesis of **4** (and **3**) had been unsuccessful.[1,8]

Lienhard studied some aspects of these problems with compounds which contain functional groups similar to the substituted thiazolium moiety of α-lactylthiamin.[9,10] He found that solvents with a lower dielectric constant than that of water markedly increase the rate of decarboxylation of 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride, suggesting that pyruvate decarboxylases may have hydrophobic active sites. He also predicted a rate constant for the decarboxylation of α-lactylthiamin. The rate of elimination of ethyl pyruvate from 2-(1-carbethoxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride was also measured, but no rate for the elimination of pyruvate was determined.

The model studies did not provide any information about the thermodynamic and kinetic properties of the equilibrium in which pyruvate and thiamin react reversibly to form α-lactylthiamin. These are key pieces of information for comparing the nonenzymic system with enzymic catalysis. Therefore, our goals were to synthesize α-lactylthiamin and to measure all rate and equilibrium constants associated with it in the catalytic cycle.

In this paper we describe the first synthesis of α-lactylthiamin, along with methods we developed and results we obtained which reveal directly its stability and reactivity during the catalysis of decarboxylation of pyruvate. We compare the results with those

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Table I. ^{1}H NMR Chemical Shift Values of Characteristic Peaks[a]

compd	2'-CH_3	4-CH_3	6'-H	CH_3-derived from pyruvate
thiamin	2.50	2.60	8.06	not present
α-lactylthiamin	2.26	2.36	7.24	1.93
ring-open thiamin	2.36	1.66	7.97	not present
hydroxyethylthiamin	2.36	2.46	7.26	1.63[b]
pyruvate	not present			2.25

[a] Singlets unless noted. [b] Doublet.

predicted from models and what is now known about the related enzyme system, so that the function of the protein in catalysis becomes more apparent.

Experimental Section

Materials. α-Lactylthiamin (2-(1-Carboxy-1-hydroxyethyl)thiamin Hydrochloride) (4). Thiamin hydrochloride (5.0 g, 15 mmol) was suspended in 100 mL of absolute ethanol and cooled to −5 °C under dry nitrogen. Ethyl pyruvate (5 mL) was added to the mixture, followed by ethanolic sodium ethoxide (formed from 0.7 g of sodium in 50 mL of ethanol). After 30 min of stirring at −5 °C, the solution was made acidic with anhydrous hydrogen chloride (prepared by adding drops of concentrated sulfuric acid to concentrated hydrochloric acid and passing the resultant gas through concentrated sulfuric acid). The precipitate, consisting of sodium chloride and thiamin, was removed by filtration. Solvent was removed from the filtrate by rotary evaporation at 25 °C (20 torr). The ethyl ester of α-lactylthiamin was left as a slightly yellow solid in 85% yield (5.8 g). In some preparations a thick oil remains which becomes a solid on standing overnight. Repeated recrystallization from acidic ethanol gave pure material: mp 224–226 °C dec; UV $\lambda_{max}^{H_2O}$ (pH 7) 272 nm (ε 13 900), 231 (ε 15 300), $\lambda_{max}^{H_2O}$ (pH 2) 265 nm (ε 14 700), 246 (ε 15 000); ^{1}H 60-MHz NMR (D_2O relative to internal DSS) δ 1.27 (3 H, t, ^2J = 7 Hz, CH_3CH_2O–), 2.20 (3 H, s, –(CH_3)COD), 2.53 (3 H, s, CH_3-pyrimidine), 2.67 (3 H, s, $CH_3C(4)$), 3.27 (2 H, t, ^2J = 6 Hz, –$CH_2C(5)$), 4.0 (4 H, m, –CH_2OD, –CH_2OC(=O)–), 5.7, (2 H, dd, ^1J = 17 Hz, –$H_2CN(3)^+$), 7.42 (1 H, s, H-pyrimidine). Anal. for $C_{17}H_{26}Cl_2N_4O_4S$: C, H, N.

