Thiamin Diphosphate: A Mechanistic Update on Enzymic and Nonenzymic Catalysis of Decarboxylation

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Contents

Ronald Kluger was born in New Jersey in 1943. While an undergraduate at Columbia he was told by Gilbert Stork to devise a way to make deuteriochloroform so it would be cheap enough to use for NMR spectra, which he did (*J. Org. Chem.* **1964**, *29*, 2045). His graduate work with Elkan Blout (in peptide chemistry) and then with Frank Westheimer at Harvard (on the mechanism of phosphate ester hydrolysis) led to his current interest in bioorganic reaction mechanisms (Ph.D., 1969). He was a postdoctoral fellow at Brandeis with Robert Abeles where he learned mechanistic enzymology. He joined the Department of Chemistry at the University of Chicago (1970) and moved to the University of Toronto (1974) where he is a Professor of Chemistry. He has been an Alfred P. Sloan Fellow and received the Merck Sharp and Dohme Lecture Award of the Chemical Institute of Canada in 1982. He is a coauthor of about 60 research papers and holds patents for some novel sleep drugs. His current interests are reaction mechanisms and stereochemistry of catalysis involving coenzymes, quantitative aspects of catalytic reactivity in phosphate and acyl-transfer reactions, and the design of biologically active molecules.

I. Introduction

A. Purpose and Scope

The elucidation of biochemical reaction mechanisms in terms of principles of organic chemistry is a major activity in modern bioorganic chemistry. The purpose of this review is to summarize the application of these principles to a system that has provided us with continued mechanistic interest: catalysis by thiamin diphosphate dependent enzymes of the decarboxylation of pyruvate. The review is organized in terms of intermediates derived from thiamin. Since the area has been reviewed in the past,[1-3] primary emphasis will be on papers appearing between 1975 and 1986, although

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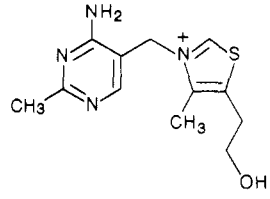
earlier work that forms an important background will be cited as well. The emphasis of the review will be on material related to work that has been done in our laboratory.

B. Metabolic Functions of Thiamin

Thiamin is best known for its function as the dietary requirement and additive, vitamin B_1.[4] In this role, it continues to be in great demand as a supplement for those who are at risk from beriberi and Korsakoff's disease[5] as well as those who are concerned about the sufficiency of their diet. The vitamin is added to many foods[6] and has been by recommended by outdoorspersons as an effective insect repellent. With such a large market for the material for human consumption, competition in its production has led to this rather complex synthetic chemical becoming readily available in pure form and at modest cost. Few materials can compete with thiamin (1) in providing such a combined appeal of medicinal relevance, structural novelty, and catalytic potency. Its reactions continue to receive a large amount of attention, and modification of its structure has been an ongoing activity throughout the world.



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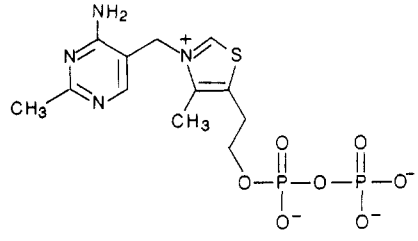

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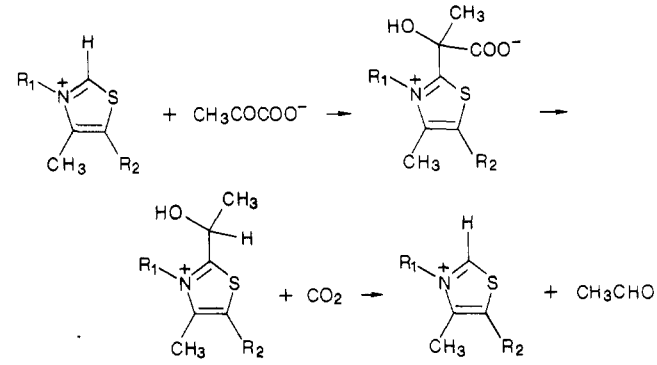
Thiamin serves a number of essential metabolic functions, and its deficiency leads to imbalances in carbohydrate status. These deficits have particularly deleterious effects on nerve function.[7] It is known that in living systems much of the thiamin that is utilized is as the diphosphate, which is formed by the reaction of thiamin with ATP.[1] The diphosphate functions as a coenzyme for enzymes involved in the metabolism of α-keto acids and related compounds. The absence of thiamin diphosphate leads to accumulation of excessive pyruvate in the blood and accompanying lactic acidosis from its reduction product.[5] In plants, thiamin diphosphate is utilized to produce acetolactate from pyruvate, a biosynthetic precursor of branched-chain amino acids. By blocking this pathway, materials that inhibit the action of acetolactate synthetase are powerful herbicides.[8]

II. Catalysis by Thiamin

A. Background: The Thiazolium Ylide Mechanism

From the time of the discovery of thiamin in the 1930s until the 1950s very little was known about the manner in which thiamin was involved in metabolic reactions.[1,4] The discovery that thiamin itself could catalyze reactions analogous to those catalyzed by pyruvate decarboxylase (the conversion of pyruvate to acetaldehyde and carbon dioxide along with the production of acetoin[9]) led to model studies with thiamin and related materials.[1] The culmination of this work was Breslow's elegant demonstration of the catalytic functional dependence upon dissociation of the proton from C-2 of the thiazolium ring, producing an ylide (the structure is discussed later) that can add to the carbonyl group of pyruvate.[10] The thiazolium ring can serve as an electron sink for the decarboxylation process (Scheme I).

SCHEME I



The subsequent reaction pathway then is a logical consequence of the structure of the intermediate. Soon afterward, it was demonstrated that a covalent intermediate derived from thiamin diphosphate (2) and acetaldehyde could be isolated from enzymic reaction systems.[11-14] This close relationship between enzymic and nonenzymic reactions provided an unprecedented opportunity for detailed examination of the mechanisms of enzymic catalysis. Intermediates or analogues were prepared, and their reactivity patterns have been elucidated.

B. Catalytic Role of Thiamin Diphosphate: Providing the Equivalent of a Carbonyl-Centered Carbanion

Pyruvate decarboxylase catalyzes the conversion of pyruvate to acetaldehyde and carbon dioxide, an electrophilic substitution reaction of a proton for carbon dioxide at an acyl carbon.

$$CH_3C(O)COO^- + H^+ \rightarrow CH_3C(O)H + CO_2$$

The electronic properties of a carbonyl center are conducive to nucleophilic substitution, rather than electrophilic substitution. If the enzyme catalyzed a direct electrophilic substitution reaction at an acyl center, this would be chemically unprecedented. Chemists have overcome this problem by the use of a variety of synthetic equivalents to the acyl carbanions.[15] The inventor of pyruvate decarboxylase used thiamin diphosphate to achieve a similar result. In the enzymic system, thiamin diphosphate adds to pyruvate to provide a material that stabilizes increased electron density at the carbon atom that was originally in the carbonyl group of pyruvate. It should be noted that although the functional groups of proteins are diverse, they do

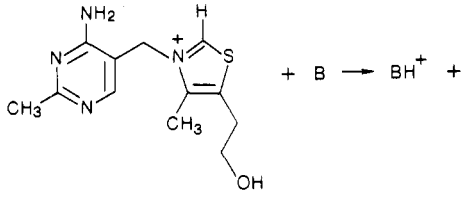
not include any that might assist in the efficient generation of acyl carbanion equivalents. This is the special domain in which thiamin diphosphate functions as a coenzyme.

C. Reaction Intermediates Derived from Thiamin Diphosphate

Thiamin diphosphate undergoes a number of conversions during its function as a coenzyme for the decarboxylation of pyruvate and in its reactions with other substrates. This review will examine each of these in terms of the pyruvate decarboxylase reaction and nonenzymic analogies.

