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Thiamin Diphosphate Catalysis: Enzymic and Nonenzymic Covalent Intermediates

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Contents

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1. Introduction

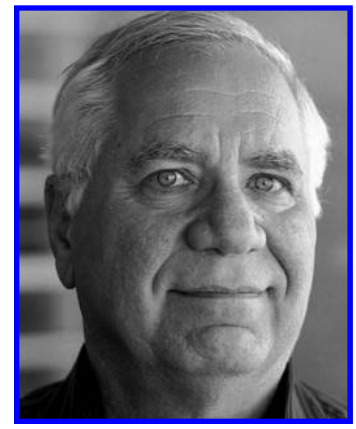
1.1. Acyl Carbanions and Thiamin Diphosphate

The idea of "reasonableness" of organic reactions is based on recorded evidence of reaction patterns. The logical patterns are organized based on structural and functional group relationships. Thermal decarboxylation ordinarily requires that the departing carboxyl group be in conjugation with an unsaturated functional group, permitting transition state stabilization of the incipient delocalized carbanion which becomes protonated (Scheme 1).[1–4] (In practical terms of synthesis, decarboxylation in the absence of conjugation requires a procedure involving silver salts, halogens, and free radical mechanisms.[5]) Biochemical pathways are understood as direct analogies to organic chemical reactions, with enzymes serving as effective catalysts in reducing the energies of transition states.[6–8] Decarboxylation of pyruvate is a common primary metabolic process whose outcome amounts to electrophilic substitution at an acyl carbon. The early discovery of this reaction was the source of extensive chemical speculation since it is outside the established pattern of spontaneous decarboxylation processes.[9] If such a reaction were to occur by a simple exchange of electrophiles, the intermediate would be an acyl carbanion, a species that is too high in energy to exist as an intermediate (Scheme 2). While enzymes may stabilize high energy species they also utilize indirect pathways that involve stabilized conjugates and associated transition states. In the case of the decarboxylation of pyruvate, addition of enzyme-bound thiamin diphosphate (TDP, Scheme 3) to the carbonyl group of the substrate provides an intermediate whose decarboxylation produces a stabilized intermediate (Scheme 4).[10–13]

1.1.1. TDP Is a True Catalyst

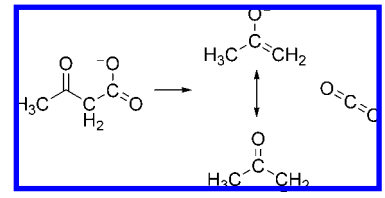
While many coenzymes are coreactants (NADH, folate), TDP is truly catalytic, remaining within the protein site.[9] It is clear that one important function of TDP within the enzyme is stabilizing transition states associated with reactions involving the equivalent of the conjugate base of C-1 of an aldehyde. This carbanion equivalent can be protonated (as in pyruvate decarboxylase), oxidized (pyruvate oxidase, pyruvate: ferredoxin oxidoreductase), or combined with other bound molecules (pyruvate dehydrogenase, transketolase). At the end of the transformation of the substrate, the bond to TDP is cleaved and the product is released. The best known enzymic reaction involving TDP generates the equivalent of the acyl carbanion of acetaldehyde in pyruvate decarboxylase from the addition product of TDP and pyruvate (Scheme 5).

In enzymes, the acyl carbanion intermediate is most often generated by the spontaneous loss of carbon dioxide from the conjugate of an α-ketoacid and TDP, with a proton, electrophile, or oxidant accepting the electrons left by the departure of carbon dioxide to complete the reaction. Recognizing that acyl carbanions are inherently useful for synthetic transformation, Stetter showed that deprotonating thiazolium conjugates of aldehydes creates acyl carbanion equivalents that undergo 1,4-addition of the carbanion to an α,β-unsaturated carbonyl compound (Michael acceptor).[14,15] This gives an efficient and uniquely effective route to valuable 1,4-dicarbonyl compounds, including the key precursor to the pyrrole of atorvastatin (Lipitor).[16]

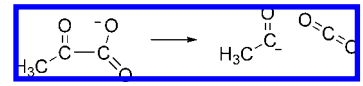


Ronald Kluger is a professor of chemistry at University of Toronto in Toronto, Canada, where he has worked since 1974. His research coordinates studies of organic reaction mechanisms with their biochemical applications. His interests in organic chemical research began when he was an undergraduate with Gilbert Stork at Columbia University (AB, 1965) and continued with graduate studies in bioorganic chemistry at Harvard University (PhD, 1969) with Frank Westheimer. His project delineated the sources of reactivity and product variation in the hydrolysis of cyclic phosphate esters. He learned enzymology as a postdoctoral fellow with Robert Abeles at Brandeis University and began his independent research career at the University of Chicago in 1970. His first research proposals suggested that synthetic intermediates derived from thiamin and TDP would provide important insights into the basis of enzyme catalysis and inhibitor design. He continued in Toronto with this as well as projects in biotin activation, phosphate reactivity, acyl phosphate-based reagents, and site-directed protein modification (with an emphasis on hemoglobin). He has been honored for his work with national and international awards, including the CIC Medal (2006), which is the highest research distinction offered by Canada's national chemical society. He is a Fellow of the Royal Society of Canada, the Chemical Institute of Canada, and the American Association for the Advancement of Science. In 2005, following in the footsteps of Richard Schowen, he was a visiting professor at Martin Luther University in Halle, Germany, where he and Kai Tittmann shared their interests in the subject of the present review.

Scheme 1

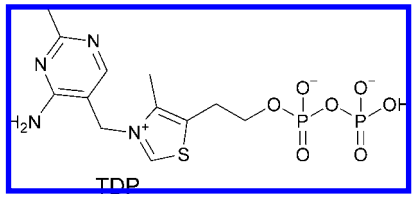


Scheme 2



Kai Tittmann studied biochemistry at the Martin-Luther University Halle, Germany, where he obtained his graduate degree ("Diploma") in 1996 and Ph.D. degree (2000). His doctoral thesis focused on the mechanistic analysis of thiamin diphosphate- and flavin-dependent enzymes under the co-supervision of Professor Gerhard Hübner at Halle and Professor Sandro Ghisla at Konstanz University, Germany. Since 2003, he has been an independent group leader ("Junior professor", the German equivalent of an Assistant Professor) at the University of Halle, where he enjoyed a vivid (and still ongoing) collaboration with visiting Professors Richard Schowen from Kansas and Ronald Kluger from Toronto. In 2003, he was a visiting scientist in the laboratories of Professor Frank Jordan at Rutgers University, New Jersey. He was also a Visiting Associate Professor at the Ben- Gurion-University of the Negev, Beer-Sheva, Israel, with Professor David Chipman. His primary research interests include the mechanistic and structural analysis of thiamin enzymes and electron transfer reactions in proteins by means of kinetics, redox potentiometry, NMR spectroscopy and X-ray crystallography. He has been honored for his work with the "Dorothea-Erxleben Award" (best doctoral thesis of the Martin-Luther University) and with the "Award for excellent basic research" by the Ministry of Education at Saxony-Anhalt.

Scheme 3



1.2. The Quest for TDP Catalysis Mechanisms

While the structure and overall mechanism of TDP-promoted processes are now well-established, both were the results of remarkable insights that were far ahead of their time.

1.2.1. The Chemical Nature of TDP Catalysis

As noted above, the decarboxylation of pyruvate is common in metabolism but is not known at all as a spontaneous reaction. The idea that an enzyme, pyruvate decarboxylase, could promote such a reaction presented an early challenge to the idea that biochemical reactions can be understood as extensions of conventional chemical phenomena. In the 1930s the structure of thiamin and TDP were being discovered, and proposals for mechanisms were based on analogies from known reactions. Since no known reactions corresponded to the later-discovered patterns involving TDP, proposals were necessarily based on purely speculative analogies.[9]

1.2.2. Ideas Based on Reaction Patterns

In 1933, based on observations that primary amines catalyzed decarboxylation of α-ketoacids, Langenbeck predicted that the reactive site in the cofactor of pyruvate decarboxylase would be a primary amine. This would function by forming a Schiff base derivative of the substrate, undergoing tautomerization prior to decarboxylation (Scheme 6).[17] The mechanism is similar to tautomerization in the intermediates derived from substrates in pyridoxal phosphate-dependent enzymes.[18]

1.2.3. Thiamin's Structure and More Ideas about TDP

The structure of thiamin (vitamin B1) was deduced by Williams based on the products from the destruction of thiamin by sulfite (Scheme 7).

The results were reported in 1935 and 1936.[19,20] Williams and Waterman then used established heterocyclic chemistry to synthesize thiamin, proving the structure and also giving a practical, commercially viable route.[21] Although Williams and Waterman were employed at Bell Laboratories, their thiamin research was done privately at Columbia Teachers College. They donated the income from the patents to fund the Research Corporation, with the proceeds to be used for eradicating diseases caused by nutritional deficiencies. The income continued to be used to support research in chemistry until 1978. Waterman went on to found the New England Nuclear Corporation, and the proceeds from its sale in 1981 continue to support the Research Corporation.[22]

In 1937, Lohmann and Schuster established that the coenzyme "cocarboxylase" in pyruvate decarboxylase is TDP.[23,24] Melnick noted that elucidation of the structure of TDP showed that it contained an amino group. Since this was predicted to be the active functional group in TDP by Langenbeck, it was taken as proof that the cofactor functions through a Schiff base tautomerization mechanism (Scheme 6) in a conjugate with the substrate.[25,26] However, Melnick found that the amino function in thiamin is chemically unreactive and unlikely to be the reaction site.[25,26] This left the puzzle as to how TDP performed its catalytic function unsolved. Melnick correctly proposed that the diphosphate group would serve to bind the coenzyme to the protein. However, he incorrectly surmised that TDP would function through oxidation and reduction (as with FAD and NAD^+).

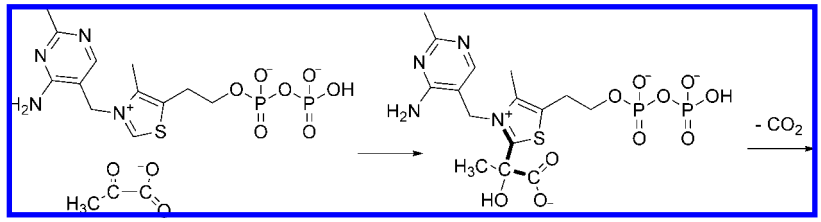
In the 1940s and early 1950s, a variety of reaction mechanisms for the role of TDP in decarboxylation were proposed that were based on increasingly complex chemical analogies that required conversions of TDP to species in which the thiazolium ring is converted to an acylic species through an addition reaction.[27]

1.2.4. Thiamin as a Catalyst

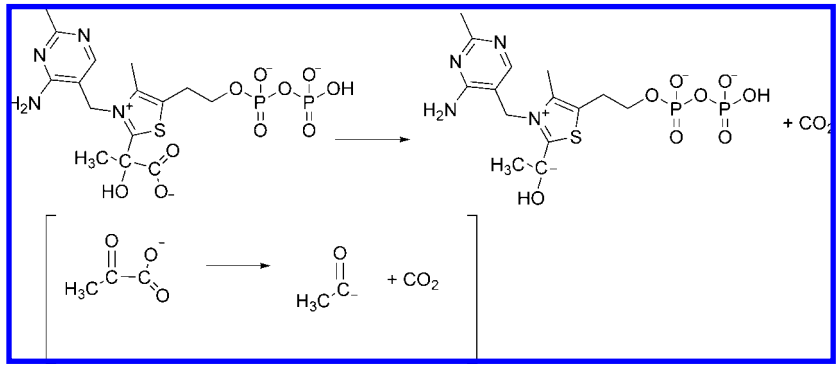
A key breakthrough came from observations of Ugai[28] and Mizuhara[29] that thiamin and other thiazolium compounds promoted α-condensation of an aldehyde to produce an α-hydroxy-ketone, which is reminiscent of formation of acetoin in the enzymic decarboxylation of pyruvate. Such a reaction formally involves formation of the acetyl C1-carbanion, as in the decarboxylation of pyruvate (Scheme 8).

This focused attention on the thiazolium group of TDP. It also established the validity of studying reactions of thiamin without a protein, assuming that the protein provides

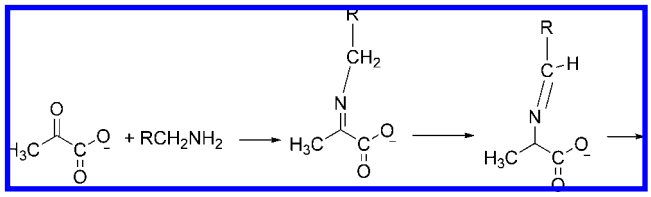
Scheme 4



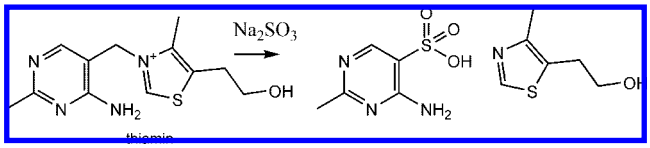
Scheme 5



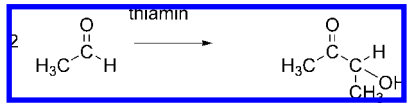
Scheme 6



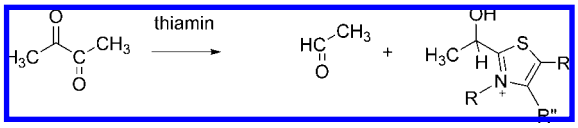
Scheme 7



Scheme 8



Scheme 9



specificity and improved catalysis but not the inherent alteration of reactivity.