The ethyl ester was converted to the acid (quantitatively) in 12 M hydrochloric acid during 18 h at room temperature. After concentration (20 °C (25 torr)) to remove excess hydrogen chloride and lyophilization, the chloride hydrochloride of **4** was obtained as a white solid (stored dry at −17 °C): mp 85 °C (gas, CO_2, evolves; resulting solid (**6**), mp 228–230 °C dec[7]); UV $\lambda_{max}^{H_2O}$ (pH 6.8) 274 nm (ε 12 200), 230 (ε 11 900), $\lambda_{max}^{H_2O}$ (pH 2) 263 nm (ε 12 700), 247 (ε 12 800); ^{1}H NMR (D_2O) δ 2.12 (3 H, s, >(CH_3)COD), 2.46 (3 H, s, CH_3-pyrimidine), 2.61 (3 H, s, $CH_3C(4)$), 3.21 (2 H, t, J = 6 Hz, –$CH_2C(5)$), 3.91 (2 H, t, ^2J = 6 Hz, –CH_2OD), 5.65 (2 H, dd, ^1J = 17 Hz, –$H_2CN(3)^+$), 7.29 (1 H, s, H-pyridine).

Spectroscopic Methods. All ^{1}H NMR spectra were recorded on a Varian T-60 spectrometer. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) was used as an internal reference for δ 0. A Varian Cary 210 spectrophotometer, equipped with a thermostated automatic sample changer and multiwavelength accessory, was used to record UV spectra and to follow changes in absorbance of samples as a function of time. Temperature was maintained at 25.0 ± 0.1 °C or 50.0 ± 0.1 °C with a Neslab Exacal circulator.

Product Analysis. ^{1}H NMR spectroscopy and UV spectroscopy were used to identify the products resulting from the reactions of α-lactylthiamin under different conditions. According to eq 1 and by our analyses, two sets of initial products are possible: hydroxyethylthiamin and carbon dioxide or thiamin and pyruvate. NMR spectra and UV spectra of genuine samples of thiamin and hydroxyethylthiamin were compared to those obtained from the reactions of α-lactylthiamin. For NMR analysis, the characteristic peaks listed in Table I were used and coincide with assignments reported by others.[11,12] It should be noted that the singlet methyl signals in the δ 2.2 and 2.6 region have never been unambiguously assigned specifically to the groups indicated but have become accepted to be in the order given (which is different at high pH, as in our study, than at low pH). Positions are numbered according to the system in which the pyrimidine ring is primed.

Decomposition Reactions of Thiamin and Hydroxyethylthiamin. It is well-known that thiamin undergoes a reaction in basic solutions which leads formally to addition of one atom of oxygen and opening of the thiazolium ring.[13] Spectral data for the "ring-open" compound are given in Table I. In basic solutions, thiamin that was generated from α-lactylthiamin became equilibrated with the "ring-open" product. Thiamin could be completely recovered when the solutions were made acidic.

We found that hydroxyethylthiamin is not produced from α-lactylthiamin at high pH (>10); therefore its decomposition was not a complicating factor at pH >10. Below pH 10 and above pH 8.5, both hydroxyethylthiamin and thiamin are produced, giving complex spectral results. Data from this region were not utilized.

Below pH 8.5, hydroxyethylthiamin is the principal product but its decomposition occurs at a rate that complicates the measurement of the rate of its production, so a kinetic procedure was developed which compensates for this precisely (discussed under Kinetic Methods).

Kinetic Methods. An amount of α-lactylthiamin hydrochloride was added to 3 mL of a buffer solution to give an absorbance of ca. 1.0 at 272 nm. Buffers used were 0.1 M sodium acetate (pH 3.5–6.0), 0.1 M potassium phosphate (pH 6.0–8.5), and 0.1 M potassium carbonate (pH 10.5–11.0). Ionic strength was adjusted to 1.0 with potassium chloride. In each pH range, reaction rates measured with 0.3 M buffers were identical with those measured with 0.1 M buffers.

Reactions were recorded on the UV spectrophotometer at single or dual wavelengths for 10 half-times to obtain final absorbance values. The final values were used as infinite time points in conventional first-order plots to obtain observed rate constants from data generated in the first 3 half-times. Some data had to be subjected to a two-wavelength procedure described below and justified in the Appendix when subsequent reactions were a kinetically complicating factor. The rate constants were reproducible (three trials) with an absolute uncertainty of less than 1.5%.

Below pH 6.5, the decarboxylation of α-lactylthiamin was followed by observing the change in absorbance at 270 nm as hydroxyethylthiamin is produced. Between pH 6.5 and 8.5, where decomposition of hydroxyethylthiamin occurs at a rate comparable to that of its production, the problem of separating rates of consecutive reactions is encountered. There is no spectral isosbestic point for the decomposition reaction of hydroxyethylthiamin, so the simple expedient of observing such a wavelength as a built-in correction does not exist. Therefore, in order to obtain the rate constant for production of hydroxyethylthiamin, we developed a method which utilizes the differences in changes in absorption at two wavelengths (253 and 285 nm). The rate of production could be extracted by subjecting the data to an algorithm based on the known extinction coefficients of α-lactylthiamin and hydroxyethylthiamin at these wavelengths. Details of the method and a proof of the algorithm are described in the Appendix.