1. Ylide Form of Thiamin Diphosphate

The first step in any catalytic cycle that involves thiamin diphosphate is removal of the proton from the 2-position of the thiazolium ring. The remarkable



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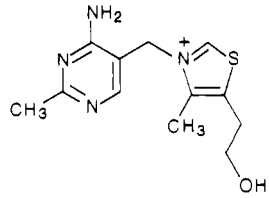

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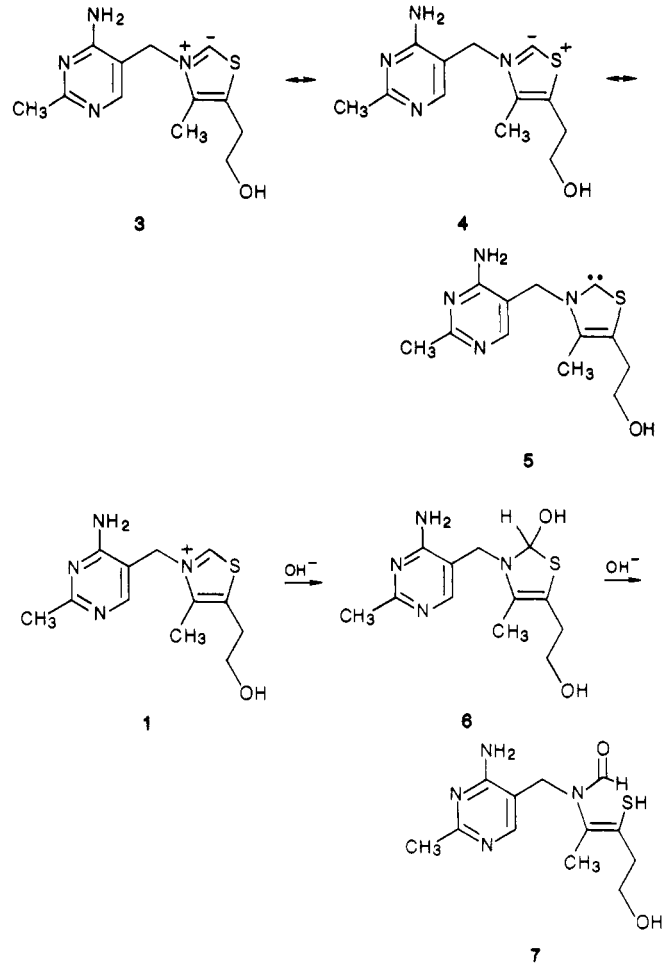
facility of ionization of this C–H species has been the subject of experimental and theoretical investigations. The exchange of the proton for a deuteron in neutral solution is evidence for formation of the ylide (3) as an intermediate.[10] However, if removal of the proton is the rate-determining step in the exchange process, the rate of addition of a proton to the ylide is not revealed in such an experiment. Determination of the pK_a of the carbon acid should provide the necessary information, but such a measurement is not routine, as discussed below.

a. Structure of the Ylide. The ability of thiamin to form an ylide can be rationalized in terms of molecular orbital theory and resonance structures.[16-19] Although the ylide is commonly portrayed as a structure with a formal positive charge on nitrogen (3), an important resonance structure can be drawn in which the positive charge resides on sulfur and nitrogen is neutral (4). In both cases, the electronegative elements stabilize the adjacent anion by inductive effects.[20,21] A neutral "carbene" structure also plays an apparent role in the observed reactivity of this species (5).[22,23]

b. Determination of the pK_a of Thiamin. Since thiamin is able to transfer the proton from the 2-position of the thiazolium ring to a Brønsted base, it might be expected that in a basic solution thiamin will be in equilibrium with significant amounts of the ylide form. However, this is not the case, since thiamin and thiamin diphosphate also undergo formation of a pseudobase (6) by addition of hydroxide to the thiazolium ring at



C-2. The pseudobase reacts further with hydroxide to form a ring-opened derivative (7).[24-29] At equilibrium, the ylide is undetectable in the presence of thiamin and the ring-opened material.

Since the pK_a of thiamin cannot be determined by direct titration, alternative procedures must be devised. A number of indirect methods for determination of pK_a are well established, and each has its own potential for error. Most of these depend on kinetic procedures to determine this thermodynamic quantity. The status of the problem with respect to several of these procedures will be summarized.

If the rate constant for removal of the C-2 proton from thiamin as well as that for reprotonation under the same conditions is known, then the pK_a' (the apparent pK_a under the conditions of the experiment) can be found by a combination of arithmetic and the use of solution constants. As explained below, the exchange of a proton for an isotope of hydrogen from C-2 gives the rate constant for proton removal but not the rate constant for reprotonation. The problem of the effect of the solvent isotope further complicates matters. Kemp and O'Brien measured the rate of the hydroxide-catalyzed detritiation of the C-2 position of thiamin.[30] The reaction is fast, and thus the kinetic isotope effect should be small. Since the reaction was conducted in water, no solvent isotope effect complicates the measurement.

The exchange reaction is a two-step process with the thiamin ylide serving as a steady-state intermediate. In [2-^{3}H]thiamin is T–H*, the thiamin ylide is T^-, and thiamin is T–H.

$$OH^- + T\text{-}H^* \xrightarrow{k_1} T^- (+HOH^*) \quad (1)$$

$$HOH + T^- \xrightarrow{k_2} T\text{-}H + OH^- \quad (2)$$

The rate law for the observed hydroxide-catalyzed exchange is

$$v = k_{obsd}[OH^-][T\text{-}H^*] = k_2[T^-] \quad (3)$$

Since the ylide is a steady-state intermediate

$$d[T^-]/dt = 0 \quad (4)$$

$$k_1[T\text{-}H^*][OH^-] = k_2[T^-][HOH] \quad (5)$$

$$[T^-] = k_1[T\text{-}H^*][OH^-]/k_2[T^-] \quad (6)$$

Therefore

$$v = k_1[T\text{-}H^*][OH^-] = k_{obsd}[OH^-][T\text{-}H^*] \quad (7)$$

$$k_{obsd} = k_1 \quad (8)$$

Since the second-order rate constant for the hydroxide-catalyzed detritiation of thiamin is 4×10^5 M^{-1} s^{-1}, the value of k_1 is the same. For the purposes of obtaining an approximate pK_a, the isotope effect will be ignored.

The equilibrium constant for the hydroxide-catalyzed proton removal followed by reprotonation is given by k_1/k_2 in the preceding formulations and is the inverse of the basicity constant K_b.

$$K_b = [OH^-][T\text{-}H]/[T^-] = k_2/k_1 \quad (9)$$

$$pK_b = \log k_1 - \log k_2 = 5.6 - \log k_2 \quad (10)$$

$$pK_w - pK_a = pK_b \quad (11)$$

At 30 °C pK_w is 13.8, therefore

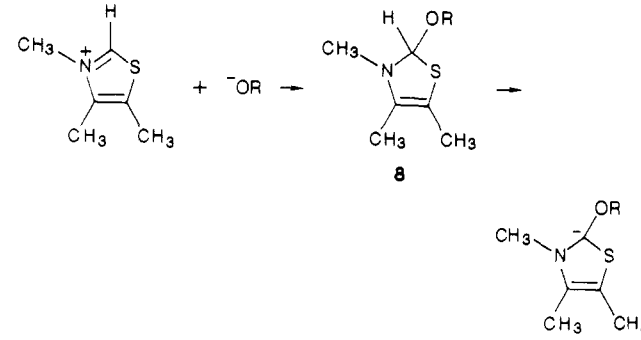
$$pK_a = pK_w - pK_b = 13.8 - 5.6 + \log k_2 \quad (12)$$

$$pK_a = 8.3 + \log k_2 \quad (13)$$

The value for the pK_a of thiamin at 30 °C is known if the rate constant for protonation of the thiamin ylide by water is known for these conditions. Since this value has not yet been determined directly, it must be estimated. If thiamin is considerably more basic than hydroxide, then one would expect the rate constant for proton transfer from water to occur at the maximum rate for protonation of a localized carbanion. Recently, Bednar and Jencks measured the rate of protonation of cyanide ion by hydronium ion and found it to be 4×10^{10} M^{-1} s^{-1} at 20 °C.[31] Other acids whose pK_a values are below that of HCN react with cyanide at rates approximating 10^9 M^{-1} s^{-1}. As a localized carbanion, the thiamin ylide can be considered an analogue of cyanide. If we assume that the rate constant for protonation at 30 °C is 10^9 M^{-1} s^{-1} and the concentration of water is 55 M, the apparent first-order rate constant for protonation of the thiamin ylide by water is about 5×10^{10} M^{-1} s^{-1} (i.e., $\log k_2 = 10.7$). This gives a pK_a for thiamin of 19. If the rate constant for protonation of thiamin ylide by water is smaller, then the pK_a will also be reduced by the corresponding logarithm.

If one assumes that thiamin is a *weaker* base than water, proton transfer from water to the thiamin ylide will occur with a smaller rate constant than does transfer from thiamin to hydroxide. Therefore, the rate constant of 4×10^5 M^{-1} s^{-1} for proton transfer from thiamin to hydroxide corresponds to that for a process in the thermodynamically favored direction. This is considerably below the value for thermodynamically favorable transfer from HCN, but the analogy may not be appropriate. In this case, the pK_a should be 13 or less, and the upper limit for k_2 is about 10^5 M^{-1} s^{-1} (the pK_a could also equal that of water, and the second-order rate constants in both directions would be equal).