The result was followed up in 1954 by Mizuhara and Handler who reported that the cleavage of the α-dicarbonyl compound biacetyl is promoted by thiamin and that 2-(1-hydroxyethyl)-thiamin is formed (Scheme 9).[30]

Surprisingly, they suggested that this supports a mechanism for thiamin catalysis in which a pseudobase is the first intermediate, from addition of hydroxide to C2 of the thiazolium ring. They proposed that this would change the thiazolium moiety to a nonaromatic heterocycle with an amino group that serves as a nucleophile to add to the carbonyl group of the substrate, a variant of the general mechanism proposed by Langenbeck.[17] They also dismiss a mechanism involving ring-opening from the pseudobase derived from thiamin that was proposed by Karrer[31] that also is a variant of the Langenbeck proposal. Soon after those proposals, Wiesner and Valenta once again proposed the Langenbeck mechanism in terms of the amino group of the pyrimidine[17] based on a wider range of model reactions[32] despite Melnick's earlier dismissal of that approach.[25,26] At this point, every mechanism that had been proposed, many of which were supported by critical experiments, did not lead to a reasonable conclusion.

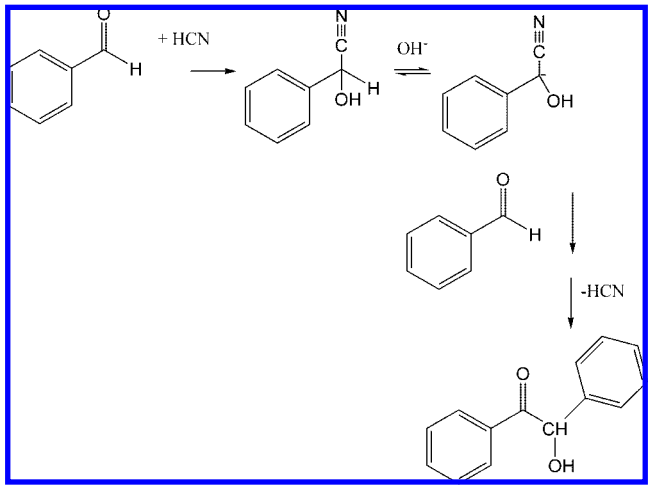
1.2.5. C−H Ionization As a Source of Catalysis

The mechanisms proposed up to 1956 involved amines derived directly or indirectly from TDP functioning as catalytic nucleophiles involving Schiff base tautomers, following established analogies. It turned out that the amine-carbonyl reaction was not the correct analogy. The matter of discovering which amine was responsible for catalysis would lead nowhere. The more appropriate analogy had been completely missed despite the fact it was widely known in organic chemistry since 1840. The cyanide-catalyzed formation of benzoin from benzaldehyde in basic solution had been studied by Lapworth in the early 1900s. He demonstrated that a kinetically competent cyanohydrin is formed by addition of the carbon of cyanide to the carbonyl of benzaldehyde, stabilizing the equivalent of the conjugate base of the aldehyde group (Scheme 10).[33–35]

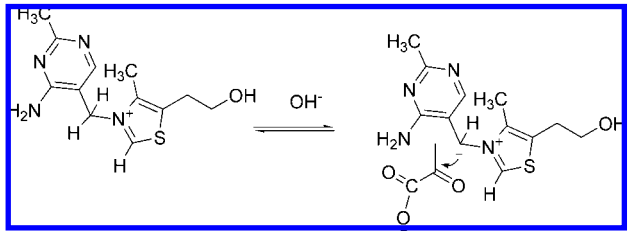
The typical TDP enzyme reaction involves decarboxylation of a 2-ketoacid, leaving the equivalent of the aldhyde-derived carbanion to be stabilized, exactly as in the benzoin condensation.

1.2.5.1. Ionization of the Methylene Bridge. In a departure from previous proposals, Breslow noted that an analogue of cyanide could be produced by ionization of TDP from a carbon adjacent the thiazolium nitrogen. The Lapworth addition mechanism could then be adapted to the particular reactants. On the basis of this model, Breslow proposed that the site of ionization from the nucleophilic carbanion could be the methylene group that joins the heterocyclic constituents of thiamin. This nucleophilic carbanion would

Scheme 10



Scheme 11



be stabilized by the adjacent thiazolium nitrogen, making the species an ylide or carbene, an analogue of cyanide. In the next step, addition of the carbanion to the carbonyl of the substrate will accelerate decarboxylation since the resulting carbanion is stabilized by the adjacent positively charged nitrogen (Scheme 11).[36]

Breslow's proposed ionization site was ruled out when Westheimer showed that neither thiamin (in nonenzymic reactions) nor TDP (in enzymic reactions) incorporate deuterium from solvent deuterium oxide at the methylene bridge position,[37] a condition required by the formation of the carbanion. Westheimer's analysis involved cleaving thiamin with bisulfite by Williams' procedure[38,39] and mass analysis of the resulting methanesulfonic acid derivative of the pyrimidine of thiamin (Scheme 12).

The procedure did not include an examination of the deuterium content of the thiazole product, which would have revealed the actual site of reaction. Many years later, in responding to a question of whether he had ever made a scientific error, Westheimer said that he regretted focusing on disproving Breslow's proposal instead of searching for the correct answer.[40] (In the work that disproved the Breslow proposal, the sample containing the part of thiamin that does undergo H−D exchange was not analyzed.[37])

1.2.5.2. Ionization of the Thiazolium C−H. Thus, with ionization on the methylene adjacent to the thiazolium nitrogen ruled out, Breslow looked at exchange at the adjacent C−H in the thiazolium ring. Using an early ^{1}H NMR spectrometer, he was able to observe the exchange of the C2 protons of thiazolium compounds directly (implicating the site shown in Scheme 12). He found that they exchange with solvent deuterium and that this occurs with a half-life of about 20 min in neutral solution, a remarkably fast exchange process for a carbon acid.[41,42]

Thus, Breslow showed that formation of the C2-carbanion is likely to be the first step involving TDP, since the material would be an effective nucleophilic analogue of cyanide. Addition of the anion from TDP to the α-carbonyl group of pyruvate in decarboxylases would give an intermediate with appropriate electronic and resonance properties. This overall mechanism has since been confirmed by many other experiments: the C2-derived carbanion from TDP adds to the α-carbonyl group of an α-ketoacid substrate, creating an analogue of a cyanohydrin as in the benzoin condensation.[43] This changes the electronic properties of the substrate, making subsequent anion formation by decarboxylation much lower in energy for the overall electrophilic substitution processes. As well, the addition and release of TDP to and from the carbonyl compound occur readily, permitting a defined intermediate to be held in a precise location within the protein without introducing significant thermodynamic barriers to its formation or decomposition.

Breslow's revelation came from the simplest, yet most appropriate of models for its purpose. It was universally accepted with enthusiasm. The proposal was confirmed repeatedly in more detailed models and in examination of many enzymes that utilize TDP as a cofactor.[43]

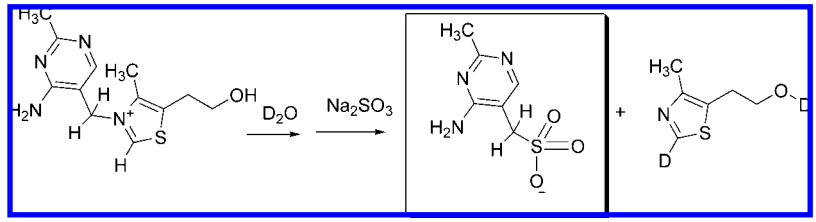
The discovery of probable, structurally defined intermediates derived from the substrate and TDP provides a unique opening into the workings of proteins as catalysts; the reaction of the very same intermediates that occur on the TDP catalytic pathways can be compared to precise models that could undergo reaction without the protein. This would put the catalytic role of a protein into quantitative perspective. An excellent and extensive analysis of this aspect has been provided by Schowen.[12]

1.2.5.3. The C−H Acidity of Thiamin. Breslow had shown that the C2 proton of a thiazolium compound is exchanged for deuterium in deuterium oxide with a half-life of about 20 min at 28 °C.[41] The exchange rate is about the same as that of acetone, whose p$K_a \sim 20$. At equilibrium, the ylide is undetectable in the presence of much greater amounts of both thiamin and the ring-opened material that forms from initial addition of hydroxide to C2 of thiamin.[27] The rate of exchange does not measure the pK_a of thiamin: the rate constant for protonation of the carbanion would have to be available for calculating the overall equilibrium. If the pK_a is available, the concentration of conjugate base at any pH can be determined from the Henderson–Hasselbalch expression.

Kemp and O'Brien examined the rate of base catalyzed detritiation of thiazolium compounds that contain a tritium tracer in place of H at C2. They observed that the exchange reaction is general base catalyzed with a Brønsted coefficient of greater than 0.9 for the process, indicative of a localized conjugate base and very fast, diffusion-controlled protonation with rate constants of about 10^{10} s^{-1}.[44,45] They observe equilibrium isotope effects (H/T) of 2.7 and 4.8 and calculate that the pK_A of thiamin at C2 is between 17 and 19.

On the basis of this estimate and the Henderson–Hasselbalch equation, the fraction of TDP in the C2 ionized state (ylide) of thiamin diphosphate at pH 6 will be one part in 10^{12} of the TDP present. If we consider that k_{cat} for pyruvate decarboxylase is about 50 s^{-1} (pH 6, 30 °C), then if pyruvate is held in place perfectly by the enzyme, the effective molarity[46] of TDP could be as high as 10^8 M with pyruvate and TDP present at the concentration of the enzyme.[47]The small amount of ionization of TDP to the ylide form suggests that the maximum possible rate is about 10^4 less than the observed overall rate.[44] Kemp and O'Brien note

Scheme 12



that the equilibrium concentration of the ylide (the conjugate base at C2), rather than the rate of proton removal to form the ylide, is the critical issue in the addition of the ylide to the carbonyl group of pyruvate. Since the presence of Brønsted bases will not affect an equilibrium controlled by the solution pH and the pK_A of acid, they propose that the enzyme must alter the environment in the protein to change the thermodynamics to favor ionization.[44] They also note that the nonenzymic rate for addition of an adjacent ylide to a carbonyl requires no acceleration to be enzymatically competent.

While Kemp and O'Brien had presented excellent evidence from models that the pK_a of thiamin at C2-H would be around 18, contradictory conclusions from direct measurements appeared several years later. Hopman and Brugnoni stated that the pK_a at C2-H is 12, five units lower than the value estimated by Kemp and O'Brien.[48] Therefore, the concentration of free ylide would be sufficient at its normal equilibrium concentration.[48] These workers used a pH-jump method (perturbing the equilibrium by addition of base and observing the rate of return to equilibrium from which the forward and reverse rate constants can be calculated) and rapid spectral scanning.[49] This gave that pK_a = 12 for ionization of C2-H of the thiazolium ring of thiamin. Since the pK_a of HCN = 10, this value might not seem unreasonable. However, the relatively slow exchange (reported by Breslow[42]) is consistent with a much less acidic site. Hopman and Brugnoni also reported that the pK_a for dissociation of a proton from the conjugate acid of the 4′ NH_2 group of the pyrimidine of thiamin is 4.75.[48] (In 1977, Roberts and coworkers established by ^{15}N NMR that the actual site of protonation on the pyrimidine is the 1′ nitrogen within the ring,[50] and the 4′-NH_2 is a much weaker base.)

In 1988, Washabaugh and Jencks reported another study of the C2-H acidity of thiamin and obtained a value close to what had been implicated in Kemp's report, disputing the lower value.[44,51] They determined the rate of exchange of C−H for C−D by NMR under different catalytic conditions. They calculated the pK_a data with a range of bases to give Brønsted plots, similar to the method used by Kemp. Their results led them to assign the pK_a = 17.7, with the observation that there is no participation or assistance from the pyrimidine in the nonenzymic reaction. This makes thiamin a much weaker acid than HCN. The exchange rate and pK_a once again led to the conclusion that the rate constant for protonation of the conjugate base is very fast, near the diffusion-controlled limit. This is unlike other carbon acids where the anionic charge is delocalized.[52]

The problem of the rate of formation of the ylide from TDP on an enzyme was addressed successfully by Kern and co-workers who observed the exchange rates at the thiazolium C2-H in pyruvate decarboxylase and transketolase.[53] On the basis of the H/D exchange kinetics and the known X-ray structures of several TDP enzymes, the authors suggested a strictly conserved Glu residue to facilitate ylide formation (Scheme 13). The X-ray structures suggest that the side chain of the conserved Glu is uncharged in the resting state with its carboxyl hydrogen engaged in a hydrogen bond to N1′ of the pyrimidine. Eventual proton transfer from Glu to N1′ aids in the amino−imino tautomerization of the aminopyrimidine. Internal transfer of a proton from C2-H to the imino nitrogen produces the ylide.