Above pH 10.5, thiamin rather than hydroxyethylthiamin is principally produced by the loss of pyruvate from α-lactylthiamin. Under these conditions, thiamin undergoes rapid ring opening so that the observed rate of production of "ring-open" product is identical with the rate of production of thiamin. The "ring-open" form (**7**) undergoes a further, slow decomposition reaction. This reaction complicates the observation of production of **7** since its rate is comparable to the rate of conversion of α-lactylthiamin. Again, the two-wavelength procedure (264 and 270 nm) solved this problem.

Ring-Opening Reaction of Thiamin. The rate of attainment of equilibrium between thiamin and its "ring-open" form, **7**, was determined by following the change in absorbance at 270 or 254 nm using first-order plots as described earlier.

Equilibrium Constant for Formation of α-Lactylthiamin. ^{1}H NMR analysis, using the data in Table I and the known equilibrium between thiamin and **7**,[13] which could be observed separately, was done for a sample kept at 25.0 °C. Thiamin (0.2 mmol) in 0.8 mL of water was combined with 0.47 mmol of sodium hydroxide. After equilibrium was attained, the relative amounts of thiamin and the "ring-open" form were determined by integration of peaks in Table I. Then, 1 mmol of sodium pyruvate was added to give 1 mL of solution (now pH 9.8, as measured with a Radiometer pH meter 26, calibrated with NBS buffers). The ^{1}H NMR spectrum was recorded every 30 min until an unchanging (equilibrium) condition resulted (ca. 3 h). The relative amounts of species present were determined by integration of peaks listed in Table I. A similar experiment was carried out at pH 9.6 by using 0.40 mmol of sodium hydroxide.

Results

The rate constants for conversion of α-lactylthiamin to thiamin and pyruvate (k_{-1} in eq 1) or hydroxyethylthiamin and carbon dioxide (k_2 in eq 1) and for conversion of thiamin to an equilibrium

(11) Mieyal, J. J.; Bantle, G.; Votaw, R. G.; Rosner, I. A.; Sable, H. Z. *J. Biol. Chem.* **1971**, *246*, 5213.
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(13) (a) Clarke, H. T.; Gurin, S. *J. Am. Chem. Soc.* **1935**, *57*, 1876. (b) Maier, G. D.; Metzler, D. E. *Ibid.* **1957**, *79*, 4386. (c) Haake, P.; Duclos, J. M. *Tetrahedron Lett.* **1970**, 461. (d) Zoltewicz, J. A.; Uray, G. *J. Org. Chem.* **1980**, *45*, 2104.

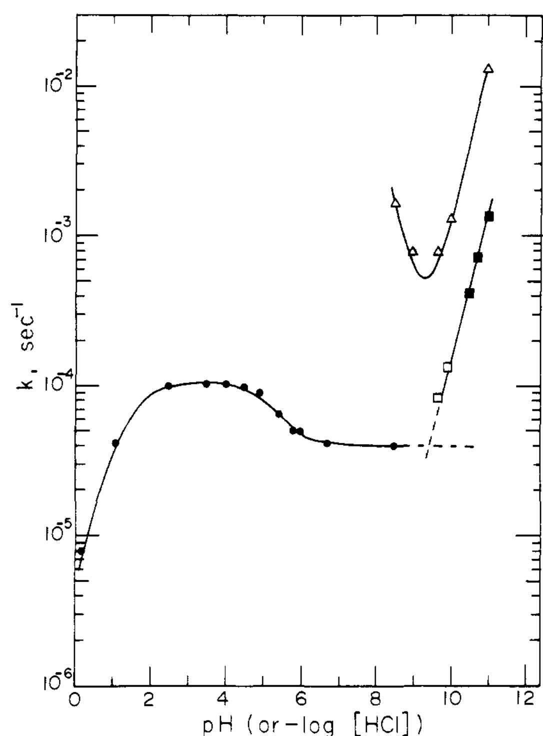
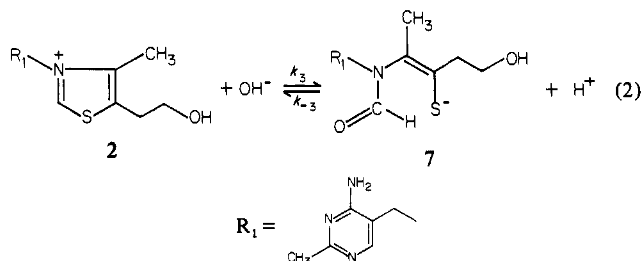
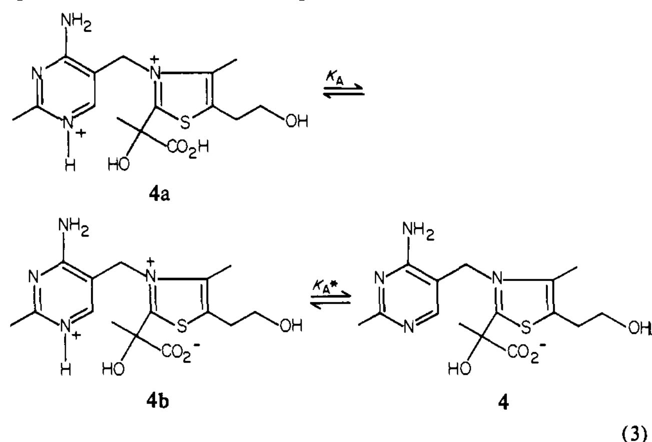