SCHEME II

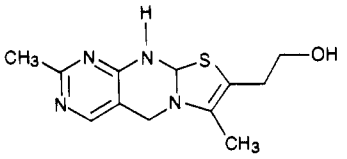


In order to determine the pK_a for the dissociation of thiamin to form the ylide, Hopmann and Brugnoni used rapid-reaction techniques.[32] It is expected that ionization of thiamin to yield the ylide will be the kinetically favored process, while addition of hydroxide and ring opening are thermodynamically favored. During the time before a significant amount of addition of hydroxide has occurred, an unstable equilibrium between thiamin and its ylide may be established. Thus, by rapidly scanning the spectrum of thiamin that is mixed with base (in this case borate buffer) in a stopped-flow apparatus, Hopmann and Brugnoni reported that they could observe establishment of the unstable equilibrium. They assigned a pK_a of 12.7 to thiamin on the basis of data from these experiments. The possible general-base catalysis by the buffer and identification of the species observed spectrophotometrically are not considered in detail, so the interpretation is somewhat uncertain. However, if we accept the value reported, this makes thiamin a stronger acid than water and the value for k_2 approximately 3×10^4 M^{-1} s^{-1}. In terms of the experimental and theoretical information so far available, the relatively low value for the pK_a may be a reasonable one. However, Bunting suggests that the pK observed by Hopmann is pK_{R^+} for pseudobase formation and the actual pK is near 20.[33] The problem that needs explanation is the small size of the rate constant for protonation of hydroxide by thiamin, if this is the thermodynamically favored process. Direct determination of the rate constant for protonation of the thiamin ylide or a study of the rate constant for deprotonation of thiamin by Brønsted bases with pK_a values straddling the proposed pK_a should be of significant interest.

c. Ylide Formation in Nonaqueous Solutions. The possibility that the ylide form of thiamin may not be responsible for the observed catalytic patterns was suggested by Karimian on the basis of his studies on the reactions of thiazolium compounds with alkoxides in nonaqueous solvents.[34] In those cases, a strong steric effect was observed for the exchange reaction of the proton at the 2-position of the thiazolium ring. It is proposed that in nonaqueous conditions an alkoxide must add to the thiazolium ring and the resulting adduct (8) is the species that undergoes exchange (Scheme II).

Haake noted that such a result could not account for the behavior of thiamin in aqueous solution since the rate of pseudobase formation is slower than the rate of exchange.[35] Furthermore, it was noted by Karimian that thiamin does not undergo the nucleophilic addition reactions in nonaqueous solvents that are observed for other thiazolium species. Zoltewicz's demonstration that thiamin reacts with bisulfite through an initial reaction at its pyrimidine ring indicates the complexity of the reactivity patterns of thiamin.[36] Thus, although it is clear that thiamin exchanges protons at C-2 via the ylide whose structure was proposed by Breslow, the behavior in nonaqueous solutions has not been established. Since the hydrophobicity of the active site of thiamin diphosphate dependent enzymes has been demonstrated,[37] one might be tempted to suggest that other mechanisms might be operative, but this is unlikely since the medium is aqueous.

d. Ionized Thiamin: Isolation of the Ylide and Dimer. The ylide derived from thiamin should be accessible by proton removal from C-2 of thiamin itself under conditions in which reprotonation cannot occur. If the ylide could be isolated, determination of its reactivity and structure would answer many important mechanistic questions, including a possible determination of the rate constant for protonation discussed above. It has recently been reported that treatment of thiamin hydrochloride with 2 equiv of sodium ethoxide in ethanol (5 °C, overnight, under nitrogen) yields a material that precipitates in 3.8% yield.[38] It has been proposed that the material has spectroscopic properties expected for the ylide derived from thiamin. However, the authors note that Maier and Metzler reported the same reaction and identified the product as the tricyclic material **9**.[25,26] Risinger and co-workers also investigated the reaction and proposed that the structure is the further cyclized product **10**.[39]



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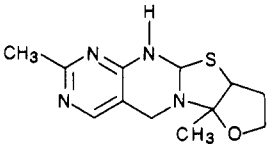

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The NMR peaks can be assigned to match the ylide structure, but the recent analysis is not presented in sufficient detail to make a critical judgment as to the correctness of the assignments. The fact that the ^{13}C NMR spectrum does not permit an assignment of all signals limits the usefulness of the analysis. Upon learning of these results of the isolation of the proposed ylide, Michael Brandl in our laboratory attempted the synthesis. However, he was not able to isolate the material that was reported, and we must await publication of further experimental details to know whether this material can be studied as an isolated species.

2. Thiazolium vs. Oxazolium vs. Imidazolium

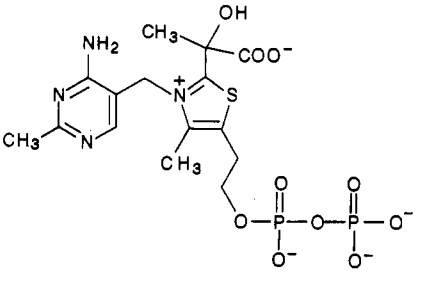
It is a natural activity for chemists to speculate about why a biochemical system has evolved to utilize a particular functional group. In the case of thiamin, it is easy to see the potential role of a positively charged nitrogen in stabilizing an ylide and to assume that no other stable positively charged ion is readily available in a biochemical system. As noted earlier, the role of the sulfur atom in stabilizing the ylide carbanion is probably significant, but would another atom work better?

Several authors have compared the thiazolium compound's reactivity with that of the related oxazolium and imidazolium compounds. Imidazolium compounds are much less acidic than thiazolium compounds, and so an insufficient concentration of ylide is available in neutral solution.[2,18] Lowe and Ingraham noted that although oxazolium ions are more acidic than thiazolium ions, they do not add to benzaldehyde.[40] They concluded that the anion is also less reactive than thiazolium and thus a poorer catalyst. Such a conclusion requires assuming that basicity and nucleophilicity are not directly related, whereas one would normally expect such a correlation.

A more probable reason has been identified by other workers. Sable and co-workers noted that the "azolium" compounds are all subject to addition reactions by hydroxide, leading to formation of a pseudobase.[2] The pseudobase form does not undergo carbon ionization but rather undergoes a ring-opening reaction, which is catalyzed by a second mole of hydroxide. Therefore, the ratio of the rate constant for proton removal to that for hydroxide addition is an important factor in the kinetic control of ylide formation. Sable noted that oxazolium compounds tend to ring open rather than ionize and that this corresponds to an advantage of about 10^2 in favor of thiazolium as a catalyst. Since the rate of the catalytic reaction depends on the concentration of the active form of the catalyst, the tendency of oxazolium ions to undergo ring opening by reaction with hydroxide suppresses the accumulation of ylide.

D. 2-(2-Lactyl)thiamin and the Diphosphate Derivative

The adduct formed by the addition of the ylide derived from thiamin diphosphate to pyruvate is named as a derivative of lactic acid with thiamin diphosphate connected from C-2 of the thiazolium ring to C-2 of lactate. We will refer to the adduct as lactylthiamin diphosphate (**11**) and the parent compound lacking the diphosphate as lactylthiamin (**12**) (lactyl = 2-hydroxy-2-carboxylatoethyl).



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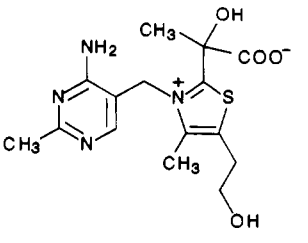

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1. Isolation of Lactylthiamin Diphosphate

Lactylthiamin diphosphate is expected to have properties that will permit facile loss of carbon dioxide (making it an "active pyruvate"). Its decarboxylation product is 2-(1-hydroxyethyl)thiamin diphosphate, the precursor of acetaldehyde. Both intermediates were sought soon after Breslow proposed the now accepted mechanism, and reports of the isolation of both intermediates appeared. However, as explained below, it is unlikely that lactylthiamin diphosphate has been isolated from the pyruvate decarboxylase system.

Holzer and Beaucamp added [2-^{14}C]pyruvate to pyruvate decarboxylase and quenched the enzyme reaction with hot methanol.[14,41] Material was isolated by paper chromatography that was identified as [^{14}C]-2-(1-hydroxyethyl)thiamin diphosphate. This released [^{14}C]acetaldehyde upon incubation with the apoenzyme. A second material was isolated whose mobility was similar to that of thiamin diphosphate and that upon incubation with apoenzyme also gave [^{14}C]-2-(1-hydroxyethyl)thiamin diphosphate. When [1-^{14}C]pyruvate was used, the second material isolated upon reincubation with the apoenzyme released $^{14}CO_2$. The yield of the second material was less than 1%, while 2-(1-hydroxyethyl)thiamin diphosphate was isolated in over 10% yield. On the basis of these results, Holzer identified the second material as lactylthiamin diphosphate. Later, Lienhard and co-workers prepared an analogue of lactylthiamin diphosphate and showed that it is extremely reactive in methanol and ethanol.[42] Treatment with hot methanol would lead to complete decarboxylation in a matter of minutes. Even in water, the material spontaneously is converted to 2-(1-hydroxyethyl)thiamin diphosphate in a few hours. Thus, genuine lactylthiamin diphosphate would not survive the conditions under which the second material had been isolated. Later work from our laboratory with synthetic lactylthiamin diphosphate and lactylthiamin confirmed Lienhard's general predictions of reactivity. It is likely then that the material Holzer isolated was probably another adduct of thiamin diphosphate. One possibility that would account for the observed results is isolation of the thiamin diphosphate adduct of acetolactate, which forms from the condensation of the conjugate base of 2-(1-hydroxyethyl)thiamin diphosphate and pyruvate followed by release from the enzyme and partial decomposition of the acetolactate.