We can see that since this is a tautomeric equilibrium combined with the relay of a proton through the pyrimdine, the concentration of the ylide could be much higher than is the case where it is transferred to solvent–water. The Glu side chain that hydrogen bonds to N1′ of the aminopyrimidine has been associated with C2 deprotonation in TDP enzymes and is seen widely in crystal structures (Glu51 in PDC,[54] Glu418 in TK[55]). Replacement of the conserved Glu residue by other amino acids impairs ylide formation resulting in the lack of enzymatic activity. However, a recent study revealed that there is no such Glu residue in the TDP enzyme glyoxylate carboligase (GCL),[56] and therefore the role of the Glu side chain is not obligatory for TDP catalysis. Additional mutagenesis on this enzyme surprisingly showed that introduction of an acidic side chain close to N1′ abolishes catalysis. It is reasonable to assume that interaction with Glu can stabilize the imino tautomer of TDP through hydrogen-bonding, permitting the amino nitrogen to function as a proton acceptor without requiring any specific function in catalysis of GCL.[56]

2. Covalent Intermediates in TDP Catalysis

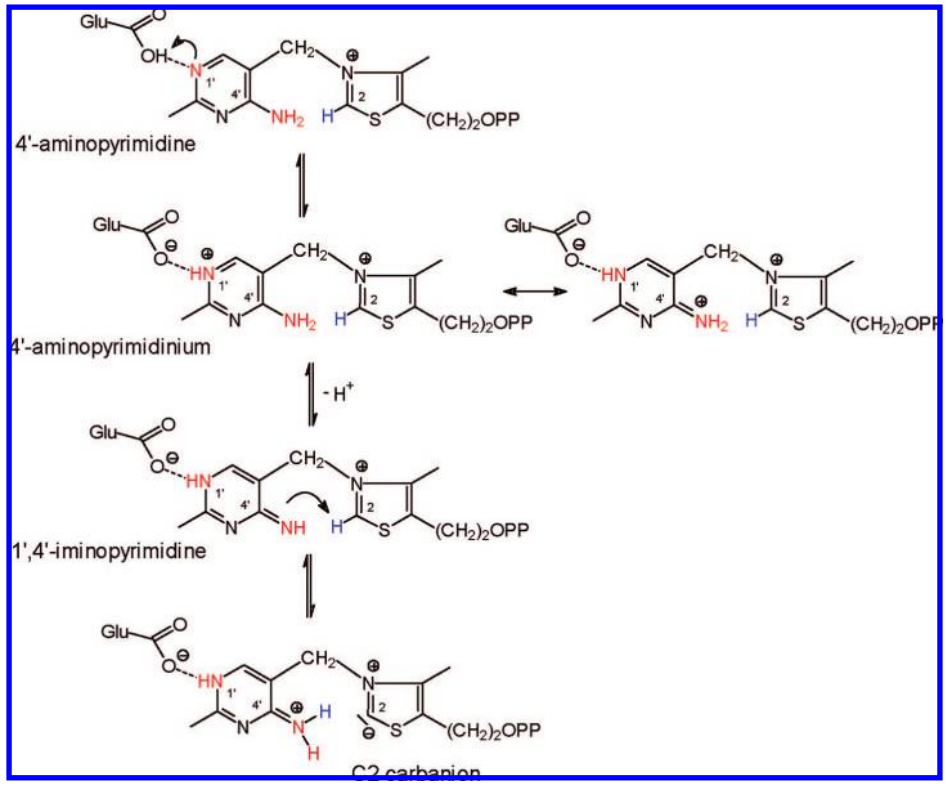
TDP always functions in enzymes by losing the C2 proton, with the resulting carbanion adding to a carbonyl group of a substrate. The addition holds the substrate in a precise location and alters its electronic properties. With this common background a very diverse set of unusual catalytic processes can be achieved.

2.1. Intermediates Derived from the Addition of TDP or Thiamin to 2-Ketoacids

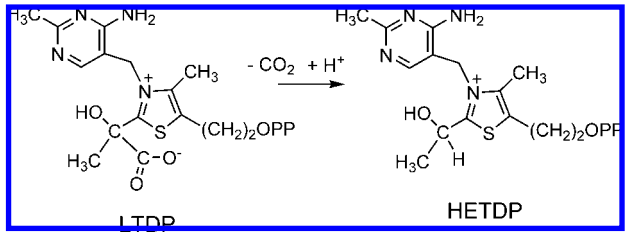
Pyruvate decarboxylase from wheat germ binds TDP more weakly than other TDP enzymes, permitting the coenzyme to be released at an observable rate. According to the proposed mechanism, the C2-carbanion derived from enzyme-bound TDP would add to the α-carbonyl of pyruvate to form a covalent conjugate, α-lactyl-TDP (LTDP). Loss of carbon dioxide and protonation of the resulting carbanion leads to formation of the TDP conjugate of acetaldehyde, 2-(1-hydroxyethyl)-TDP (HETDP) (Scheme 14). It should be noted that LTDP and HETDP are chiral, while the reactants and products are not.

The enzyme would then promote the release of acetaldehyde from HETDP, regenerating enzyme-bound TDP. Krampitz prepared exogenous HETDP with a ^{14}C label in the acetaldehyde-derived portion (by reacting ^{14}C-acetalde-

Scheme 13



Scheme 14



hyde with TDP) and showed that the apoenzyme (lacking TDP) catalyzed the release of ^{14}C-acetaldehyde to produce the catalytically active holoenzyme.[57] Using high concentrations of the apoenzyme along with labeled HETDP, Krampitz observed that acetaldehyde was released in a rapid burst, followed by a slower, continuing release of acetaldehyde that occurs as the TDP generated by the release of acetaldehyde dissociates from the enzyme. The molar amount of acetaldehyde in the initial release from HETDP on the enzyme must equal the molar concentration of TDP binding sites on the enzyme. However, since the reported nonenzymic preparation of HETDP generates a racemic mixture, it is likely that only one enantiomer is reactive providing an early piece of evidence for chiral intermediates in thiamin enzymes.

2.2. Common Reaction Patterns

Although TDP-dependent enzymes catalyze a wide variety of reactions, there is a common pattern of analogous pathways that proceed via similar TDP-derived covalent intermediates.[10,58–60] Important general lessons can be deduced by focusing on reactions of pyruvate (and other 2-keto acids) and of phosphate derivatives of monosaccharides.

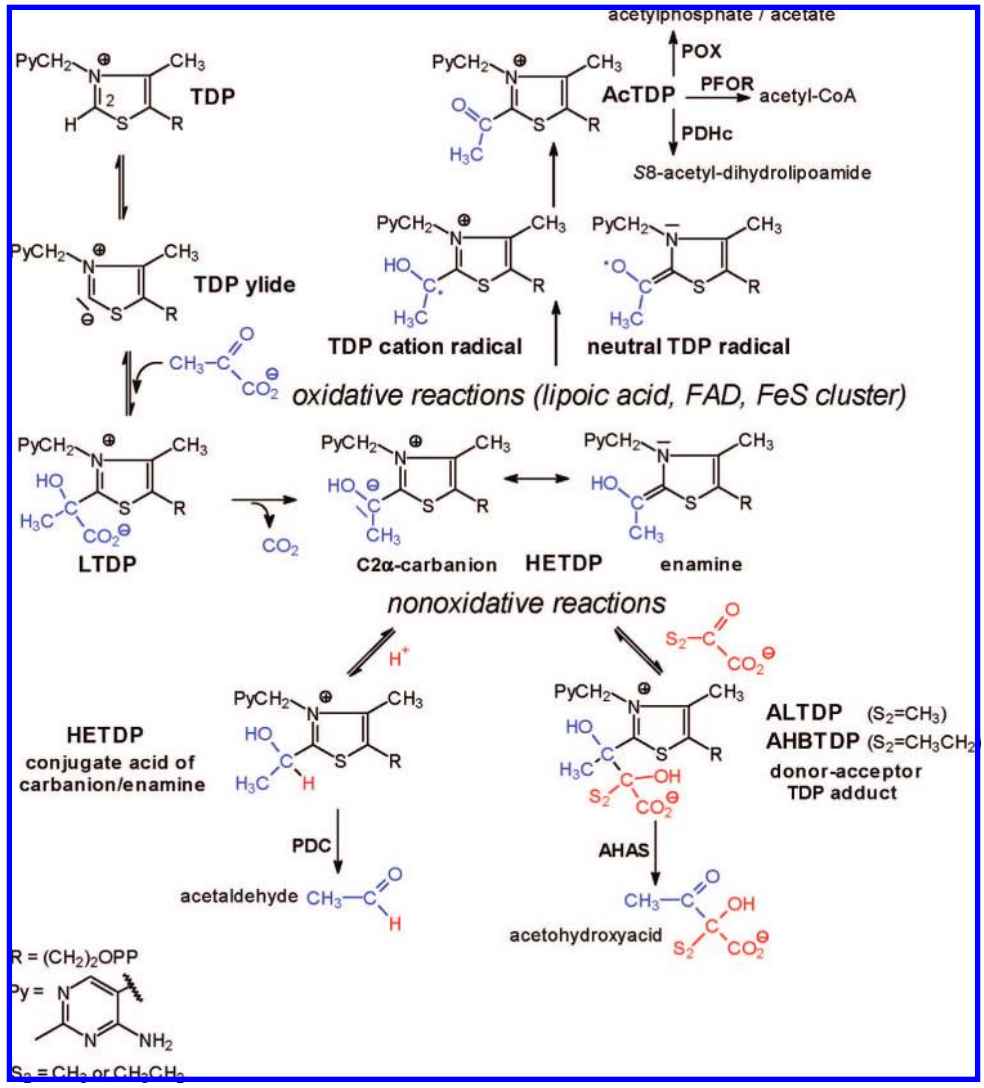
2.2.1. Covalent Intermediates in the Enzyme-Catalyzed Conversion of Pyruvate

A generalized reaction sequence for enzymes utilizing pyruvate is depicted in Scheme 15. The sequence remains consistent with the proposals made by Breslow, beginning over 50 years ago.[41,61] After formation of the TDP ylide, the substrate carbonyl is attacked by the thiazolium C2 carbanion to form the tetrahedral substrate-TDP adduct 2-(2-lactyl)-TDP, LTDP. Loss of carbon dioxide gives the carbanion/enamine form of 2-(1-hydroxyethyl)-TDP, HETDP. This is a central intermediate and branching point of catalysis. The carbanion/enamine is subject to subsequent nonoxidative or oxidative conversion. For example, it may be oxidized by neighboring redox-active cofactors such as flavins (pyruvate oxidase, POX), Fe_4S_4 clusters (pyruvate:ferredoxin oxidoreductase, PFOR), or lipoamide (pyruvate dehydrogenase multienzyme complex, PDHc). Depending on the protein and oxidizing cofactor, there may be transient formation of a radical TDP intermediate, or simultaneous transfer of two electrons (see section 3.3). The resulting 2-acetyl-TDP intermediate can react to produce acetate, acetylphosphate, acetyl-CoA, or 8-*S*-acetyl-dihydrolipoamide.

The intermediate can also react without oxidation by being protonated at C2α to give HETDP with subsequent elimination of acetaldehyde (pyruvate decarboxylases, PDC). Addition of the carbanion/enamine to the carbonyl of another keto acid (pyruvate, 2-ketobutyrate) leads to the formation of a conjugate resulting from both substrates and TDP (acetohydroxyacid synthase, AHAS).

At least six covalent TDP-derived intermediates result from the initial formation of LTDP and its conversion to the conjugate base of HETDP. In recent years, application of a variety of physical methods has made possible the direct observation of many of the proposed covalent intermediates,

Scheme 15. Reaction Sequence and Involved Intermediates of Pyruvate-Processing TDP Enzymes



enabling analysis of not only their structure within the enzyme but also their reactivity. The reaction sequence and intermediate pattern of enzymes acting on other aliphatic, branched-chain, or aromatic keto acids correspond in principle to these given for pyruvate.

2.2.2. Intermediates from Phosphate Esters of Monosaccharides

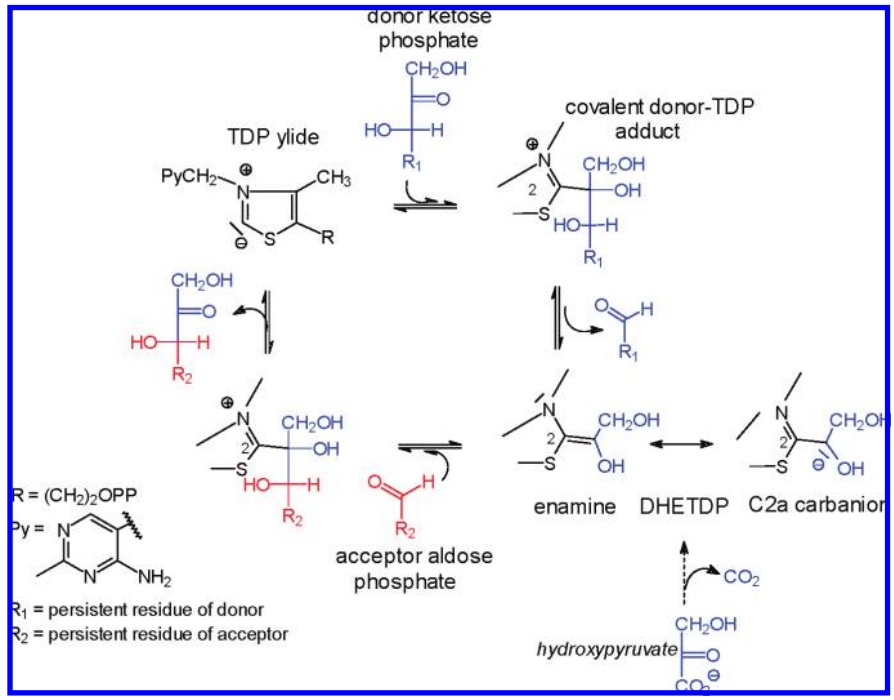
In cellular carbohydrate metabolism, the TDP enzyme transketolase (TK)[62,63] catalyzes the transfer of a 2-carbon dihydroxyethyl fragment from a ketose phosphate to the C1 position of an aldose phosphate (Scheme 16). The reaction pattern is very similar to the nonoxidative carboligation of two pyruvate molecules catalyzed by AHAS as outlined above. The C2 carbanion of enzyme-bound TDP first adds to the C2 keto function of a donor ketose (D-xylulose 5-phosphate, X5P; D-fructose 6-phosphate, F6P) yielding a covalent cofactor−substrate adduct. In the next step, which is reversible, the C2−C3 bond of the sugar–phosphate is cleaved to give the 1,2-dihydroxyethyl-ThDP carbanion/enamine intermediate and a 3-carbon (donor X5P) or 4-carbon (donor F6P) aldose phosphate. After binding another acceptor, such as ribose 5-phosphate (R5P) to the active site, the carbanion/enamine covalently adds to the C1 *aldo* function of this acceptor, followed by the elimination of the resultant ketose. Apart from the physiological donor ketoses X5P and F6P, the 2-keto acid β-hydroxypyruvate is commonly employed as an artificial donor substrate for kinetic and structural studies since the quasi-irreversible nonoxidative decarboxylation of this compound generates the DHETDP carbanion/enamine intermediate on the enzyme with sufficient lifetime for a biophysical characterization.