Figure 1. Rate constants for decarboxylation of α-lactylthiamin (●), expulsion of pyruvate from α-lactylthiamin (■), equilibration of thiamin and its ring-open form (△), and addition of thiamin to pyruvate (□).

mixture with its "ring-open isomer" ($k_3 + k_{-3}$ in eq 2) are presented in Figure 1 as a function of solution conditions.



Rate of Decarboxylation of α-Lactylthiamin. The rate data can be understood in terms of differing reactivities of the various protonation states of α-lactylthiamin. From Lienhard's model studies we know that **4a** will be unreactive[9] since in order for carbon dioxide to be able to leave, the proton on the carboxyl group must be removed first. The rate of decarboxylation of **4b** should be larger than that for **4** because the inductive effect of the protonated pyrimidine group is favorable for decarboxylation.



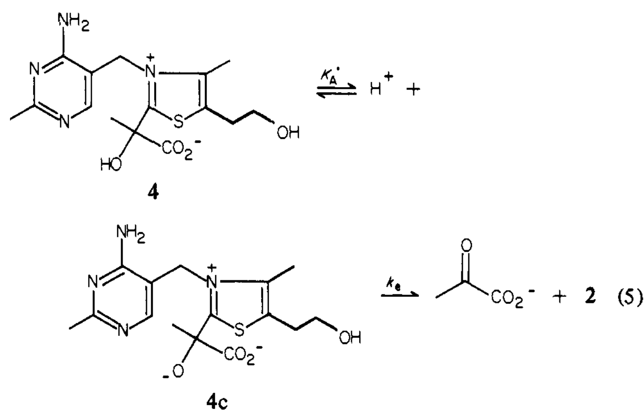
(3)

With $K_A = 5 \times 10^{-2}$ and $K_A{}^* = 1.6 \times 10^{-5}$, the rate constant for decarboxylation of **4b** being $k_2 = 1.1 \times 10^{-4}$ s^{-1} and that for **4** being $k_2{}^* = 4.0 \times 10^{-5}$ s^{-1}, the pH rate profile for decarboxylation in Figure 1 was constructed from eq 4.

$$k_{obsd} = \frac{K_A k_2{}^* K_A{}^* + k_2 a_{H^+} K_A}{K_A{}^* K_A + K_A a_{H^+} + a_{H^+}{}^2} \quad (4)$$

Rates were also measured at 50.0 °C, giving $k_2{}^{50°C} = 3.36 \times 10^{-3}$ and $k_2{}^{*50°C} = 1.28 \times 10^{-3}$ s^{-1}. The value of $k_2{}^*$ is close to what Lienhard had predicted by model studies and estimates of substituent effects.[9] However, the higher values of k_2 had not been anticipated.