2. Synthesis of Lactylthiamin Diphosphate

Although 2-(1-hydroxyethyl)thiamin and 2-(1-hydroxyethyl)thiamin diphosphate were readily prepared by condensation of acetaldehyde with thiamin or thiamin diphosphate,[1] attempts to prepare lactylthiamin and lactylthiamin diphosphate were unsuccessful. Krampitz remarked that he expected lactylthiamin and lactylythiamin diphosphate to be very unstable, since they are analogous to malononitrile, which is quite unstable.[1] This suggested that the material could be an intermediate on the reaction pathway, but it would be very short lived since it would readily decarboxylate.

Attempts to use a complex synthesis in which the lactyl group was first prepared with a precursor of the thiazolium moiety attached were reported to be unsuccessful.[43] Thus, although there was little doubt that lactylthiamin and lactylthiamin diphosphate are intermediates in the nonenzymic and enzymic reactions, they remained elusive.

As mentioned earlier, Lienhard and co-workers used model compounds to predict the reactivity of lactylthiamin. They studied the reactions of thiazolium compounds that contained a lactyl side chain at the 2-position.[42,44] They found, as expected from what was known from the reaction patterns of carboxylated pyridines, the undissociated form of the carboxylic acid in aqueous solution is resistant to decarboxylation. Dissociation of the proton as well as transfer to a solvent less polar than water accelerates the decarboxylation rate. Since carbon dioxide is the leaving group in this reaction, the proton on the carboxyl group must be removed or the conjugate acid of carbon dioxide, a high-energy species, will be produced. The transition state for decarboxylation of the conjugate base involves less charge separation than does the reactant, and thus the reaction is accelerated by transfer to a less polar solvent. Lienhard's group also measured the rate at which the ethyl esters of these analogues undergo elimination to produce ethyl pyruvate.[44] They found that the reaction is subject to base catalysis, and one could expect that the unesterified material would also undergo base-catalyzed elimination, although no specific model for that process was studied.

Lienhard's work provided guidance for the synthesis of lactylthiamin and lactylthiamin diphosphate. The resistance of Lienhard's compounds to decarboxylation while the carboxyl group remains protonated suggested that lactylthiamin and lactylthiamin diphosphate should be isolable in strongly acidic solutions. Thiamin itself is stable in acid. Thus, an attempt was made in our laboratory to produce lactylthiamin under acidic conditions. Pyruvic acid will condense with thiamin under alkaline or neutral conditions, and under these conditions, lactylthiamin rapidly decarboxylates, so direct synthesis is ruled out.

Therefore, an alternative indirect route was used for the synthesis of lactylthiamin. Risinger had shown that the use of ethanol as a solvent rather than water prevents the well-documented decomposition of thiamin by reaction with hydroxide.[45] We prepared the ethyl ester of lactylthiamin by condensation of ethyl pyruvate and thiamin in ethanolic sodium ethoxide.[28] Ethyl lactylthiamin was readily isolated and crystallized. Dissolving the material in concentrated hydrochloric acid leads to hydrolysis of the ester group without decarboxylation of the resulting lactylthiamin. The material was isolated by evaporation of the hydrogen chloride. Lactylthiamin can be recrystallized and is stable as long as it is protonated, as predicted by Lienhard's work. The conjugate base decarboxylates more rapidly in solvents less polar than water. In methanol, it has a half-life of a few minutes.[28] A modification of this synthesis led to the preparation of lactylthiamin diphosphate from thiamin diphosphate and *tert*-butyl pyruvate.[46] The properties of lactylthiamin and lactylthiamin diphosphate are clearly incompatible with the material isolated by Holzer's group that was reported to be lactylthiamin diphosphate.

3. Reactivity of Lactylthiamin

As expected, lactylthiamin readily undergoes decarboxylation, producing 2-(1-hydroxyethyl)thiamin in

high yield. At high pH (>10), lactylthiamin also can undergo an elimination reaction to produce pyruvate and thiamin. We reported a quantitative study of the reactivity of lactylthiamin as a function of solution acidity.[28] The decarboxylation rate very closely follows Lienhard's predictions based on his model studies. The elimination of pyruvate from lactylthiamin was not predictable from the models. The elimination is base catalyzed, and at pH 9.5 the rates of elimination and decarboxylation are equal. At higher pH, elimination is the dominant process. These results show unambiguously that in the thiamin-catalyzed decarboxylation of pyruvate formation of lactylthiamin is rate limiting below pH 9.5, while at higher pH, decarboxylation of lactylthiamin is rate limiting.

The rate of decarboxylation of lactylthiamin is maximal between pH 3 and 4. In solutions of this acidity, the pyrimidine ring is protonated but the carboxyl group of lactylthiamin is not. The protonated pyrimidine provides polarization that stabilizes the increase in electron density in the molecule as carbon dioxide is lost from lactylthiamin. Protonation of the carboxyl group slows the reaction, since breaking the carbon–carbon bond necessary for decarboxylation would generate protonated CO_2, a high-energy species. In the unprotonated state, CO_2 is readily generated, and the residual electron density is dissipated into the thiazolium ring.

4. Isotope Effects

The reactions of lactylthiamin can be studied by using isotope effects as a probe of mechanism. The CO_2 lost from pyruvate during catalysis by pyruvate decarboxylase was monitored for ^{13}C content in studies by three groups.[47-49] Although the intermediates were not observed directly, the $^{12}C/^{13}C$ ratio in the product compared with that in the reactant during the early stages of conversion gives an accurate kinetic isotope effect, which indicates to what extent loss of CO_2 from lactylthiamin is rate limiting. The results indicate that the decarboxylation is only partially rate limiting in the enzyme-catalyzed process leading to the loss of CO_2. The formation of lactylthiamin is also partially rate limiting.

In addition to the primary isotope effect, secondary isotope effects have been observed by Alvarez and Schowen in the pyruvate decarboxylase reaction.[50] They used [*methyl*-$^3H_3$]pyruvate as a substrate and found that the secondary isotope effect on V/K is 0.88, consistent with an activation process occurring by addition of a group of the protein to the carbonyl group of pyruvate. The isotope effect on V_{max} is 1.08, but interpretation was uncertain since the intrinsic isotope effects on individual steps were not known. Obviously, interpretation of the secondary isotope effect can be done with confidence if good models for the intrinsic isotope effects are known. This motivated us to prepare [$^3H_3$]lactylthiamin and measure the secondary isotope effects on the rate constants for its decarboxylation and for elimination of [$^3H_3$]pyruvate.[51] We observed isotope effects of 1.09 for decarboxylation and 1.10 for elimination. In a less polar medium the isotope effects do not change significantly. Therefore, whatever the polarity of the enzymic environment, one can safely predict the magnitude of the intrinsic isotope effect for each step and conclude that one or both steps is rate determining in the enzymic reaction.