Thus, the reaction sequence of TK includes several TDP-derived intermediates. Significantly, the central carbanion/enamine and the initial tetrahedral carbohydrate-TDP conjugates have recently been characterized by structural analysis and spectroscopy.

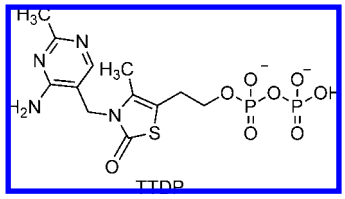
2.3. Inhibitors Related to Intermediates

The specificity and mechanism of an enzyme can be used as the basis for creating highly effective inhibitors. A dramatic idea in this area is the concept of "suicide inhibitors," where an enzyme converts an unreactive analogue of the substrate to a reactive entity that inactivates the enzyme.[64–66] With TDP enzymes, a compound that can react with TDP to form an intermediate will serve such a purpose if this generates a more reactive species that can combine with a protein side chain.

Scheme 16. Reaction Sequence and Involved Intermediates of Transketolase



Scheme 17



Alternatively, if an intermediate can form but not react further, the enzyme's catalytic site will be blocked. Another approach is to replace TDP with an analogue that is incapable of catalysis and which also resembles a key transition state.

2.3.1. Transition State Analogues Related to the C2α Carbanion - Thiamin-Thiazolone Diphosphate (TTDP)

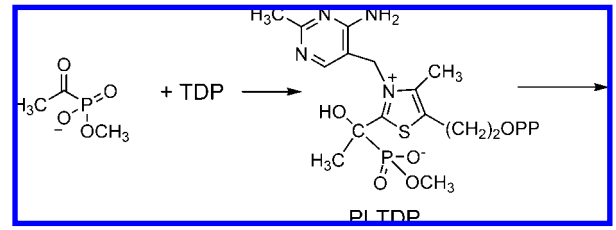
Lienhard and Wolfenden proposed that based on Pauling's application of transition state theory to enzymic reactions,[67] stable analogues of (transient) transition states would bind very tightly to their cognate protein.[8,68] On the basis of the proposed nature of the transition state for decarboxylation, Gutowksi and Lienhard produced thiamin-thiazolone diphosphate (TTDP, Scheme 17) as an analogue of that transition state.[69]

They observed that TTDP bound very tightly to pyruvate dehydrogenase, in accord with their prediction.[69] This was an early piece of evidence for the value of the concept of transition state analogues in designing powerful inhibitors. However, later work with other enzymes that catalyze pyruvate decarboxylation showed that TTDP binding is not universally tight in these systems and is therefore not related to the transition state, which should be similar in all cases. [70]

2.3.2. Enzyme-Generated Intermediates and Analogues: Conformations and Least-Motion

Another approach to producing a transition state analogue is to let the enzyme produce its own. If the formation of

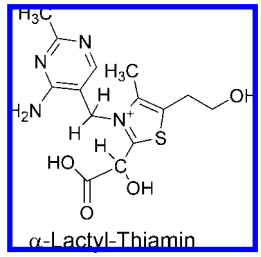
Scheme 18



LTDP from pyruvate is a central part of the catalytic function of an enzyme that promotes its decarboxylation, then addition of a pyruvate analogue could generate an analogue of LTDP. Methyl acetylphosphonate is a 2-ketoacid that exists exclusively as a monoanion in neutral solution; conceptually, the phosphonate group replaces the carboxyl group of pyruvate. This material is a powerful inhibitor of pyruvate dehydrogenase, presumably because the enzyme forms α-(methyl)phosphonolactyl-TDP (PLTDP) (Scheme 18).[71]

The addition product of methyl acetylphosphonate and thiamin was later synthesized,[72] and its crystal structure was determined.[73] The stable conformation of the C2 substituents has the methylphosphono group perpendicular to the plane of the thiazolium ring. Loss of carbon dioxide from LTDP in a similar conformation would lead to overlap of the carbanion-derived electrons with the thiazolium π-system, delocalizing the electrons in the anion into the available orbitals of the thiazolium cation. This would be a mechanism for decarboxylation that fits the general physical principle of least motion. Recently, Furey and Jordan have determined a high resolution crystal structure and spectroscopic data on a similar phosphonolactyl-TDP that is bound in the pyruvate dehydrogenase E1-subunit.[74,75] The conformation with respect to the C−P bond is similar to that in the nonenzymic compound, while the coenzyme itself is in the V-conformation that is found in TDP on the enzyme prior to reaction. Similar observations hold true for phosphonolactyl-TDP in pyruvate oxidase.[76]

Scheme 19



2.4. Pyruvate-TDP Addition Intermediate

As noted above, the initial covalent intermediate from TDP and pyruvate on an enzyme is expected to be the addition product, LTDP. Krampitz noted that this would decarboxylate very rapidly and might only exist as a transition state.[13] In order to estimate the reactivity of such an intermediate, Crosby and Lienhard prepared an analogue of thiazolium group of LTDP, 2-(1-carboxy-l-hydroxyethyl)-3,4-dimethylthiazolium chloride (CHDT), and showed that the rate constant for its decarboxylation is at least 10^5 times smaller than k_{cat} for pyruvate decarboxylase.[77] This indicates that LTDP would most likely be an intermediate, not a transition state on the enzyme, and that the enzyme would have to enhance its reactivity. On the basis of the observed increase in the rate of decarboxylation of CHDT in solvents less polar than water, Lienhard imagined that the enzyme would achieve the observed acceleration by desolvating the intermediate and transferring it into a hydrophobic region of the protein.[77] Since pyruvate is well-solvated in water, considerable energy would be needed to achieve the desolvation, leading to an unfavorable equilibrium, which is counterproductive for decarboxylation.

2.4.1. Lactyl-Thiamin - Synthesis and Reactivity

The rates and patterns of reactions of lactyl-thiamin (LT, Scheme 19),[78] formally the conjugate of thiamin and pyruvate, provided an excellent quantitative comparison with those of enzyme-bound LTDP.[79] Since decarboxylation of this compound electronically requires that the carboxylic acid group be dissociated, it was expected that the material would be stable in strongly acidic solutions. Condensation of ethyl pyruvate with thiamin in ethanol containing sodium ethoxide gives the ethyl ester of LT.[78] Hydrolysis of the ester in concentrated HCl gives the free acid. The pH−rate profile for decarboxylation of α-lactyl-thiamin shows that this material is at least 10^6 times less reactive than LTDP is when it is bound to pyruvate decarboxylase (Without the protein, LTDP loses carbon dioxide not faster than does LT.) The reaction was slower than had been reported for the supposed decarboxylation of pyruvate catalyzed by thiamin,[80] indicating that some of the earlier observations were incorrectly interpreted.

The complete kinetic and thermodynamic profile for LT were obtained by kinetic and equilibrium measurements.[78] Later, Alvarez and Schowen provided a similarly detailed view of the enzymic reaction and were able to correlate the points where the enzyme produced the stabilization necessary for the reaction to exceed the nonenzymic decarboxylation.[79]

2.4.2. Lactyl-TDP - Synthesis, Reactions with Enzymes and Isolation

LTDP was produced in a reaction that is analogous to the synthesis of LT, starting from TDP instead of thiamin.[81] The *t*-butyl ester of pyruvate (from the reaction of isobutylene and pyruvic acid[82]) was combined with TDP in a weakly basic solution of *t*-butoxide in DMF. The *t*-butyl ester was cleaved in trifluoroacetic acid. The material binds very slowly to the apoenzyme of wheat germ pyruvate decarboxylase. Because HETDP does bind, any nonenzymic decarboxylation produces HETDP, which binds faster. Recently, Jordan has shown that LTDP binds very slowly to an apoenzyme of a mutated pyruvate decarboxylase,[83] enabling a detailed structural study, and that a ketophosphonate forms a stable intermediate, similar to a reactive intermediate.[71,84] Tittmann and co-workers produced mutated pyruvate oxidases that allowed the in situ observation of LTDP and PLTDP by X-ray crystallography.[76]

2.5. Fluoropyruvate and Its Reactions on TDP Enzymes

Flournoy and Frey examined the reactions of fluoropyruvate with pyruvate dehydrogenase.[85] They observed that fluoride is eliminated, with acetate as the major product. The results are consistent with addition of TDP to the ketocarbon of fluoropyruvate, forming the monofluoro analogue of LTDP. Upon loss of carbon dioxide, the resulting carbanion eliminates fluoride faster than it is protonated, leaving acetyl-TDP as the product (Scheme 20).

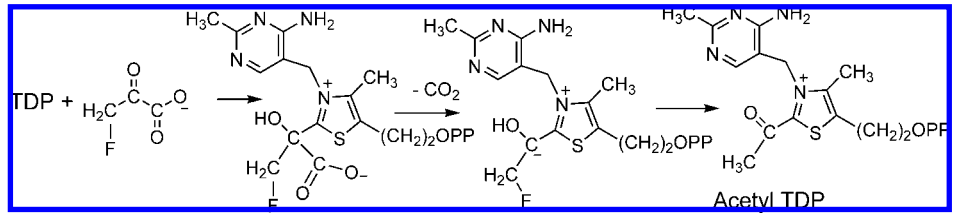
This undergoes hydrolysis to acetate and TDP (Scheme 21). The hydrolysis of acetylthiazolium compounds occurs at a sufficient rate to account for the observed reaction patterns.[86,87] This process uses only the first part of the enzyme's catalytic cycle, with no catalysis provided by the enzyme for the hydrolysis reaction, whereas in the normal cycle, HETDP is converted by the enzyme to acetaldehyde and TDP.[88,89] Since pyruvate decarboxylase is subject to allosteric substrate activation, the fluoropyruvate reaction was used to probe the steps subject to activation.[88,89] It was concluded that allosteric effects occur only in the steps that involve elimination of acetaldehyde.

Frey also observed that enzymes that produce oxidized products also produce acetyl-TDP as an intermediate: phosphoketolase, pyruvate dehydrogenase, pyruvate oxidase and pyruvate oxidoreductases.[90] Acetyl-TDP was characterized by isolating it from quenched enzymic reaction mixtures. This species is chemically competent as an intermediate in the decarboxylation and dehydrogenation of pyruvate by the PDH complex. In these cases, the acetyl group is transferred to an acceptor.

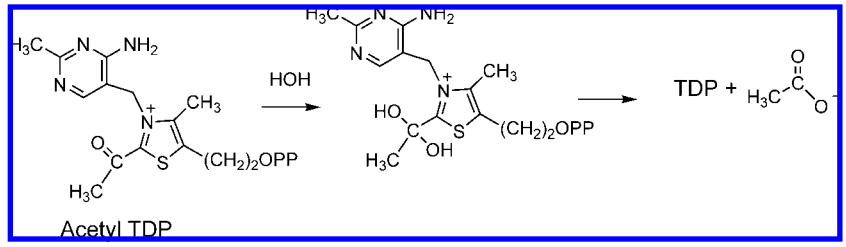
2.6. Benzoylformate Decarboxylase and Its TDP Intermediates

Kenyon, with his co-workers and collaborators, has investigated many of the fascinating enzymes in the mandelate pathway of *Pseudomonas putida*, systems that had been discovered by Hegeman.[91] Their analysis has led to the understanding of the most fundamental aspects of the nature of enzyme catalysis.[92–101] Oxidation of mandelate produces benzoylformate, an α-ketoacid. Decarboxylation of benzoylformate produces benzaldehyde, a reaction that parallels the decarboxylation of pyruvate. Benzoylformate

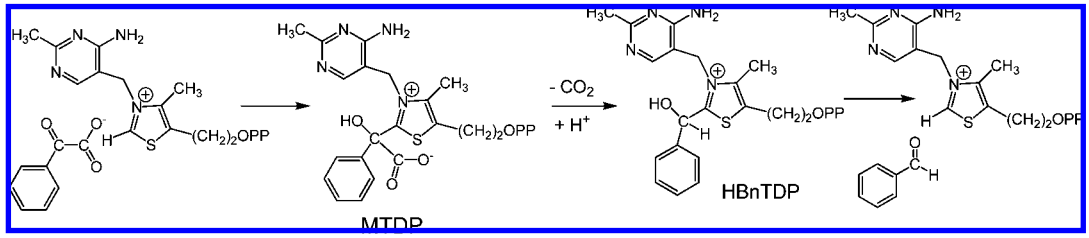
Scheme 20



Scheme 21



Scheme 22



decarboxylase (BFD) contains TDP as a cofactor, consistent with other α-ketoacid decarboxylases.[91,92,94,96] Therefore, it would be logical to assume that mechanism involves addition of C2 of the conjugate base of TDP to the 2-keto group of enzyme-bound benzoylformate. The addition produces mandelyl-TDP (MTDP), an analogue of LTDP in which a benzene ring replaces the acyl-derived methyl of LTDP. Like LTDP, MTDP is set up to lose carbon dioxide and accept a proton to form 2-(1-hydroxybenzyl)-TDP, prior to release of benzaldehyde. The overall process is summarized in Scheme 22.