Expulsion of Pyruvate from α-Lactylthiamin. The rate constant (k_{-1}) for this reaction could be derived from the data in Figure 1. The observed rate constant is proportional to hydroxide concentration and is not affected by buffer concentration. Therefore the process is specific base catalyzed: $k_{obsd} = k_{-1}[OH^-]$ (where $k_1 = 1.3$ M^{-1} s^{-1}). In terms of mechanism, this suggests that loss of the hydroxyl proton of **4** to give **4c** precedes elimination. Since



(5)

the pyruvate expulsion reaction is base catalyzed and the decarboxylation reaction is not, at high pH α-lactylthiamin produces more thiamin and pyruvate than it does hydroxyethylthiamin and carbon dioxide. In terms of the thiamin-catalyzed decarboxylation of pyruvate, which proceeds via α-lactylthiamin, there is a change in rate-limiting step (for 1 M standard states since k_1 describes a third-order process, k_{-1} second-order, and k_2 first-order) from thiamin pyruvate combination to decarboxylation of α-lactylthiamin when $k_{-1}[OH^-] = k_2{}^*$. This occurs at pH 9.5. Model studies had not anticipated this situation since reactions above pH 7 were not considered.[9,10]

Ring-Opening Reactions. Thiamin is well-known to react with hydroxide ions to become in equilibrium with a form in which the thiazolium moiety is not intact[12,13] (eq 2). The mechanism of this reaction involves initially addition of hydroxide ion to C_2 of the thiazolium ring in a slow step, followed by a base-catalyzed ring opening of the pseudobase to give **7**.

The reactions of α-lactylthiamin at very high pH produce pyruvate and thiamin without any ring opening of α-lactylthiamin occurring. However, the thiamin that is produced is rapidly converted to an equilibrium mixture of **2** and **7**. The reversibility of the **2** → **7** reaction is demonstrated by the fact that acidification of the solution converts the ^{1}H NMR spectrum entirely to that of thiamin.

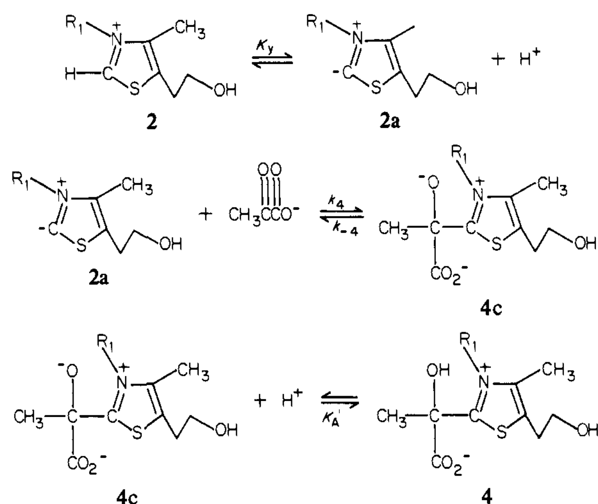
In order to obtain quantitatively reliable data on the reactions of α-lactylthiamin, we measured the rate of attainment of equilibrium between **2** and **7**. The results are plotted in Figure 1. It can be seen that equilibrium of **2** and **7** is fast compared to reactions of α-lactylthiamin to produce thiamin. The rate constant for attainment of an equilibrium mixture of **2** and **7** starting from pure thiamin is given by

$$k_{obsd} = k_3[OH^-] + k_{-3}[H^+]$$

By varying solution pH, we obtained k_3 and k_{-3} from k_{obsd}. Thus, from the data in Figure 1, $k_3 = 1.3 \times 10^1$ M^{-1} s^{-1} and $k_{-3} = 5.2 \times 10^5$ M^{-1} s^{-1}.

Solvent Effects. Lienhard noted the large increase in rate of decarboxylation of thiazolium compounds related to α-lactyl-

Scheme I



thiamin in solvents of lower polarity than water.[9] A similar observation is apparent for α-lactylthiamin. In methanol at 25 °C, we find the rate is about 100 times that in water, and in ethanol, it is about 10^4 times greater than in water.

Rate of Formation of α-Lactylthiamin. The rate constant remaining to be determined for the reaction involving thiamin in eq 1 is k_1, the formation of α-lactylthiamin from pyruvate and thiamin. Since at pH >9 α-lactylthiamin is converted more rapidly to pyruvate and thiamin than it decarboxylates, an equilibrium between **2**, **4**, **7**, and pyruvate can be established.