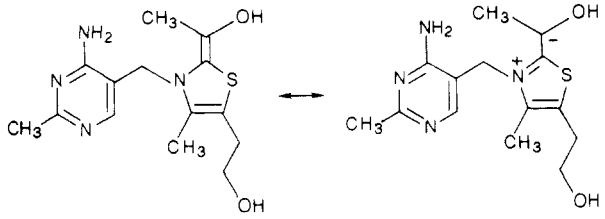
5. Enzymic Reactions of Lactylthiamin Diphosphate

The addition of pyruvate to thiamin diphosphate is a common step proposed in the mechanism of pyruvate decarboxylase, pyruvate oxidase, pyruvate dehydrogenase, acetolactate synthetase, and other thiamin diphosphate dependent enzymes.[1] This addition should generate lactylthiamin diphosphate, and it would be of interest to follow the reaction in which an apoenzyme (lacking thiamin diphosphate) combines with lactylthiamin diphosphate. When we prepared lactylthiamin diphosphate, we found that we were unable to observe such a combination process with pyruvate decarboxylase and lactylthiamin diphosphate.[46] Any association and activation appears to occur more slowly than the rate at which lactylthiamin diphosphate decarboxylates spontaneously to 2-(1-hydroxyethyl)thiamin diphosphate. Control experiments using esters of lactylthiamin diphosphate and the decarboxylated material 2-(1-hydroxyethyl)thiamin diphosphate showed that these were capable of binding to the apoenzyme.[46] We have explained elsewhere that these results suggest that the enzyme specifically sequesters lactylthiamin diphosphate generated in situ and the exogenously produced material may not find the enzyme present in a form that will bind lactylthiamin diphosphate.[3,46]

a. Generalization of the Concept of Conformational Generation by Intermediate Formation. Further studies with other enzymes are necessary to test the generality of the consideration that enzymes should not bind exogenously prepared intermediates if the formation of the intermediate in the enzymic reaction provides energy for conformational changes of the enzyme. This conclusion about enzyme-generated intermediates follows from a suggestion made Wolfenden about transition-state binding[52] and contrasts with generalizations about enzymic affinities: the fact that an enzyme will bind tightly to a species does not mean that the conformation of the enzyme that does this is present at a significant concentration. The generation of intermediates or transition states by an enzyme may be energetically coupled to the production of high-energy conformations that have catalytic advantages. Alston and Abeles observed a similar result with the binding of analogues to histidine decarboxylase.[53]

E. Conjugate Base of the Carbon Acid of 2-(1-Hydroxyethyl)thiamin: The "Second Carbanion"

Lactylthiamin in neutral solution is a betaine, having a positive charge in the thiazolium group and a negative charge in the carboxylate function. The elimination of carbon dioxide from lactylthiamin formally moves the anionic center to the α-carbon of the side chain (adjacent to the thiazolium ring) while retaining the positive center in the thiazolium group (**13**). The excess electron density at the α-carbon creates carbanionic character at this center. An important resonance structure can be drawn for this in which the charge is neutralized through transmission of electron density into the thiazolium group, creating an enamine function (**14**).[10]



14 13

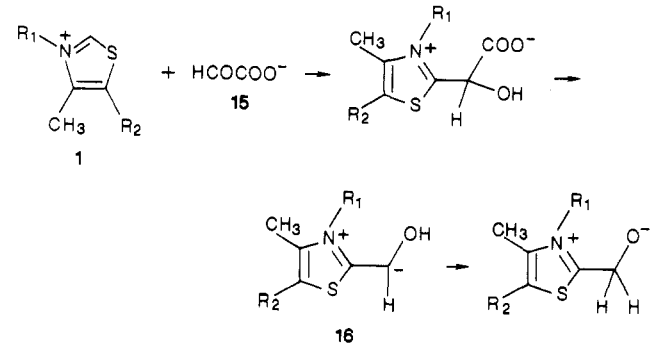
The structure and reactivity of this material have been the subject of experimental and theoretical interest. Protonation at the α-carbon produces 2-(1-hydroxyethyl)thiamin, the species depicted in the model catalytic cycle for decarboxylation of pyruvate to produce acetaldehyde. In the enzymatic reaction, the corresponding diphosphates are produced. The carbanion/enamine form is necessary for any condensation reaction to occur at the α-position, and this has led to consideration of the thermodynamic and kinetic acidities.

Sable and co-workers used proton NMR studies to demonstrate proton–deuteron exchange at the α-position of 2-(1-hydroxyethyl)thiamin and 2-(1-hydroxyethyl)thiamin diphosphate.[54,55] The reaction is much slower than the exchange of the thiazolium C-2 proton of thiamin. The half-life for exchange (pD 8.5, 50 °C) of the α-proton of 2-(1-hydroxyethyl)thiamin is 5 h, corresponding to an observed first-order rate constant of 4×10^{-5} M^{-1} s^{-1} or a second-order rate constant of about 40 M^{-1} s^{-1}. For comparison, the rate constant reported by Kemp and O'Brien[30] for the hydroxide-catalyzed detritiation of [2-^{3}H]thiamin at 30 °C is 7.5×10^5 M^{-1} s^{-1}. Correcting for the temperature difference and the isotope effect, one can estimate that the proton of thiamin is removed approximately 10000 times faster than the proton from the α-position of 2-(1-hydroxyethyl)thiamin. If the rate of protonation of the two species is the same, then thiamin has a pK_a 5 units lower than 2-(1-hydroxyethyl)thiamin. Since the conjugate base was not observed and the rate-determining step for exchange is certainly proton removal, the thermodynamic basicity of the product can only be estimated. This conjugate base is a key intermediate in both enzymic and nonenzymic catalysis and, therefore, further information is needed if a quantitative understanding of its reactivity patterns is to be available for application.[42]

1. Tautomerization of the Carbanion–Enamine

The conjugate base of 2-(1-hydroxyethyl)thiamin contains a hydroxyl group adjacent to the carbanionic site. If the rate of exchange of the α-position in the parent compound reflects its thermodynamic acidity, then the species is much more basic than an alkoxide group, and one would expect to observe that protonation on the α-carbon accompanied by deprotonation of the hydroxyl group should occur. This tautomerization would significantly reduce the concentration of the species capable of undergoing reactions characteristic of the carbanion. The alkoxide thus generated should release acetaldehyde. In nonenzymic reactions, release of acetaldehyde does not occur, while it occurs efficiently in pyruvate decarboxylase. Sable and co-workers considered this problem and suggested that the enzyme controls the course of the reaction.[55] A possible solution to this problem is control of the conformation

SCHEME III



of the side chain relative to the thiazolium ring by the enzyme so that the angle necessary for formation of a carbonyl group elimination of the thiamin ylide is maintained. This possibility should be considered in context of the information presented later in this review concerning conformational effects on reactivity. In the following section, some important work with alternative substrates, which gives further information about the stability of the α-carbanion, is presented.

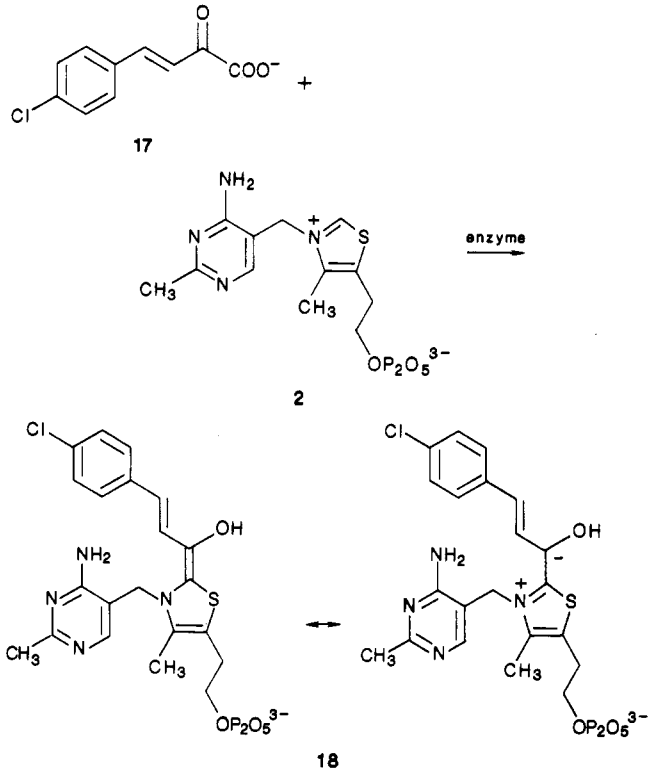
2. Enzyme-Generated Inactivators and the Carbanion Equivalent

Schellenberger and co-workers found that glyoxylic acid irreversibly inactivates pyruvate decarboxylase.[56] Through the use of radioactive labeling, these workers demonstrated that the enzyme catalyzes the loss of carbon dioxide from this substrate, but the resulting product is not released from the enzyme. Subsequent analysis of the denatured enzyme reveals formation of an adduct of thiamin diphosphate. They proposed that the resulting conjugate base of enzyme-bound 2-(hydroxymethyl)thiamin diphosphate cannot be released because it assumes a stabilized conformation that is inaccessible to species with any larger substituent. That is, the carbanion can be delocalized as the enamine, whereas for the case of the normal species with the larger hydroxyethyl side chain, the methyl group interacts with a site on the enzyme. This holds the material in a conformation that prevents formation of the enamine resonance structure, which would make the carbanion less basic than the alkoxide tautomer (Scheme III).