2.6.1. Enzyme-Catalyzed Bromide Elimination from p-Bromomethylbenzoylformate

Kozarich, Kenyon, and their co-workers found that *p*-bromomethyl-benzoylformate (BBF) is a substrate and inactivator of BFD.[94,98,102] The enzyme converts BBF to bromide ion, toluic acid, and carbon dioxide. Addition of benzoylformate after reaction of BBF shows that BFD's activity is markedly decreased as a result. Reaction of BBF with BFD involves addition of TDP to the α-carbonyl, forming the *p*-bromomethyl analogue of MTDP (Scheme 23). Loss of carbon dioxide leads to the carbanion, which undergoes an elimination reaction that competes effectively with protonation at C2α. The loss of bromide produces an intermediate xylylene, a high energy tautomer in which the aromaticity of its precursor is lost. Protonation produces 2-toluyl-TDP, an acylthiazolium compound that slowly hydrolyzes to *p*-toluic acid and TDP.

The implied route indicates that enzymic protonation of the carbanion at C2α from decarboxylation of the TDP conjugate of BBF is slower than the elimination reaction that generates the substituted xylylene (or is formed at equilibrium), despite the higher energy nature of a xylylene component. The normal catalytic cycle requires that the C2α position is protonated prior to elimination of the aldehyde. The elimination reaction that releases bromide and produces the xylylene intermediate must occur with a unimolecular rate that is considerably faster than the enzymic protonation process, which is at least as fast as would be indicated by the magnitude of k_{cat}. Since it is expected that xylylenes are high energy species that require the loss of aromaticity of a benzene ring, the immediate decarboxylation product must also be a high energy species.

2.6.2. Reactions of Benzoylphosphonate with Benzoylformate Decarboxylase

Benzoylphosphonic acid (Scheme 24) is an analogue of benzoylformic acid that parallels the similarity of acetylphosphonate and pyruvate.[71–73,103]

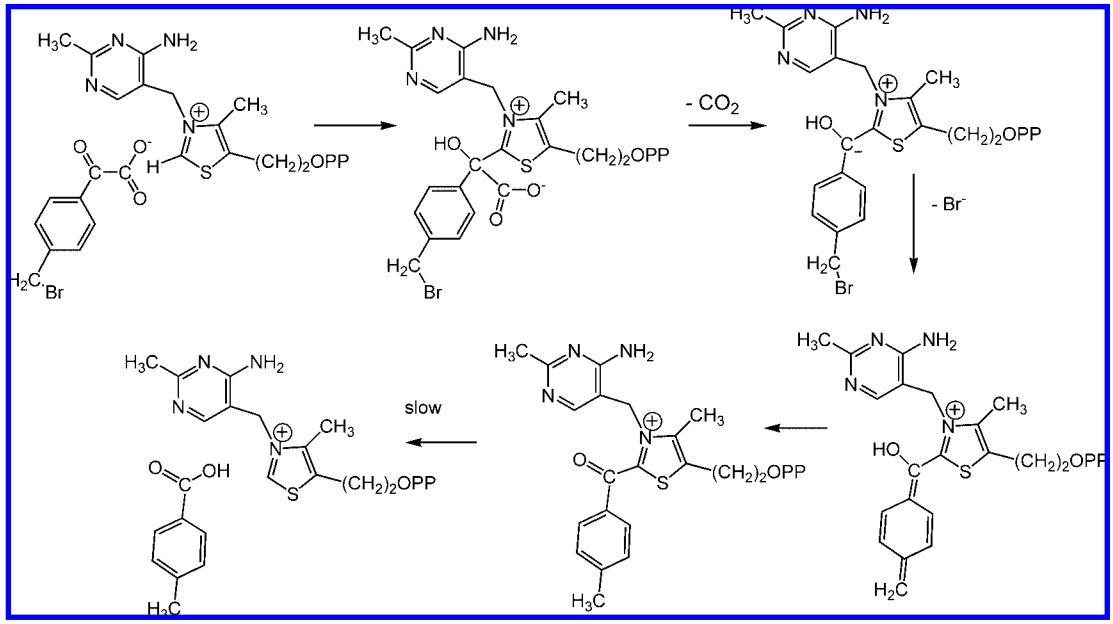
The addition product of methyl acetylphosphonate and TDP is stable on an enzyme, providing reversible inhibition,[71] and the nonenzymic addition analogue has been the subject of crystallographic analysis.[73] The methyl derivative is expected to be more stable than free acid.[104] Unesterified β-ketophosphonic acids are subject to thermal decomposition with C−P bond cleavage (Scheme 25).[104]

This reactivity pattern is consistent with the finding that benzoylphosphonate inactivates BFD, forming a TDP conjugate that transfers a phosphono group to an active site serine hydroxyl to yield a phosphate ester (Scheme 26).[93]

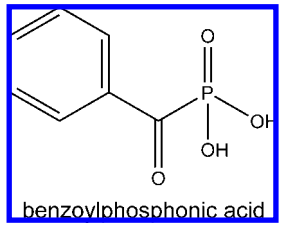
2.7. The Unexpected Reactions of 2-(1-Hydroxybenzyl)-thiamin (HBnT)

The decarboxylation of benzoylformate in BFD should involve initial formation of a conjugate of TDP followed by loss of carbon dioxide and protonation to give 2-(1-

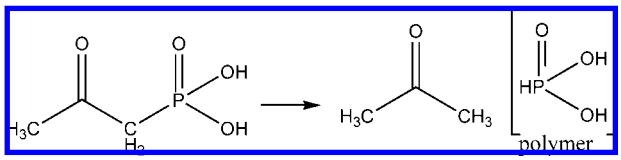
Scheme 23



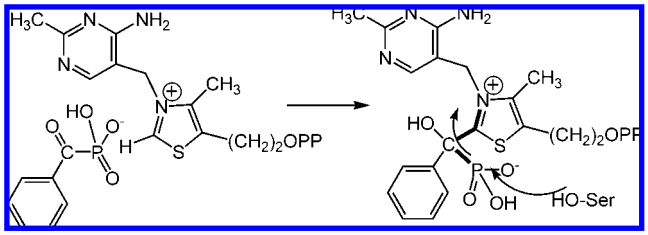
Scheme 24



Scheme 25



Scheme 26



hydroxylbenzyl)-thiamin diphosphate (HBnTDP). The corresponding molecule without a diphosphate, HBnT, became the subject of attention for the purposes of elucidating the reactivity of intermediates in the enzymic and nonenzymic processes. The expected reaction is base-catalyzed formation of benzaldehyde and thiamin. This was so obvious that it took some effort to discover that the reaction is eclipsed by an alternative reaction that destroys TDP.

2.7.1. The Carbon Acidity of HBnT

The decarboxylation of MTDP should produce 2-(1-hydroxylbenzyl)-TDP. Sable had examined this material and had shown that the C2-α proton can be exchanged for deuterium in deuterium oxide, establishing the existence of the conjugate base of the this carbon acid.[105] Bordwell and co-workers produced an analogue to estimate the acidity at C2α (pK_a = 14.1 from studies in DMSO).[106]

2.7.2. General Base-Catalyzed Reactions of HBnT

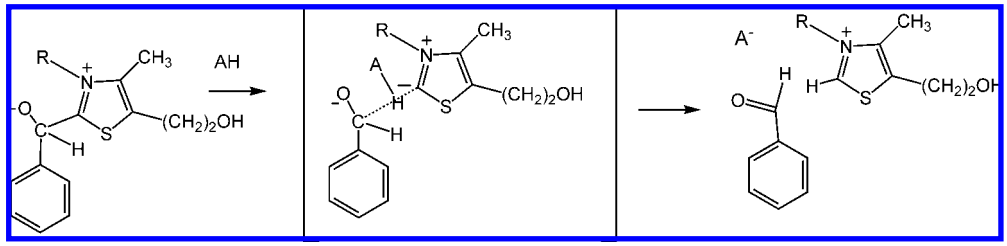
Crane and Washabaugh reported a very detailed study of the reactions of the C2α conjugate base of HBnT.[107] These include the C−H ionization that leads to aldol condensations and the competing O−H ionization that leads to elimination of benzaldehyde and release of thiamin. The latter is a direct analogue of the last step in catalysis by BFD. These authors emphasize the role of general base-catalysis in the decomposition of HBnT, concluding that the mechanism that leads to the observation is kinetically equivalent to formation of the conjugate base of the hydroxyl and general acid-catalysis of the step that releases the thiamin ylide and benzaldehyde. This mechanism implies that the proton from a Brønsted acid adds to the ylide in the transition state (Scheme 27).

This is a very surprising and implausible mechanism since the expulsion of the ylide creates a localized carbanion with no site at which to insert a proton prior to completion of the reaction. Yet, there is no likely alternative within the scheme. The observed rate constants for general base catalysis were derived from initial rate analysis of formation of a carbonyl derivative, but the products were not isolated and identified.

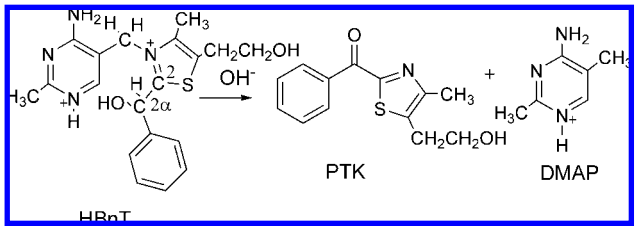
2.7.3. Products in General Base-Catalyzed Reactions of HBnT

While the kinetics of the observed general base catalyzed reaction of HBnT were reproducible, the lack of a product study suggested a possible source of an explanation. In an alternative approach, the buffer-catalyzed decomposition of HBnT in neutral solution was observed directly by following changes in the UV–vis spectrum without addition of carbonyl reagents.[108] The product from the reaction in neutral solution had a strong absorbance with a maximum at 328 nm, which corresponds to neither thiamin nor benzaldehyde. Since no UV–vis spectrum of the products had been reported, this

Scheme 27



Scheme 28



result suggests that the assumption about the actual products being benzaldeyhde and thiamin is incorrect. The observed absorbance indicates that the buffer catalysis involves a reaction that competes with elimination of benzaldehyde.[108] The products from the decomposition of HbnT in neutral solution were isolated and identified as 4-amino-2,5-dimethylpyrimidine (DMAP, Scheme 28) and a ketone, 2-benzoyl-5-(2-hydroxyethyl)-4-methylthiazole (PTK, Scheme 28). These products result from removal of the C2α-proton with subsequent structural reorganization. Therefore, the buffer-catalyzed process in neutral solution does not produce benzaldehyde and thiamin to any signficant extent.

2.7.4. Fragmentation Products of the C2α Conjugate Base of HBnT

Oka and co-workers had reported that they attempted the reaction of thiamin with aromatic aldehydes in ethanol containing triethylamine. This was expected to generate the C2α carbanion of HBnT, a species that would be useful as an acyl carbanion synthon. Instead, the reaction gave products that are the result of cleaving the methylene bridge and the thiazolium ring of thiamin (the products in Scheme 28).[109–111] These are the same products observed in the fragmentation of HBnT in neutral solution.[108,112] Hydrogenation of the carbonyl of the product to the alcohol gives a product that is identical with the nonpyrimidine product that arises from the sulfite cleavage of HBnT (Scheme 7).[39] Oka and co-workers were well aware that the reaction must proceed via C−H ionization of HBnT: "It is reasonable to assume that benzaldehyde first reacts to give HBnT followed by further rearrangements of the reaction product to give [the fragmentation products]."

Oka also reported the UV–vis spectrum of the ketonic fragmentation product (in ethanol) with maxima at 263 and 326 nm. Both are within a few nanometers of the absorbance maxima in the spectrum of the material from the fragmentation of HBnT in water. They also confirm that the material is a ketone that forms a derivative with carbonyl reagents, which could have been the material tracked by Crane and Washabaugh.[107] In contrast, benzaldehyde is eliminated from HBnT at a higher pH through a specific-base catalyzed process, while the neutral process is entirely Oka's fragmentation.[112]

2.7.4.1. Protonation of N1′ Promotes Fragmentation of HBnT. A kinetic analysis indicated that N1′-protonated HBnT undergoes the fragmentation reaction very rapidly. [112] In neutral solution, the first-order fragmentation reaction from the conjugate base at C2α is several orders of magnitude faster than the expected formation of benzaldehyde and thiamin, which results from reaction of the conjugate base of the 2α-OH. In more alkaline solution, pH 9 and higher, elimination of benzaldehyde becomes the dominant process. If the positive charge on the protonated pyrimidine directs ionization to C2α, fixing the positive charge on N1′ by alkylation should direct the reaction fragmentation even in alkaline solution.

Alkylation of the N1′ position creates a permanent local positive charge at N1′.[113] In this case, fragmentation is the exclusive process even at high pH.[112] The basis of this distinction was not apparent at the time it was noticed. Later work showed that the fragmentation results from lower energy ionization of the C2α hydrogen compared to ionization of the hydroxyl proton when N1′ is positively charged.[114] This may be the result of the ability of the carbanion to delocalize the charge, so that the overall thiazolium region is uncharged (Scheme 29).