The measured value of k_1/k_{-1} from ^{1}H NMR analysis is the same at pH 9.6 and 9.8 and is 1.0 M^{-1}. Therefore the value of k_1 is 1.3 $M^{-2}\ s^{-1}$. The rate constant for this process has not been measured by this direct procedure before. However, a rate constant for the same process at 30 °C can be extracted from measured rates of proton uptake for a solution of thiamin and pyruvate at pH 7.8 as reported by Jordan and Mariam.[12] Under those conditions, our data require that formation of α-lactylthiamin is rate limiting so that the observed rate constant should yield k_1. However, Jordan and Mariam's data yield a value of k_1 that is larger by a factor of about 200 than the value we report. Professor Jordan has reinvestigated his experiments and has now concluded that the rate they measured is complicated by side reactions.[14]

Discussion

Reactivity of α-Lactylthiamin. The conversion of α-lactylthiamin to hydroxyethylthiamin and carbon dioxide (k_2 in eq 1) is first order and is fastest for the species in which the pyrimidine ring is protonated. The two plateau rate constants are related by a pK_a of 4.9, a value close to that which has been determined for thiamin itself.[15] The rate constant at 25 °C, pH 7.0 of 4 × $10^{-5}\ s^{-1}$, corresponds to a half life of 4.8 h, obviously too slow to be competent for an enzymic process.

The other process available to α-lactylthiamin is reversion to pyruvate and thiamin (k_{-1}). Since this is a specific-base-catalyzed process, the barrier to expulsion is reduced by increasing hydroxide concentration and the competition with decarboxylation becomes favored. At pH 9.5, the rate of expulsion of pyruvate equals the rate of decarboxylation. This equivalence point had not been anticipated and other studies had ignored this aspect of reactivity, assuming only decarboxylation would occur or a decomposition of α-lactylthiamin by some other (ring-opening) process.[9] In fact, ring-opening of α-lactylthiamin is so slow that it does not compete with either reaction under all conditions of our study. Obviously, the steric and/or electronic character of the large anionic substituent at the C(2) position prevent the normally facile addition of hydroxide to form the pseudobase of the thiazolium salt.

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(15) Cain, A. H.; Sullivan, G. R.; Roberts, J. D. *J. Am. Chem. Soc.* **1977**, *99*, 6423.

Stability of α-Lactylthiamin. The equilibrium constant for formation of α-lactylthiamin from pyruvate and thiamin is 1.0 M^{-1}. This indicates that in dilute solution, α-lactylthiamin will be unstable with respect to dissociation into pyruvate and thiamin. The implications for enzymic catalysis via this species are discussed later.

Reaction Mechanism: Formation of α-Lactylthiamin. The pK_a of thiamin for proton dissociation at C(2) to form an ylid (**2a**) is 12.7.[16] The anion must be the species that adds to pyruvate in the specific-base-catalyzed process. The observed rate constant can be related to the likely mechanism presented in Scheme I.

$$\frac{d\mathbf{4}}{dt} = k_4[\mathbf{2a}][CH_3COCO_2^-] = k_1[OH^-][\mathbf{2}][CH_3COCO_2^-]$$

$$k_4 = k_1[OH^-][\mathbf{2}]/[\mathbf{2a}]$$

$$K_y = [\mathbf{2a}][H^+]/[\mathbf{2}] = 2.0 \times 10^{-13}\ M$$

$$[\mathbf{2}]/[\mathbf{2a}] = [H^+]/2.0 \times 10^{-13}$$

$$k_4 = k_1 \times 0.5 \times 10^{-1}$$

$$k_4 = 6.5 \times 10^{-2}\ M^{-1}\ s^{-1}$$

For the reverse reaction, K_a' has been estimated to be 2.5 × 10^{-14} M.[10]

$$\frac{d\mathbf{2}}{dt} = k_{-4}[\mathbf{4c}] = k_{-1}[\mathbf{4}][OH^-]$$

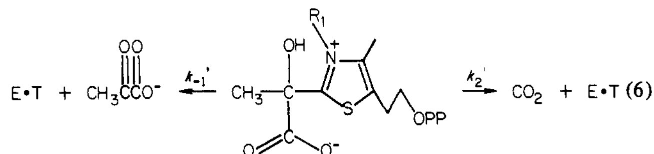
$$k_{-4} = k_{-1}[\mathbf{4}][OH^-]/[\mathbf{4c}] = 0.4k_{-1}$$

$$k_{-4} = 0.5\ s^{-1}$$

Effects of an Enzyme. The conversion of pyruvate to acetaldehyde by yeast pyruvate decarboxylase is described by a first-order rate constant (at saturation) of 3.9 × 10 s^{-1}, at pH 6.8.[10] In this enzyme, the rate-determining step is probably conversion of hydroxyethylthiamin diphosphate to acetaldehyde, regenerating enzyme-bound thiamin diphosphate.[5] Therefore, the rate constant for decarboxylation must be larger than the observed overall value.[10]