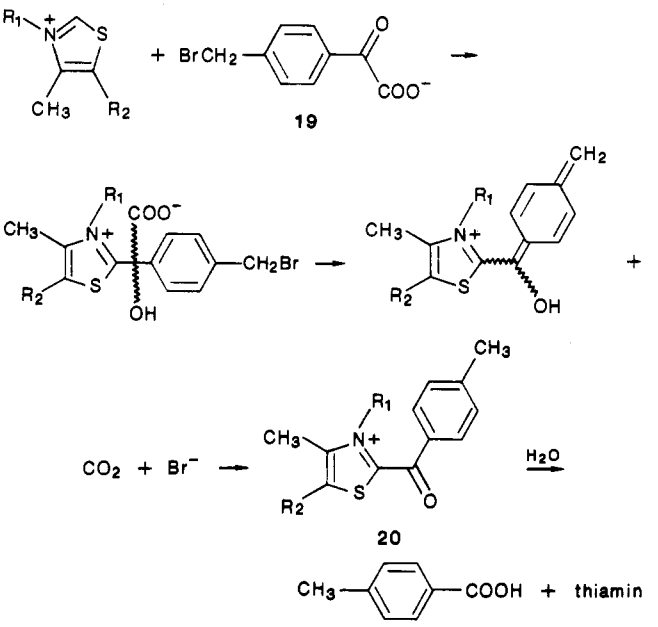
In the normal enzymic reaction, tautomerization by migration of the proton from the hydroxyl group of the side chain to carbon occurs rapidly, followed by elimination of acetaldehyde. Schellenberger's analysis is very intriguing, since it would explain why the nonenzymatic reaction (pyruvate and thiamin) does not produce free acetaldehyde.

Since the facts are not certain in this case, it is interesting that using a mechanistic analysis very different from that presented in the preceding section, Kuo and Jordan were also able to design an effective inhibitor of the same enzyme.[57,58] (One may note that since two groups designed effective inhibitors using completely different mechanistic rationales for the same enzyme, this proves that effective mechanism-based inhibitors can result from consideration of different mechanisms for the same enzyme, at least one of which must be disprovable.) Kuo and Jordan reasoned that the enzyme specifically stabilizes the carbanion equivalent as the enamine and that more stable analogues of this

SCHEME IV



SCHEME V



species would bind irreversibly if generated on the enzyme. These workers studied the binding and reaction of (*E*)-4-(4-chlorophenyl)-2-keto-3-butenoic acid (**17**) with pyruvate decarboxylase. Addition of the thiamin diphosphate ylide to this compound followed by decarboxylation should generate the enamine with extended conjugation (Scheme IV). In the enzyme reaction, they observe a new absorbance, which they assign to the extended enamine (**18**).[57,58]

It is proposed that protonation of the material is retarded because of the stabilization that is available to the conjugate base by the extended delocalization. This leads to irreversible inactivation of the enzyme. The kinetic implications of such a phenomenon are complex, but the hypothesis is attractive and should have further applications. One must be cautious since the material that is used as inhibitor is very different from the normal substrate, it is turned over very slowly, and the source of the inactivation has not been proven.

Kozarich, Kenyon, and their co-workers recently reported that benzoylformate decarboxylase can generate an enzyme-bound intermediate that appears to render thiamin diphosphate temporarily inactive.[59,60] Using *p*-(bromomethyl)benzoylformate (**19**) as a substrate, these workers found that the enzyme catalyzes the elimination of bromide and the production of toluic acid via the proposed intermediacy of benzoylthiamin diphosphate (**20**), which hydrolyzes slowly (Scheme V). This intermediate results from tautomerization of the product of the bromide elimination reaction. Interestingly, the fluoro analogue does not undergo the elimination reaction but is processed as a normal substrate, producing *p*-(fluoromethyl)benzaldehyde.[60]

3. Evidence for a Delocalized Carbanion

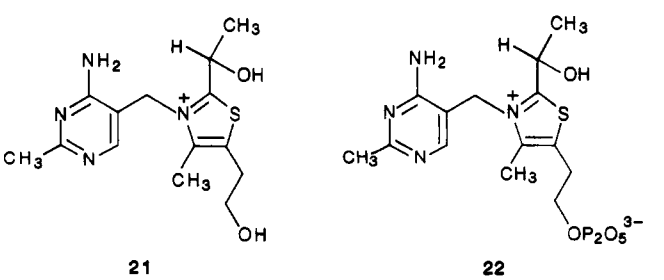
The structure of the intermediate is of some interest. Is the hydroxyethyl side chain planar, or is the carbanion localized at a tetrahedral center?[3,65] During the course of the decarboxylation, is protonation concerted with loss of carbon dioxide to avoid generation of the high-energy intermediate? Ullrich isolated 2-(1-hydroxyethyl)thiamin diphosphate from the reaction of pyruvate with the E1 subunit of pyruvate dehydrogenase and showed that the material is optically active.[62] Since this compound is not normally formed during the catalytic process of this enzyme, its formation comes as a result of a quenching of the immediate decarboxylation product with a proton source. If 2-(1-hydroxyethyl)thiamin diphosphate is added to pyruvate dehydrogenase lacking thiamin diphosphate, the material is not accepted for further reaction by the enzyme.

F. 2-(1-Hydroxyethyl)thiamin

As noted earlier, the acetaldehyde adduct of thiamin was the first intermediate prepared after Breslow proposed its intermediacy in the Mizuhara reaction.[1,10]

1. Preparation of 2-(1-Hydroxyethyl)thiamin

Krampitz, Sprague, and co-workers prepared 2-(1-hydroxyethyl)thiamin (**21**) and showed that the material will react with acetaldehyde under the Mizuhara conditions to produce acetoin.[12] Later, Risinger developed a convenient synthesis of 2-(1-hydroxyethyl)-thiamin in which the material is prepared by condensation of acetaldehyde and thiamin in ethanol with



sodium ethoxide.[45] The use of ethanol and ethoxide avoids the reaction of thiamin with aqueous base, which degrades the thiazolium ring.[24] The reaction of ethoxide with thiamin does not promote the decomposition reaction since this requires removal of a proton from the adding nucleophile.

2. Resolution of 2-(1-Hydroxyethyl)thiamin

2-(1-Hydroxyethyl)thiamin is a chiral molecule with the stereocenter located at the 2-position of the hydroxyethyl side chain. Japanese workers reported that the enantiomers of (hydroxyethyl)thiamin both promote growth in rats and that both are converted to thiamin in rat liver.[63,64] Although the physical properties of the resolved materials are reported, the procedure for the resolution is not. Since the absolute stereochemistry of reactions involving thiamin and thiamin diphosphate are of interest in enzymic and nonenzymic reactions, the resolution of 2-(1-hydroxyethyl)thiamin was reinvestigated in our laboratory. Victoria Stergiopoulos was able to resolve the material as the dibenzoyl tartrate salt by fractional crystallization in ethanol.[65] Subsequently, Professor Khashayar Karimian improved the procedure while on his first visit to our laboratory (subsequently, Professor Karimian left the University of Tehran on an extended basis). In order to determine the absolute stereochemistry of the resolved materials, Gerald Gish attempted a series of oxidation, reduction, and hydrolysis reactions that would be expected to convert the stereocenter to the 2-position of lactic acid. Unfortunately, despite many reaction attempts, no lactic acid was obtained.[17] Therefore, an alternative was needed, and Karimian found that 2-(1-hydroxyethyl)thiamin could be readily converted to a crystalline material that retained the stereochemistry of interest.[66] Dr. George Detitta and Walter Pangborn at the Medical Foundation of Buffalo determined the absolute stereochemistry of the derivative by X-ray crystallography. The result of this determination permitted the assignment of the *R* configuration to the (+) enantiomer of 2-(1-hydroxyethyl)-thiamin.[66]

G. 2-(1-Hydroxyethyl)thiamin Diphosphate

1. Preparation and Isolation

Krampitz and co-workers prepared the diphosphate of racemic (hydroxyethyl)thiamin by condensation of thiamin diphosphate with acetaldehyde under mildly basic conditions. They showed that the apoenzyme of wheat germ pyruvate decarboxylase will convert this material to acetaldehyde and thiamin diphosphate.[11] Krampitz reported that no attempt was made to convert 2-(1-hydroxyethyl)thiamin to 2-(1-hydroxyethyl)-thiamin diphosphate, since there was considerable uncertainty about the stability of 2-(1-hydroxyethyl)thiamin under the reaction conditions.[11]

Radioactively labeled 2-(1-hydroxyethyl)thiamin diphosphate was isolated from yeast pyruvate decarboxylase by Holzer and Beaucamp who incubated [2-^{14}C]pyruvate with yeast pyruvate decarboxylase.[41] The isolated material was shown to comigrate on paper chromatography with genuine 2-(1-hydroxyethyl)thiamin diphosphate. Krampitz has commented on some of the complications of these experiments.[1]

2. Stereochemical Studies of 2-(1-Hydroxyethyl)thiamin Diphosphate

The material is produced in the enzymic decarboxylation of pyruvate and accumulates in significant quantities. It is chiral, but in only one reported case has the optical activity of the product been noted. As mentioned earlier, Ullrich and Mannschreck isolated (−)-2-(1-hydroxyethyl)thiamin diphosphate from the E1 subunit of pyruvate dehydrogenase.[62] This material can now be assigned the absolute stereochemistry of the *S* configuration at the stereocenter.[66]