2.7.4.2. Kinetics of the Fragmentation of HBnT. The buffer catalyzed fragmentation reaction shows a nonlinear dependence of rate on buffer concentration (Figure 1).[115] The curvature is indicative of a change in rate-determining step in a multistep process (Scheme 30).[116]

At high buffer concentrations, $k_{-1}[BH^+] > k_2$, so the second step is rate-determining and the reaction appears to be independent of buffer concentration ($k_{obs} = k_1k_2[B]/(k_{-1}[BH^+] + k_2)$. At low buffer concentrations, k_2 is rate-determining and ($k_{obs} = k_1[B]$ and increases with buffer contration). Protonation of the intermediate reverses the reaction. Transfer of a deuteron is slower than transfer of a proton, so the forward reaction is favored in deuterium oxide

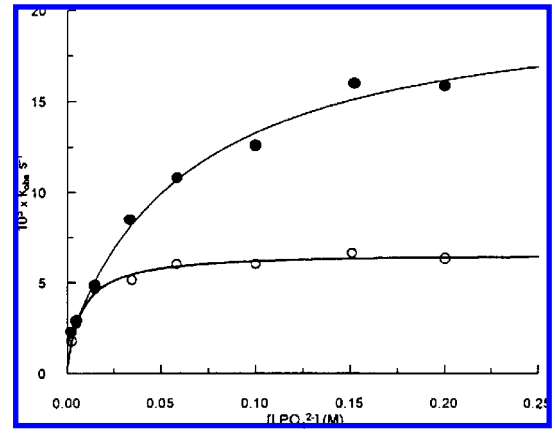
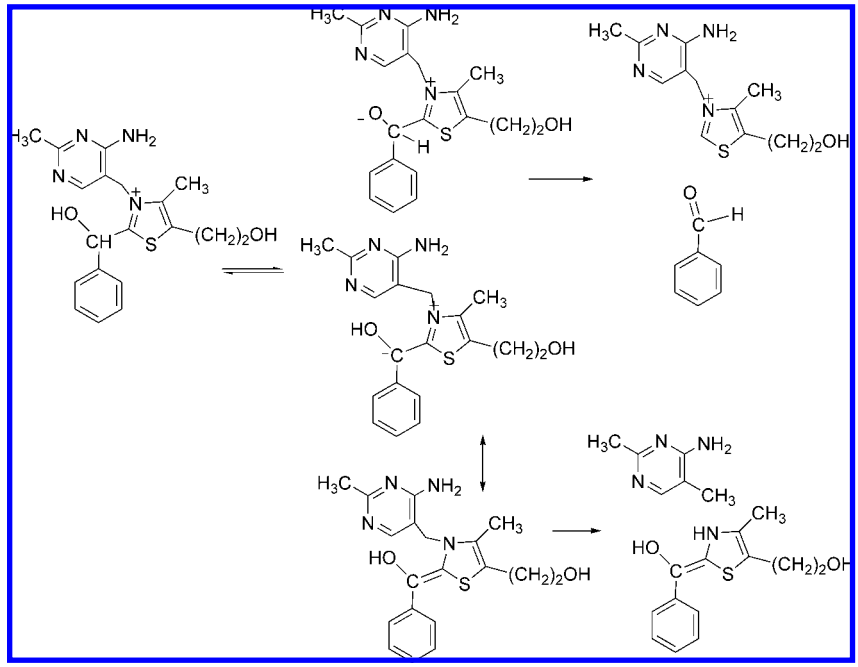
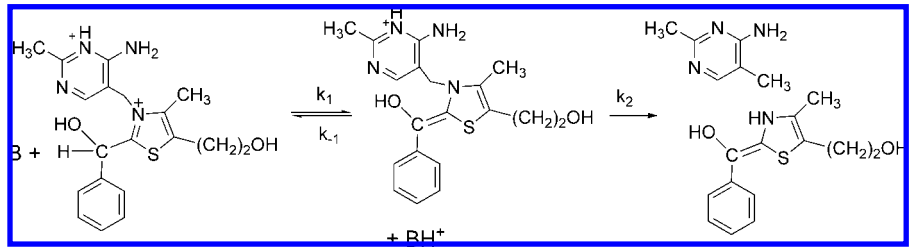


Figure 1. Fragmentation of HBnT as a function of buffer concentration in water (○) and in deuterium oxide (●).

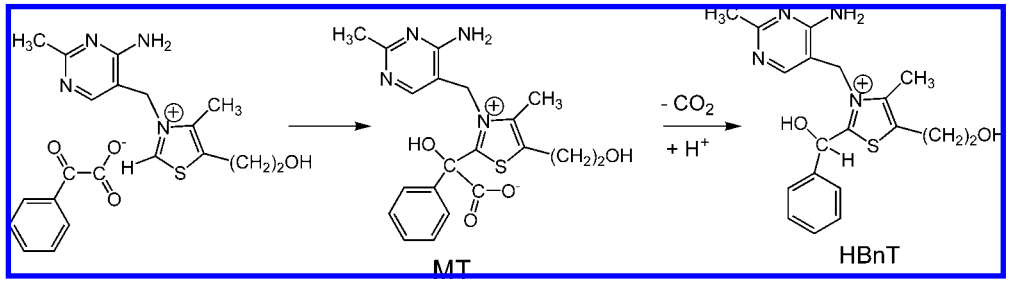
Scheme 29



Scheme 30



Scheme 31



(Figure 1). At the midpoint of the curvature in the plot, $k_{-1}[BH^+] = k_2$. With the pK_a estimated to be about the same as in HET[106] and $k_1$determined directly, we arrive at values for k_{-1} and k_2. The data were fit to the derived equation for reaction via an E1$_{cb}$ reaction intermediate.[117,118] The results give the rate constant for the fragmentation step at 40 °C as being on the order of 10^4 s^{-1}, which is larger than the enzymic decarboxylation reaction ($k_{cat} = 10^2$ s^{-1}).

2.7.4.3. Inverse Solvent Isotope Effect. The maximum rate of fragmentation of HBnT in deuterium oxide is significantly larger than it is in water, with an inverse solvent isotope effect on the maximum rate ($k_{DOD}/k_{HOH} = 3.0$). The solvent isotope effect in the region where the rate increases linearly with base concentration is also inverse ($k_{DOD}/k_{HOH} = 1.3$). This is a classic indication of reversible formation of a carbanionic intermediate. The system can be treated quantitatively by fitting the data to the Keeffe-Jencks equations, resulting in accurate rate constants for each step.[117]

2.7.5. Avoiding Fragmentation on the Enzyme

Since the nonenzymic fragmentation rate exceeds the normal enzymatic turnover rate, it is tempting to speculate on devices that the enzyme might have evolved to avoid the fragmentation. However, theories of enzyme evolution suggest that the process involves selection for faster processes, not avoidance of inherently fast processes.[119] Adding constraints on conformation and other clever inventions can be invoked without evidence, but this does not serve to answer the reality of the situation, which became apparent after study of other intermediates: the enzyme accelerates decarboxylation by a process that incidentally suppresses fragmentation.

2.8. The Reactivity of α-Mandelyl-thiamin (MT)

Kinetic analysis of the reactions of α-lactyl-thiamin enabled a quantitative analysis of the catalytic enhancement

provided to LTDP by pyruvate decarboxylase.[78,79] It would be logical that the nonenzymic decarboxylation of the conjugate of benzoylformate and thiamin, α-mandelylthiamin (MT) (Scheme 31), would provide a basis for a similar study for the enzymic reactions of MTDP.

2.8.1. Synthesis of MT

The synthesis of LT had been achieved by forming the conjugate base of thiamin in ethanol containing sodium ethoxide and adding ethyl pyruvate to form the ethyl ester of LT. Hydrolysis of the ester in concentrated HCl produces the free acid, which is slow to decarboxylate in acidic solutions. The condensation of thiamin and ethyl benzoylformate does not occur under the same conditions. Ethyl benzoylformate is either less reactive, more sterically hindered, or a combination thereof. The synthesis was eventually achieved by adding magnesium chloride to the condensation reaction solution.[120] The yield is very low, but sufficient material is available for product and kinetic studies.

2.8.2. Decarboxylation of MT

The conversion of MT to carbon dioxide and HBnT initially produces the C2α conjugate base of HBnT. This is also the product of the first step of the fragmentation reaction of HBnT. Therefore, the decarboxylation of MT will give the fragmentation products in competition with the formation of HBnT. Consistent with the formation of HBnT resulting from reaction of the conjugate base with Brønsted acids, the yield of HBnT relative to fragmentation is proportional to buffer concentration and is consistent with microscopic reversibility.[114] The pH−rate profile for decarboxylation of MT is shown in Figure 2 along with the base-catalyzed elimination of benzoylformate from MT at high pH.

The scheme used to fit the data in Figure 2 is shown below (Scheme 32).

The overall rate law that was used to generate the curve in the region where decarboxylation is the major process is

$$K_{obs} = K_{a1}K_{a2}k_2 + K_{a1}k_1[H^+]/K_{a1}K_{a2} + K_{a1}[H^+] + [H^+]_2$$

2.8.3. Pyridinium Catalysis of Decarboxylation of MT

In examining the effects of buffers, it was discovered that the decarboxylation of MT is accelerated by the acid component of pyridine and C-alkyl pyridine buffers but not other Brønsted acids[121,122] (Figure 3).

Examination of the structure of MT does not reveal any site for addition of a proton in the transition state that produces CO_2. *N*-Alkylpyridinium salts are not effective, ruling out an electrostatic basis for the observed acceleration.[122] Consideration of reaction energy profiles for decarboxylation reactions revealed that the bond breaking process leads to an associated pair of CO_2 and the residual carbanion (Scheme 33).[123–125]

In the case of catalysis by pyridines, it is likely that the catalyst is able to participate in a π-stacking arrangement that provides the needed proton efficiently (Scheme 34).

If this process occurs, then the resulting product will be protonated more rapidly than where an acid diffuses into the reaction site. Since the fragmentation occurs from the C2α conjugate base of HBnT, efficient protonation will lead to more efficient competition against the fragmentation reaction. This has been observed to be the case (Figure 3a).[121,122,126]

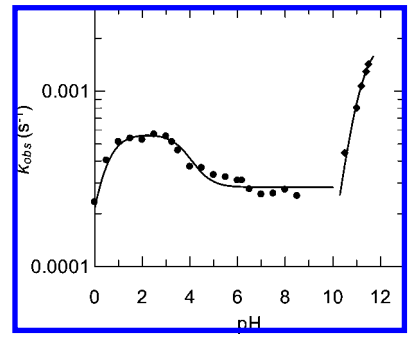


Figure 2. Decarboxylation (•) and elimination (♦) rate−pH profile for MT.

2.8.4. Internal Return of CO_2

There is no activation barrier for the reverse reaction when CO_2 is associated with the carbanion.[123,124] On the basis of transition rate theory, the rate constant for a reaction with no barrier is on the order of magnitude of a C−C bond vibration, about 10^{13} s^{-1}. With diffusion of CO_2 having a rate constant of about 10^8 s^{-1}, reforming the C−C bond is favored over release of CO_2 by a factor of about 10^5. The idea that association of unhydrated carbon dioxide and a nucleophile will lead to carboxylation was originally proposed by Sauers, Groh, and Jencks in their study of the reaction of monoesters of carbonates as models for the carboxylation of biotin.[127]

2.8.5. Overcoming the Return of CO_2

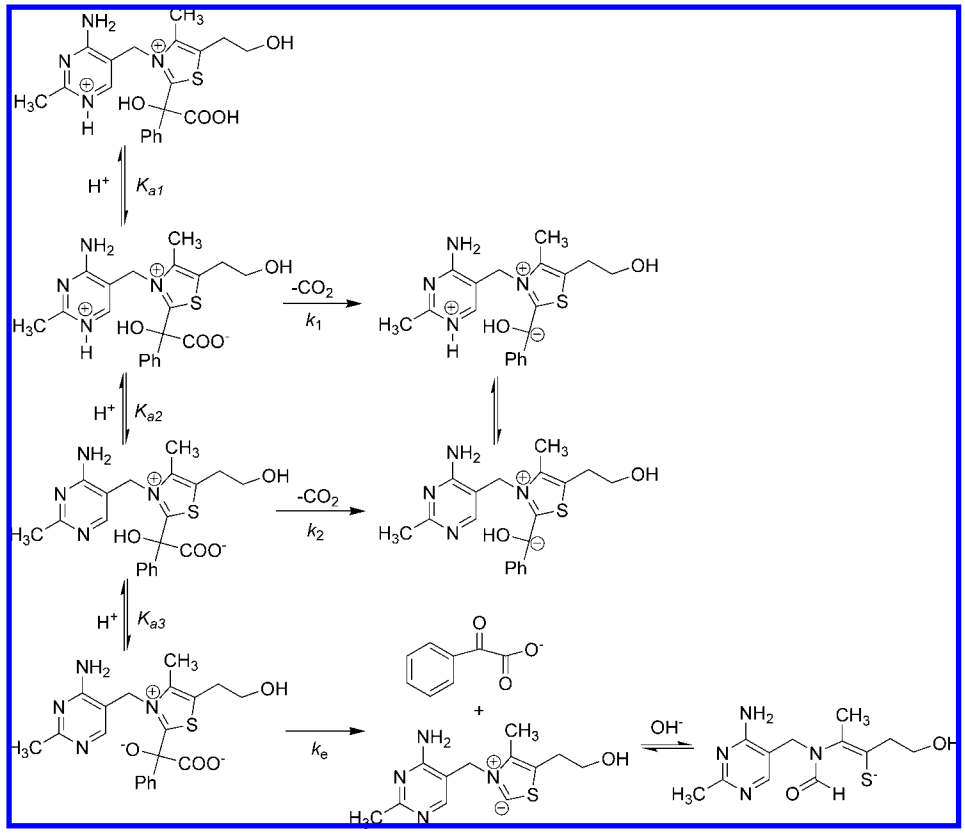
Protonation of the carbanion will produce HBnT. Therefore, CO_2 can escape by diffusion. Since diffusion is much slower than the recombination process, a successful competitor must overcome the need for diffusion to arrive at the reaction site. Jencks describes this form of catalysis as "preassociation",[128] while Schowen uses the anthropomorphic term "spectator catalysis".[129] Enzymes have built-in acids that can block the return of CO_2.