Under the same conditions, the rate constant for nonenzymic decarboxylation of α-lactylthiamin is 4 × $10^{-5}\ s^{-1}$. Therefore, the enzymic rate constant is larger by a factor of at least 10^6, agreeing with Lienhard's analysis based on model compounds.[10] Lienhard has suggested that part of this enzymic acceleration can result from the reaction occurring in an enzymic region of low polarity, since nonpolar solvents markedly increase the rate of reaction. Others have suggested that another source of acceleraton could be the involvement of a Brønsted acid which could protonate the very basic incipient hydroxyethylthiamin diphosphate carbanion which is formed as carbon dioxide is lost.[17,18]

Carbon isotope effect studies indicate that the value of the ratio k_2'/k_{-1}' in eq 6, the ratio of the rate constant of the decarboxylation



step to that for the steps competing with it, is about 5.[19,20] This information enables us to analyze the effects of the enzyme on the reaction producing pyruvate and, by microscopic reversibility, the formation of the adduct as well. The enzyme increases the rate of expulsion of pyruvate from the adduct by about a factor of 10^8 at pH 6.8 (primed rate constants refer to enzymic processes).

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$$\frac{k_{-1}'}{k_{-1}[\mathrm{OH}^-]} = \frac{k_2'}{k_2^*}\left(\frac{k_2/k_{-1}[\mathrm{OH}^-]}{k_2'/k_{-1}'}\right)$$

$$\frac{k_{-1}'}{k_{-1}[\mathrm{OH}^-]} = 10^6\left(\frac{500}{5}\right) = 10^8$$

It has been suggested that general-base catalysis by a group on the enzyme may be involved in converting α-lactylthiamin diphosphate to pyruvate and thiamin diphosphate.[17,18] The upper limit of a general-base-catalyzed process can be approximated by the rate constant for elimination from the conjugate base of the substrate (a very late transition state with respect to proton transfer). Since our data give a value for the rate constant of such a process of 0.5 s^{-1}, it would appear that the enzymic rate may nearly be accounted for. An additional factor that could promote elimination would be the enzyme's ability to bind the adduct in such a restricted manner that no rotational entropy loss occurs in proceeding to the transition state (giving a rate factor of up to about 5).[21]

In the direction of addition of the thiamin diphosphate derived ylid to pyruvate, the second-order rate constant of the corresponding nonenzymic process involving thiamin (k_4) is 6.5×10^{-2} M^{-1} s^{-1}. If we assume that the effect of uncompensated loss of translational entropy on binding to an enzyme is typical of other bimolecular processes, the apparent concentration of one reactant with respect to the other is 10^8 M.[21] Therefore, the expected enzymic rate constant (k_4') is 6.5×10^6 s^{-1}. At pH 6.8, the concentration of the ylid derived from the coenzyme would be $1/10^6$ of the total coenzyme concentration, since the coenzyme's pK_a is about 12.7.[14] This would make the overall rate constant for addition of bound ylid to bound pyruvate about 6 s^{-1} (with 1 order of magnitude of uncertainty at least). This rate constant is comparable to that for decarboxylation of the adduct and the reversion of its anion. The equilibrium constant for formation of **3** from enzyme-bound species is $1.0\ M^{-1} \times 10^8\ M = 1.0 \times 10^8$. The corresponding dissociation constant is then 1.0×10^{-8} M. The dissociation constant of pyruvate from the Michaelis complex in the case of pyruvate oxidase, a thiamin diphosphate-dependent decarboxylase, is 10^{-4} M, and the same value probably applies to pyruvate dehydrogenase and pyruvate decarboxylase as well, on the basis of the similarity of their K_m values for pyruvate.[22] Therefore, dissociation of enzyme-bound **3** into pyruvate and enzyme-bound thiamin diphosphate is given in general by 10^{-4} M $\times$ 10^{-8} = 10^{-12} M.

It has been reported that the dissociation constant of the pyruvate analogue, methyl acetylphosphonate, from its enzymic (pyruvate dehydrogenase) adduct with thiamin diphosphate is about 10^{-8} M.[17,23] The equilibrium constant for its nonenzymic dissociation is also about 1 M. Therefore, if the adduct is a good analogue of **3**, the enzyme destabilizes the adduct by a factor in its dissociation constant of about $10^{-8}/10^{-12} = 10^4$. In terms of energy, this corresponds to about 6 kcal which can be partially utilized by the enzyme to stabilize the transition states associated with the catalytic system.[24] In particular, the energy of formation of **3** may be used to desolvate **3** on the enzyme, promoting its own decarboxylation.