Stereochemistry can provide much detailed insight into the mechanism of enzymic reactions.[67,68] It is surprising that although the intermediates in the reactions of pyruvate decarboxylase are chiral, so little was known about the stereochemical properties of the reactions. In order to exploit the information from our studies of the resolution of (hydroxyethyl)thiamin for the purposes of the elucidation of enzyme mechanisms, it is necessary to be able to convert (hydroxyethyl)-thiamin to the diphosphate with the diphosphate group specifically attached to the primary alcohol group of (hydroxyethyl)thiamin. The procedures for conversion of thiamin to thiamin diphosphate were developed in Karrer's laboratory in the 1940s,[69] and the methodology worked very well for us in the preparation of thiamin diphosphate and thiamin thiazolone diphosphate from the corresponding alcohols.[70] The conditions for the reaction involve heating 85% phosphoric acid to remove water, which converts this material into an oligomeric gum. The phosphoric acid oligomers react with the hydroxyl group of the substrate to produce pyrophosphorylated derivatives (as well as triphosphates and monophosphates, which can be separated by ion-exchange chromatography). The extension of the method to 2-(1-hydroxyethyl)thiamin was not straightforward, however.

Reaction of 2-(1-hydroxyethyl)thiamin under the Karrer conditions did not reproducibly give the desired diphosphate. Gerald Gish investigated the reaction and found that considerable phosphorylation of the secondary alcohol also occurs.[17] Although the materials can be separated by ion-exchange chromatography, a selective method was developed by Karimian, which is a modification of the Karrer procedure with the addition of a reactive cosolvent.[71] The procedure works well with (hydroxyethyl)thiamin, leading to selective pyrophosphorylation of the primary hydroxyl group. Recently, Stephen Bearne has shown that the procedure also can be used with nucleosides.[72] The procedure permitted us to synthesize (+)- and (−)-2-(1-hydroxyethyl)thiamin diphosphate by pyrophosphorylation of the resolved precursor.[66]

3. Enzymic Reactions of (+)- and (−)-2-(1-Hydroxyethyl)thiamin Diphosphate

Gerald Gish investigated the reaction of the separated enantiomers [(*R*)-(+)-(hydroxyethyl)thiamin diphosphate and (*S*)-(−)-(hydroxyethyl)thiamin diphosphate] with the apoenzyme of wheat germ pyruvate decarboxylase.[66] Activity of the enzyme with pyruvate as a substrate was determined after incubation with the enantiomers. The enzyme converts *both* enantiomers to enzyme-bound thiamin diphosphate and acetaldehyde, promoting the elimination by removal of the

hydroxyl proton. The K_m values are not identical but are similar, with that for the *R* enantiomer 1.5 times less than that for the *S* enantiomer. The activation of the enzyme involves binding of the derivative to the enzyme followed by conversion of the derivative to acetaldehyde and thiamin diphosphate. Since it is probable (but not certain) that the bound coenzyme derivative is converted to thiamin diphosphate faster than it dissociates, the K_m values depend on the rate of association of the apoenzyme with the coenzyme rather than on the rate of the conversion process.

H. Resolution of Lactylthiamin

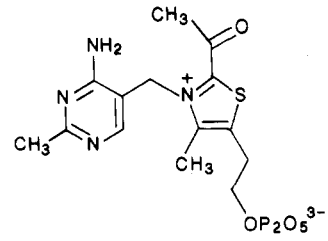
Ethyl lactylthiamin has recently been resolved as the dibenzoyl tartrate salt in acidic solution.[73] The specific rotation of the (+) enantiomer is 8.4°. The separated ethyl esters were converted by hydrolysis to lactylthiamin. (+)-ethyl lactylthiamin generates (−)-lactylthiamin. The absolute stereochemistry of these materials is unknown.

1. Decarboxylation of (+)-Lactylthiamin and (−)-Lactylthiamin

Since decarboxylation is formally an electrophilic substitution process (a proton replaces the carboxylate group), the stereochemistry of this process may reveal information about mechanism. For decarboxylases that do not use thiamin diphosphate as a cofactor, there has been no general pattern established. Reactions have been observed that occur with retention, inversion, and even racemization.[74] The stereochemical course of the decarboxylation of enzyme-bound lactylthiamin diphosphate has not been reported, but such a study might reveal the stereochemical relationship of groups within the active site. Since it is known in at least one case that enzymic decarboxylation produces a single enantiomer of (hydroxyethyl)thiamin diphosphate, it is of interest to know the significance of this stereospecificity. As a basis for comparison, the stereochemical outcome of the spontaneous decarboxylation of optically active 2-(2-lactyl)thiamin has been investigated under a variety of conditions.[73] We have found that the 2-(1-hydroxyethyl)thiamin that results from the decarboxylation is racemic (in water, alcohols, and aprotic solvents). If protonation were concerted with decarboxylation or if the carbanion were localized, then optically active product would be expected to result. This information confirms that the enamine resonance structure does contribute or that the carbanion rapidly racemizes.

I. 2-(1-Acetyl)thiamin Diphosphate

Pyruvate dehydrogenase catalyzes the conversion of pyruvate to acetyl coenzyme A.[1] The product is at a higher oxidation state than acetaldehyde, and the mechanism of the oxidation is unresolved. Two mechanistic pathways have been proposed for this conversion. Breslow and McNelis[75] as well as White and Ingraham[76] proposed that the 2-(1-hydroxyethyl)thiamin diphosphate carbanion may add to the disulfide linkage of lipoate, followed by acetyl transfer and reoxidation of dihydrolipoate to lipoate. Reed and his co-workers had suggested earlier that the carbanion is oxidized to acetylthiamin diphosphate followed by transfer of the acetyl group to dihydrolipoate and then to coenzyme A.[77]



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1. Models for Oxidation of 2-(1-Hydroxyethyl)thiamin Diphosphate

The oxidation of 2-(1-hydroxyethyl)thiamin diphosphate is expected to produce 2-acetylthiamin diphosphate. As a model for the reactions of the proposed oxidation product, Reed studied the reactions of 3,4-dimethyl-2-acetylthiazolium, showing that it will readily acetylate nucleophiles, including thiols (but not phosphate).[78,79] Bruice determined the rate constants for reaction of nucleophiles with acetylthiazolium and showed that the rates are competent for enzymic reactions.[80] Gruys and Frey prepared acetylthiamin diphosphate by chromic acid oxidation of (hydroxyethyl)thiamin diphosphate.[81] The material readily forms a stable hydrate, but it is too unstable to utilize as a substrate for enzymic reactions. It is intended to be used to identify the material isolated from an enzymic reaction (see next section).

2. Enzymic Reactions of Acetylthiamin Diphosphate

Frey and co-workers reported reactions of pyruvate dehydrogenase that are consistent with the involvement of 2-acetylthiamin diphosphate as a reaction intermediate. They have shown that pyruvate dehydrogenase will catalyze the hydrolysis of acetyl coenzyme A only in the presence of enzyme-bound thiamin diphosphate.[82] Transfer of the acetyl group from coenzyme A to dihydrolipoate should not catalyze hydrolysis, since both compounds are thiol esters and should be of similar stability. However, the thiamin diphosphate ylide should function as a nucleophilic catalyst: if the enzyme promotes transfer of the acetyl group from dihydrolipoate to the thiamin diphosphate ylide, acetylthiamin diphosphate is generated. Nonenzymic studies show that the hydrolysis of such a species is rapid and thus can account for the observed catalysis.[80,81] (Such hydrolysis is also seen in cases where 3-fluoropyruvate is a substrate and 2-acetylthiamin diphosphate is generated as an intermediate.[83]) Therefore, both model studies and enzymatic reaction patterns support this mechanism.

Flournoy and Frey have provided the most conclusive evidence in favor of the involvement of acetylthiamin diphosphate in the pyruvate dehydrogenase reaction.[84] They generated [2-^{14}C]acetylthiamin diphosphate from the reaction of [3-^{14}C]-3-fluoropyruvate with pyruvate dehydrogenase (they note that this material also inactivates the enzyme by another reaction). The [^{14}C]-acetyl group of [^{14}C]acetylthiamin diphosphate that is generated is transferred to dihydrolipoamide and then to coenzyme A. The material balance and kinetics are

consistent with the mechanism.

J. Nucleophilic Reactions of the Carbanion Derived from 2-(1-Hydroxyethyl)thiamin Diphosphate

The α-carbanion derived from 2-(1-hydroxyethyl)-thiamin is implicated in the reaction catalyzed by pyruvate decarboxylase. It is generated by the decarboxylation of lactylthiamin diphosphate and rapidly quenched by protons in situ. The possibility of this carbanion acting as a nucleophile toward other species has been considered as a mechanistic possibility in enzymic reactions.