A preassociation mechanism would be especially applicable in an enzyme's active site, where there are many sources of protons in a defined structural environment.[130] The results of mutagenesis studies on BFD suggest that acidic groups provide catalytic enhancement of the same order of magnitude as would be expected for an adjacent proton source.[101]

This mechanism should be applicable for any decarboxylation reaction that generates an aldehyde as a product. Other enzymes that generate the carbanion by decarboxylation necessarily have an acceptor as part of their inherent mechanism, and these acceptors must compete successfully with carbon dioxide. Examples include cases where the carbanion is oxidized (pyruvate oxidase[131]), converted to a radical,[132–134] adds to a carbonyl,[135–138] or reacts with a disulfide (a possible mechanism in pyruvate dehydrogenase[139,140]).

One important implication of this observation is that measurements of intrinsic values of $^{13}C/^{12}C$ isotope effects in decarboxylation reactions[141–143] may have actually measured an equilibrium process (C−C bond-breaking and formation) between bound and unbound carbon dioxide, while the enzymic process is irreversible are a result of trapping of the carbanion as discussed above.[92,141–143] As a result, estimates of the extent of the commitment factor in enzymic decarboxylation may be subject to correction.

Scheme 32



2.8.6. Competing with the Internal Return of Carbon Dioxide

The issue of internal return in carbanion chemistry has been focused on the reactions of carbanions with Brønsted acids generated by reaction with a Brønsted base that remains associated with the carbanion.[144–146] The possibility of such a return process in decarboxylation has been implicated in recent overviews of decarboxylation,[123,124,147] while the proposal of a reactive form of carbon dioxide is based on the microscopic reverse, a carboxylation reaction.[127] The observation that bromide ion can be eliminated faster than protonation occurs on an enzyme is particularly dramatic when considered in this context.[94,98,102]

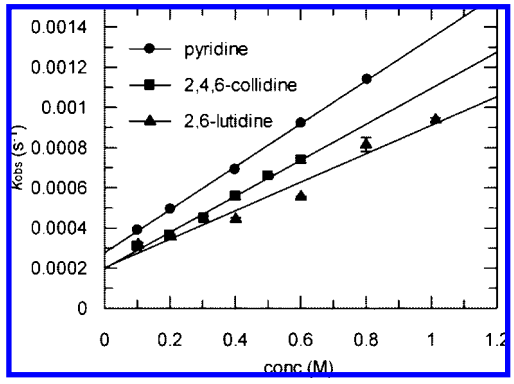
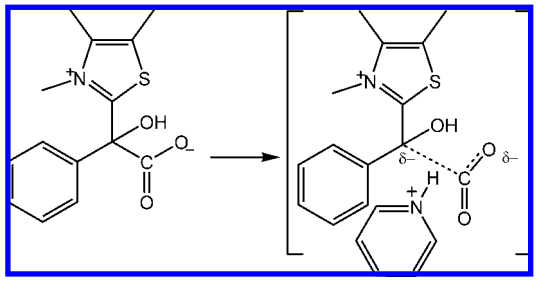


Figure 3. Buffer catalyzed decarboxylation of MT.

Scheme 33



3. Spectroscopic Detection of Covalent Intermediates in TDP Enzymes

As discussed earlier, the enzymatic conversions of pyruvate and carbohydrate substrates involve a series of different covalent intermediates derived from TDP. The unambiguous spectroscopic detection and structural analysis of these intermediates has been an important challenge that has been met with success in recent years. While intermediates of other enzyme-bound flavins and pyridoxal phosphate give rise to identifiable spectroscopic absorptions, TDP-derived inter-

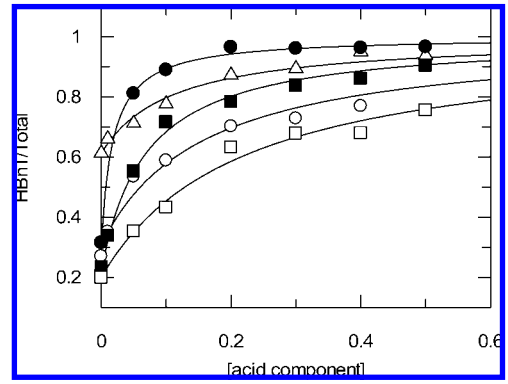
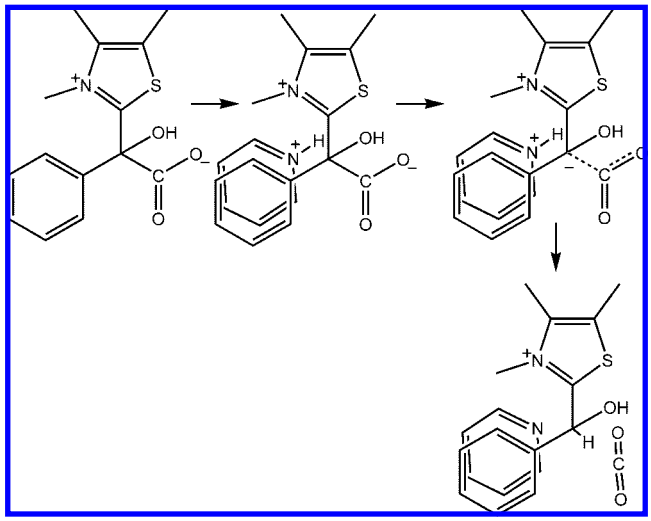


Figure 3a. Formation of HBnT versus fragmentation: pH = pK_a (○ bis-tris pH 6.7, • pyridine pH 5.4, (2,2,2)-trifluoroethylamine pH 5.7, phosphate pH 6.5, and Δ acetate pH 4.6).

Scheme 34



mediates do not produce a distinctive spectrum. A further complication is that most TDP enzymes have overall rate constants, k_{cat}, that approach 10^2–10^3 s^{-1}. This suggests that the rate constants of elementary steps in the mechanism will proceed too rapidly to observe by stopped-flow or quenched-flow methods. Also, since the overall processes of TDP-dependent decarboxylases are essentially irreversible (due to diffusion of carbon dioxide), equilibrium perturbation experiments (temperature jump, pressure jump) are not accessible methodological approaches.

In the following sections, different approaches for the spectroscopic and time-resolved observation of covalent TDP-derived intermediates will be discussed. The results obtained on the different TDP enzymes and their variants provide a wealth of information for understanding the detailed reaction mechanism and kinetics and delineate specific catalytic contributions of TDP and the enzyme's active site.

3.1. Chemical Quench and Analysis of Covalent TDP Intermediates by ^{1}H NMR

3.1.1. General Considerations in the Use of Quenching

While recent advances in NMR analysis of dynamics and structure of enzymes have made enzymes accessible for study,[148–150] the large size of TDP enzymes (≥120 kDa) and the occurrence of multiple steps within the reaction sequence render it impossible to observe directly and simultaneously the distribution of enzyme-bound reaction intermediates. An alternative approach is to quench the enzymatic reaction and analyze the in situ intermediate distribution after quenching. A major advantage of this technique is that it permits removal of the protein by acid precipitation, permitting direct observation of TDP-derived intermediates in a stable form in proportion to their enzyme-bound concentrations. While heated methanol has been employed to isolate intermediates from TDP enzymes,[151] alcohols or other solvents with low dielectric constants decompose predecarboxylation intermediates, such as LTDP, making an analysis inaccurate.[10,83,152] Owing to the inherently unstable nature of the intermediates due to decarboxylation[78] or hydrolysis[153] in neutral solution, it is fortunate that acid-quenching will stabilize the predecarboxylation intermediates by suppressing ionization of the carboxylic acid as well as reducing the rate of the hydrolysis of 2-acyl-TDP intermediates, which is base-catalyzed.

In systematic studies on TDP adducts, 0.2 M acid was found to be most suitable[154] because all C2 adducts are sufficiently stable under these conditions as is the cofactor's diphosphate moiety (acid-catalyzed hydrolysis of the diphosphate is slow). The central carbanion/enamine intermediate is rapidly protonated, even in neutral solution, and its conjugate acid will be observed. This is no limitation for the analysis of TDP enzymes where the carbanion/enamine is a transient intermediate since the protonated product will serve as an accurate surrogate. However, in decarboxylases where both the carbanion/enamine and its conjugate acid are true intermediates, additional studies are required for an independent analysis.

3.1.2. Kinetic Analysis of Steady-State Intermediate Distributions

The relative concentrations of covalent intermediates derived from TDP in enzymes can be obtained by analysis of acid-quench by ^{1}H NMR. In principle this could be done under steady-state or single turnover conditions. However, the magnitude of k_{cat} of many TDP enzymes is on the order of 10^2 s^{-1} (PDC from *Zymomonas mobilis* ≈ 150 s^{-1} at 30 °C,[154] BFD from *Pseudomonas putida* ≈ 350 s^{-1} at 30 °C[155]). As a result, the formation and interconversion of covalent intermediates are not accessible using single turnover conditions (with rapid mixing) except for specific cases where rates of interconversion of intermediates permit such an analysis. In a recent example, this was achieved with the pyruvate decarboxylase subunit of pyruvate dehydrogenase.[156]

Analysis of the intermediate distribution from a quench of a system that is present at the true steady state[154]

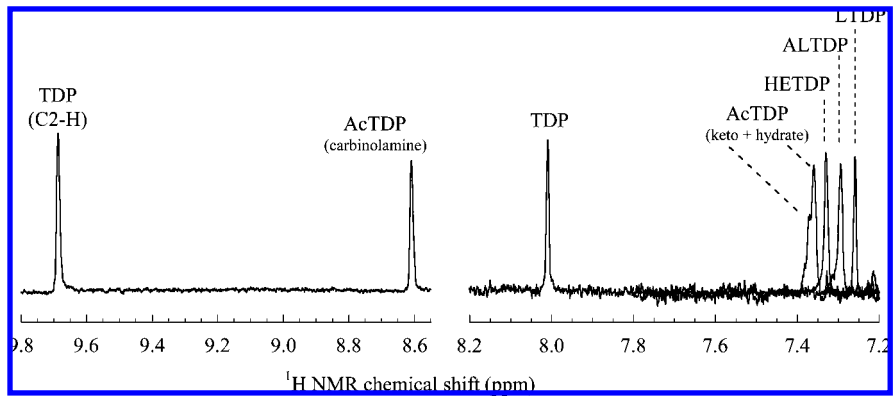


Figure 4. ^{1}H NMR-(C6′-H "fingerprint region") based detection of covalent pyruvate-derived TDP intermediates in 0.2 M acid.

necessarily gives a measure of the concentration of each covalent intermediate present during the catalytic cylce. Since the relative steady-state concentrations of the intermediates can be directly correlated with the rate constants of their interconversion, the forward net rate constants of elementary catalytic steps can be derived using Cleland's transit-time procedure.[157]

An illustrative example[154] that was the basis for analyzing more complex systems[158,159] involves the conversion of a substrate with reversible binding and three unimolecular catalytic steps involving two covalent TDP-intermediates (E-I_1 and E-I_2):

$$E + S \overset{K_S}{\Leftrightarrow} E^*S \text{ (Michaelis complex)} \xrightarrow{k_2'} E\text{-}I_1 \xrightarrow{k_3'} E\text{-}I_2 \xrightarrow{k_4'} E + P$$

At saturation, the catalytic constant k_{cat} is

$$k_{cat} = \frac{k_2' k_3' k_4'}{k_2' k_3' + k_2' k_4' + k_3' k_4'} \quad (1)$$

Another consequence of saturation at the steady state is that the formation and decomposition of all enzymatic intermediates are in balance ($d[E\text{-}I]/dt \approx 0$). Therefore,

$$\frac{[E\text{-}I_1]}{[E^*S]} = \frac{k_2'}{k_3'} = a \quad (2)$$

$$\frac{[E\text{-}I_2]}{[E\text{-}I_1]} = \frac{k_3'}{k_4'} = b \quad (3)$$

Using an experimentally determined steady-state intermediate distribution, the measured k_{cat} and equations 1−3, the forward rate constants of elementary catalytic steps are

$$k_2' = k_{cat}(1 + a + ab) \quad (4)$$

$$k_3' = k_{cat}\frac{(1 + a + ab)}{a} \quad (5)$$

$$k_4' = k_{cat}\frac{(1 + a + ab)}{ab} \quad (6)$$

The initial addition of TDP to the substrate's carbonyl is always reversible but the intermediate is present at a steady state level. The corresponding net rate constant k'_2 for this process will then be:

$$k_2' = \frac{k_{+2} k_3'}{k_{-2} + k_3'} \quad (7)$$

For an accurate estimate of the individual forward and reverse rate constants k_{+2} and k_{-2}, it is necessary to determine the forward commitment factor c_f of the decomposition of the initial covalent intermediate E-I_1

$$c_f = k_3'/k_{-2} \quad (8)$$

On the basis of reported kinetic carbon isotope effect analyses, c_f has been determined for several PDCs[79,160,161] and BFD.[92] The reported c_f values (based on comparisons with reactions used to calibrate the intrinsic isotope effect) are deduced to be 5.0 or larger, leading to the conclusion that $k_3' > k_{-2}$ and $k_2' \sim k_{+2}$.