Acknowledgment. We thank the Atkinson Charitable Foundation of Toronto for their continued support and the Natural Sciences and Engineering Research Council of Canada for an operating grant.

Appendix

The Two-Wavelength Procedure. Consider the system in eq A1. The rate constant of interest is k_1 but k_2 is comparable in

$$\mathrm{A} \xrightarrow{k_1} \mathrm{B} \xrightarrow{k_2} \mathrm{C} \qquad (A1)$$

magnitude. The value of k_1 can be obtained with only the ratio of differences of the extinction coefficients of B and C at two wavelengths being known in addition to the spectral changes with time. Initially, absorbance at an arbitrary wavelength of the solution, starting with pure A, is given by eq A2, where $\epsilon_1(\mathrm{A})$ is

$$\mathrm{abs}_1^{\,0} = \epsilon_1(\mathrm{A})[\mathrm{A}]_0 \qquad (A2)$$

the extinction coefficient of A. Absorbance at any later time is given by eq A3. The change in absorbance after any time interval

$$\mathrm{abs}_1^{\,t} = \epsilon_1(\mathrm{A})[\mathrm{A}]_t + \epsilon_1(\mathrm{B})[\mathrm{B}]_t + \epsilon_1(\mathrm{C})[\mathrm{C}]_t \qquad (A3)$$

is given by eq A4 since $[\mathrm{A}]_0 = [\mathrm{A}]_t + [\mathrm{B}]_t + [\mathrm{C}]_t$.

$$(\Delta\mathrm{abs}_1)_t = \mathrm{abs}_1^{\,0} - \mathrm{abs}_1^{\,t} = (\epsilon_1(\mathrm{A}) - \epsilon_1(\mathrm{B}))[\mathrm{B}]_t + (\epsilon_1(\mathrm{A}) - \epsilon_1(\mathrm{C}))[\mathrm{C}]_t \qquad (A4)$$

Now, if we define the ratio of extinction coefficient differences at two wavelengths as $(\epsilon_1(\mathrm{B}) - \epsilon_1(\mathrm{C}))/(\epsilon_2(\mathrm{B}) - \epsilon_2(\mathrm{C})) = Q$, then eq A5 follows. With use of eq A6, k_1 can be obtained from the

$$(\Delta\mathrm{abs}_1 - Q\Delta\mathrm{abs}_2)_t = ((\epsilon_1(\mathrm{C}) - \epsilon_1(\mathrm{A})) - (\epsilon_2(\mathrm{C}) - \epsilon_2(\mathrm{A}))Q)([\mathrm{B}]_t + [\mathrm{C}]_t) \qquad (A5)$$

$$(\Delta\mathrm{abs}_1 - Q\Delta\mathrm{abs}_2)_t = ((\epsilon_1(\mathrm{C}) - \epsilon_1(\mathrm{A})) - (\epsilon_2(\mathrm{C}) - \epsilon_2(\mathrm{A}))Q)[\mathrm{A}]_0(1 - e^{-k_1 t}) \qquad (A6)$$

absorbance data at two wavelengths since at $t = \infty$, eq A6 becomes a constant, Y.

$$(\Delta\mathrm{abs}_1 - Q\Delta\mathrm{abs}_2)_\infty = ((\epsilon_1(\mathrm{C}) - \epsilon_1(\mathrm{A})) - (\epsilon_2(\mathrm{C}) - \epsilon_2(\mathrm{A}))Q)[\mathrm{A}]_0 = Y \qquad (A7)$$

$$(\Delta\mathrm{abs}_1 - Q\Delta\mathrm{abs}_2)_t = Y(1 - e^{-k_1 t}) \qquad (A8)$$

Taking logarithms

$$\ln\,[(\Delta\mathrm{abs}_1 - Q\Delta\mathrm{abs}_2)_\infty - (\Delta\mathrm{abs}_1 - Q\Delta\mathrm{abs}_2)_t] = -k_1 t + \ln\,(\Delta\mathrm{abs}_1 - Q\Delta\mathrm{abs}_2)_\infty \qquad (A9)$$

Therefore, a plot of $\ln\,[(\Delta\mathrm{abs}_1 - Q\Delta\mathrm{abs}_2)_\infty - (\Delta\mathrm{abs}_1 - Q\Delta\mathrm{abs}_2)_t]$ vs. time has a slope of $-k_1$.

This procedure does not require the existence of an isosbestic point at either wavelength and is completely justified by the equations presented.

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