1. Addition to Lipoamide

As discussed in section II.I., the oxidation of 2-(1-hydroxyethyl)thiamin diphosphate catalyzed by pyruvate dehydrogenase in reaction with lipoamide as an intermediate process is a widely accepted mechanism. However, there is no evidence directly implicating a reaction between the conjugate base of 2-(1-hydroxyethyl)thiamin diphosphate and lipoamide, in either an enzymic or nonenzymic reaction, although significant attempts have been made to do so.

Rastetter and co-workers prepared the chemical equivalent of the 2-(1-hydroxyethyl)thiamin carbanion by the reaction of a model compound with 1,5-diazabicyclo[5.4.0]undec-5-ene in tetrahydrofuran.[85] This material was reacted with cyclic and linear disulfides as models for lipoamide in the pyruvate dehydrogenase reaction. Lipoate itself was unreactive. The failure of lipoate to react under these conditions (tetrahydrofuran, DBU) was of course of considerable concern to these workers. However, they were not able to deduce the origin of this lack of reactivity. With a less direct model, they observed transfer from a disulfide to a model for the (hydroxyethyl)thiamin diphosphate anion. For the case in which the hydroxyl group of the model for (hydroxyethyl)thiamin is converted to a methyl ether, transfer to a disulfide also occurs. Since the ether is incapable of undergoing oxidation to the acetyl state, they concluded that the mechanism involving nucleophilic attack rather than oxidation to acetylthiamin is supported. Unfortunately, these cannot be considered to be conclusive results, since the models are not very accurate structural mimics of the enzyme active site.

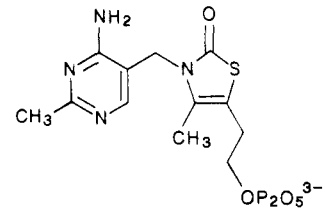
2. Addition to Pyruvate

The 2-(1-hydroxyethyl)thiamin carbanion does undergo other nucleophilic reactions. It is known to react with pyruvate to produce the thiamin diphosphate adduct of acetolactate.[1] The plant and bacterial enzyme acetolactate synthetase has been isolated and studied by Schloss and co-workers.[8] In this case, it appears that the enzyme goes to great effort to protect the carbanion or its equivalent from reaction with protons. There is a flavin requirement in that enzyme, although the mechanism does not involve oxidation. The flavin can protect the carbanion by an electron-transfer mechanism should there be a lack of pyruvate in the organism's environment. Interference with the flavin causes destruction of enzyme activity. The powerful herbicide sulfometuron-methyl appears to function by interposing itself between the flavin and thiamin diphosphate.

It is also known that pyruvate decarboxylase can produce acetoin.[86] This reaction can be rationalized as resulting from the addition of the same carbanion to acetaldehyde. Chen and Jordan showed that the acetoin produced in the enzymic reaction is chiral, so that it is likely that the reaction occurs on the enzyme and is not an artifact of the isolation procedure.[61] Thus, it has been established that the carbanion derived from 2-(1-hydroxyethyl)thiamin diphosphate does add to carbonyl groups in enzymic reactions as well as to protons.

K. Transition-State Analogues

Gutowski and Lienhard reasoned that the transition state for decarboxylation of enzyme-bound lactylthiamin diphosphate should resemble the resultant neutral enamine. On the basis of this model, they converted thiamin thiazolone to the diphosphate.[87] This material



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binds slowly and very tightly to pyruvate dehydrogenase, consistent with the assumption that the material is a transition-state analogue.[87,88] However, since the active site is hydrophobic, it is reasonable to expect that this uncharged species would bind more tightly for other reasons.[37]

Sable and co-workers found that thiamin thiazolone diphosphate does not bind particularly tightly to transketolase,[89] and similar results have been reported by other workers with other thiamin diphosphate dependent enzymes that promote the decarboxylation of pyruvate as an initial step in a catalytic sequence.[90,91] We have found that the binding of the thiazolone diphosphate to pyruvate decarboxylase is a complex process.[70] There is an initial relatively weak and reversible binding followed by a slow conversion to an irreversible state. The thiazolone is more lipophilic than is thiamin (as demonstrated by solvent partitioning[70]), and therefore, it is expected to bind to the less polar medium of the active site. It is also important to note that the analogue does not participate in any enzymic conversion other than binding, whereas thiamin diphosphate is involved in each stage of catalysis. It is clear that while the transition-state analogue hypothesis can be useful for the development of new directions in designing materials as enzyme inhibitors, the mode of action may not necessarily follow from the prediction.

L. Conformational Effects on Catalysis

The addition of the anionic center of the ylide of thiamin diphosphate to the carbonyl group of pyruvate should be subject to the known conformational requirements for addition of nucleophiles to carbonyl groups. These conformational requirements arise from a combination of steric and electronic factors. We have discussed this matter in a detailed review, which we summarize briefly here.[92] The nucleophile adds to the

carbonyl group through an anticlinal conformation with respect to the C=O axis in a plane perpendicular to that of the carbonyl group. This establishes an axis of maximal probability that still accommodates complete rotation of the plane of the thiazolium ring.[93]

Steric[94] and electrostatic effects between the reactants further define the angle of approach in addition to the binding requirements of the enzyme. In particular, the pyrimidinylmethyl substituent on N-3 of the thiazolium ring will provide steric direction, and the pyrophosphate group enables specific enzymic interaction. Elimination of acetaldehyde from the conjugate base of 2-(1-hydroxyethyl)thiamin is analogous to the reverse of the addition of the thiamin ylide to pyruvate and will be subject to conformational control as well.

The X-ray crystal structure of the ethyl ester of phosphalactylthiamin, an analogue of lactylthiamin, reveals that the substituent at the C-2 α-carbon is in the plane of the thiazolium ring.[95] This arrangement minimizes steric repulsions between the substituents and the adjacent methyl-linked pyrimidine ring. If the single interaction between the substituent and pyrimidine is large then the lowest energy conformation will have the substituent perpendicular to the plane of the ring, otherwise an arrangement where the substituents straddle the ring plane will be preferred. Each of these conformations will have a related conformer in which the thiazolium ring is rotated by 180°. For situations in which neither of the two limiting cases applies, a combination of effects should apply. The angle of the largest substituent relative to the plane of thiazolium should thus be between 90 and 60°. In the case of ethyl phosphalactylthiamin, the ethyl phosphonate group is large compared with the hydroxyl and methyl groups at the C-2 α-position and, thus, the large group is perpendicular to the plane of the thiazolium ring. In the case of lactylthiamin, the carboxylate should occupy a position similar to that of the phosphate in the analogue. To minimize heavy-atom motion, the conformation should be that of the transition state that leads to the formation of this intermediate.

1. Stereoelectronic Factors in Decarboxylation

Conversion of the carboxylate of lactylthiamin to carbon dioxide results in transfer of a pair of electrons to the α-carbon. Stabilization of the carbanion can be achieved by overlap of the orbital containing the pair of electrons with the π system of the thiazolium ring.[95] This orbital must be in a plane perpendicular to the plane of the thiazolium ring, which can be achieved in either of two conformers. These conformers are the same as two of the conformers that are associated with the formation of lactylthiamin. Therefore, the conformations for the two sequential steps are correlated with minimal motion. A substantial benefit in terms of enzymic efficiency is immediately obvious: the sequential steps will not require conformational changes in the coenzyme or its adducts. This will lead to lower energy requirements for the pathway.

III. Concluding Remarks

In this review I have emphasized the significance of being able to use accurate mechanistic data to analyze catalytic reactions involving thiamin diphosphate. Albery and Knowles have emphasized the importance of having an accurate understanding of the steps in enzymic and closely related nonenzymic systems in order to have a realistic basis for understanding the function of a protein catalyst.[96] Covalent intermediates derived from a coenzyme permit one to make accurate comparisons with the corresponding enzymic reactions. The availability of intermediates and analogues derived from thiamin and thiamin diphosphate has permitted the analysis of enzyme mechanisms with increased depth.

The three-dimensional structure of a thiamin diphosphate dependent enzyme has yet to be determined, so that the information obtained from studies of the intermediates provides an important background that will eventually be integrated with that from analyses of enzymic structures. The mechanisms by which catalysis is achieved in enzymic systems that use thiamin diphosphate as a coenzyme have been sketched in broad terms, but many specific mechanistic questions are still unsettled. The ability to convert further this qualitative understanding to a quantitative one through the availability of the key intermediates and the study of their reactions promises exciting and specific prospects for future research.

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Registry No. Thiamin diphosphate, 154-87-0.

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