3.1.3. Acid-Quench Analysis of Covalent TDP Intermediates Deriving from Pyruvate

3.1.3.1. Synthetic Intermediates. The original preparation of HETDP was by isolation of the intermediate from the protein by addition of acetaldehyde. [57] Chemical syntheses of the proposed substrate and product-derived conjugates of TDP: LTDP, HETDP, and AcTDP (see Scheme 15) had been achieved, and their properties had been established in the absence of an enzyme as the basis for comparison with enzymic systems.[78,151,153] The conjugate of TDP and acetolactate from AHAS is 2-[(1,2-dihydroxy-2-carboxy-1,2-dimethyl)-ethyl]-TDP, known as ALTDP. This was generated by an enzymatic synthesis using yeast PDC-Asp28Ala[162] under conditions where product release is rate-determining.[154]

Kinetic and spectroscopic analysis of these compounds revealed that all the C2-TDP-conjugates are stable in 0.2 M acid. Furthermore, the materials can be distinguished unambiguously by their ^{1}H NMR spectra under these conditions.[154] The ^{1}H NMR chemical shifts of the C6′-H singlets of the aminopyrimidinium moiety of TDP and the listed C2 conjugates are distinct (Figure 4). This provides a quantitative report for all TDP intermediates in conversion of pyruvate (δ ppm: C6′-H: TDP 8.01, LTDP 7.26, ALTDP 7.29, HETDP 7.33, AcTDP hydrate 7.36, AcTDP keto 7.37, AcTDP carbinolamine 8.60).[154] AcTDP is an equilibrium among three forms: keto, internal hydrate, and tricyclic carbinolamine.[153] These contributors also produce three distinct 6′-H peaks in the ^{1}H NMR spectrum.

3.1.3.2. Covalent TDP Intermediates in Pyruvate Decarboxylase. Pyruvate decarboxylase (PDC) catalyzes the decarboxylation of pyruvate to acetaldehyde and carbon dioxide (see Scheme 15), a key reaction in fermentation by yeast and in some bacteria, such as *Zymomonas mobilis*. The active site of PDC from *Zymomonas mobilis*,[163] which is virtually identical to that of yeast PDC,[54] is depicted in Figure 6.

TDP adopts the typical V conformation, juxtaposing the exocyclic 4′-amino group of the aminopyrimidine and the reactive C2 carbon of the thiazole. Jordan and co-workers suggested that in all TDP enzymes, a conserved bulky hydrophobic side chain, in this case from Ile415, acts as a fulcrum and is required to maintain the essential V conformation.[164] The enzyme-bound TDP in PDC is surrounded by an array of potential proton donors and acceptors (Glu473, Asp27, His113). The N1′ of the aminopyrimidine is hydrogen-bonded to a conserved Glu side chain, typical of the TDP enzyme superfamily,[165–168] whereas the 4′-NH_2 group interacts with the backbone carbonyl of Gly413.

At the steady-state of both the bacterial and the activated yeast PDC (the yeast enzyme is subject to homotropic substrate activation), LTDP, HETDP and TDP have been detected by the NMR quenching method.[154] Comparative intermediate analysis of wild-type PDC and various active site variants[154] suggested specific catalytic roles of individual side chains and the cofactor. This delineates the stereochemical course of the conversion of pyruvate.

The side chain of Glu473, which is positioned perpendicular to the thiazolium ring plane, is likely to have a specific supporting role in the decarboxylation of LTDP as this step is approximately 3000-fold slower in a Glu473 Asp variant ($k' = 0.13\ s^{-1}$ at 30 °C) compared to wild-type PDC ($k' = 397\ s^{-1}$). Recent studies have suggested that a proton-donating group traps the incipient carbanion to permit diffusional separation of CO_2.[121,169]

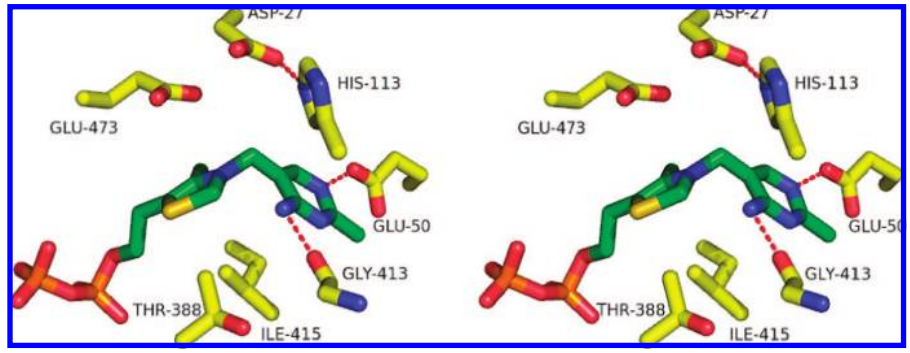


Figure 5. Active site structure of PDC from *Zymomonas mobilis* (stereoview).

Consistent with the proposal of the role of the acid being to promote diffusion of CO_2, recent work with a Glu473Gln variant (D. Meyer and K. Tittmann, unpublished) indicates that electrostatic stress between the side chain of Glu473 and the substrate-derived carboxylate of LTDP is unlikely to be a driving force for decarboxylation. In a Glu473Gln PDC variant where the side chain of residue 473 is the same size as that of the wild-type but which cannot carry a charge, decarboxylation of LTDP is almost as fast as in the wild-type enzyme. Since the carbanion/enamine is very basic, glutamine may serve as a proton relay or another nearby source may be available.

A decreased rate of elimination of acetaldehyde from HETDP has been also observed for variants of Asp27 and His113.[154] This suggests that there is a proton relay (His113, Asp27, Glu473) that quenches the incipient carbanion derived from decarboxylation to form the conjugate acid HETDP. Studies on various Asp variants of the bacterial and yeast enzyme[162,170] suggested that Asp is protonated before decarboxylation, but its role is subsequently transferring a proton to the adjacent Glu residue. As it becomes ionized, it repels a second pyruvate molecule thus avoiding a carboligation process. Hence, Glu473 and Asp27 are very likely acting as tandem acid–base catalysts, whereas His113 is critically required for structural interactions with Asp27 and the intermediates.

Substitution for the conserved Glu50, which is within hydrogen-bonding distance of N1′ of the aminopyrimidinium ring of TDP, results in variants with impaired substrate binding and product release. Since the Glu50-N1′ interaction is thought to activate the protein–cofactor tautomerization process (via Glu50-N1′-N4′-C2), it is reasonable to assume that 4′-NH_2 of the aminopyrimidinium ring of TDP is an intramolecular acid–base catalyst. This includes cofactor activation[53] and carbonyl addition/elimination of substrates and products, presumably with the 1′,4′-imino tautomer acting as a proton acceptor.[171,172]

3.1.3.3. Stereochemical Considerations for PDC. On the basis of the acid quench/NMR intermediate studies[154] and independent steady-state kinetic and carbon kinetic isotope effect studies on PDC variants,[162,167,170,173–175] a likely stereochemical course for PDC catalysis is presented in Scheme 35. The substrate-derived carboxylate of LTDP accommodates in a "carboxylate pocket" (formed by Glu473, Asp27, and His113) resulting in a perpendicular orientation in accord with a least motion-maximum overlap mechanism.[10] The experimental observation that Glu50 as the chemical trigger of the 4′-amino group of TDP is mandatory for both formation of LTDP and product release favors an (*S*)-configuration of LTDP. This places the 4′-amino/imino function of the cofactor in close proximity to the C2α-hydroxyl group where it can function locally as a base. Protonation of C2α after decarboxylation can be supported by the Glu-Asp-His triad and would occur from the face from which CO_2 is expelled. This will give (*R*)-HETDP. In line with this suggestion, optically active HETDP has been isolated from the related TDP enzyme pyruvate dehydrogenase[176] that was later shown to be the (*R*)-stereoisomer.[177] When a resonance is taken to exist between the carbanion and the enamine, the enamine intermediate will be formed as the (*E*)-enantiomer.

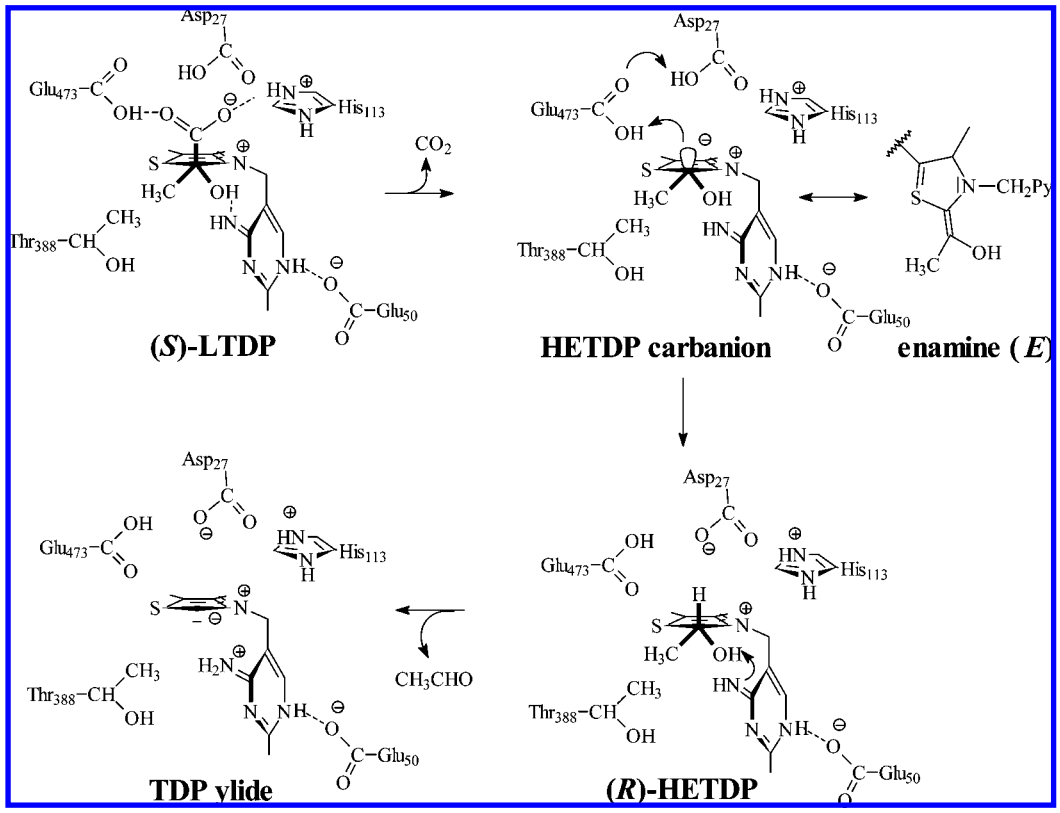
3.1.3.4. Acid-Quench Studies on TDP Intermediates in Other Pyruvate Processing Enzymes. The acid quench/^{1}H NMR approach has been applied to analysis of covalent TDP intermediates in other TDP enzymes that act on pyruvate. The NMR-based kinetic intermediate studies on human and bacterial pyruvate dehydrogenase complex (PDHc, see Scheme 15) were consistent with half-of-the-sites reactivity.[154,156,178] This supports the recent proposal of a "proton wire" that synchronizes the two remote active sites of a protein dimer by reciprocal proton transfer between both TDP molecules, with concomitant alternating cofactor activation/deactivation.[179,180]

NMR studies also revealed aspects of the regulatory mechanism of the human multienzyme complex,[181] where the activity is controlled by reversible phosphorylation of three serine side chains.[182,183] The authors reported that phosphorylation of serine 264, which is located at the entrance of the substrate channel, impairs substrate binding as well as coupling with the E2 component.[181]

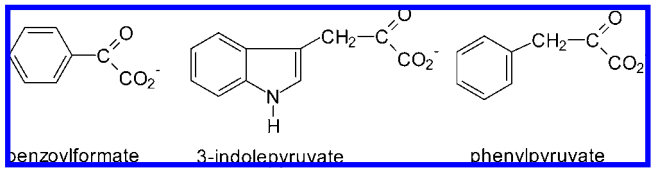
In an analysis of the mechanism of acetohydroxyacid synthase (AHAS, see Scheme 15),[158] a key enzyme in the biosynthesis of branched-chain amino acids,[184] specific side chains could be identified that mediate substrate specificity and determine the stereospecificity of the product, as was seen with PDC. The kinetic data suggested there is a proofreading step in the carboligation of the acceptor ketoacid to the enamine that could explain the exceptionally high (60-fold) acceptor substrate specificity of the AHAS isozyme II for 2-ketobutyrate compared to pyruvate.[138,158]

At the true steady-state of pyruvate oxidase from *Lactobacillus plantarum* (*Lp*POX), a TDP-dependent flavoenzyme that converts pyruvate, O_2 and inorganic phosphate to CO_2, H_2O_2 and acetyl phosphate,[185] LTDP, HETDP, and TDP were isolated as intermediates. This made it possible to estimate rate constants for formation of LTDP, decarboxylation, and the oxidation of HETDP by the flavin.[154,159] The AcTDP intermediate could only be detected when *Lp*POX was reacted with pyruvate and O_2 in the absence of phosphate. The inability to detect it at the true steady-state

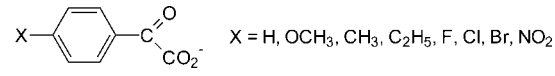
Scheme 35. Stereochemical Course of Catalysis in PDC from *Z. mobilis*



Scheme 36



Scheme 37



indicates that AcTDP may not be an intermediate in the course of acetyl phosphate formation.[159] In order for it to be on the pathway, it would have to account for less than 1% of intermediate species, an unlikely prospect. It is likely, however, that *Lp*POX generates acetylphosphate in a one-electron reaction involving a conjugate of inorganic phosphate-HETDP as free radical intermediate.

3.1.3.5. Acid-Quench NMR Studies on Covalent TDP Intermediates Derived from Aromatic Keto Acids. A mechanistically important group of TDP-dependent decarboxylases converts aromatic 2-ketoacids to the corresponding aldehydes. These include benzoylformate decarboxylase (BFD),[100,101,186] indolepyruvate decarboxylase (IPDC),[187,188] and phenylpyruvate decarboxylase (PhPDC).[189,190] The physiological substrates are shown below (Scheme 36).

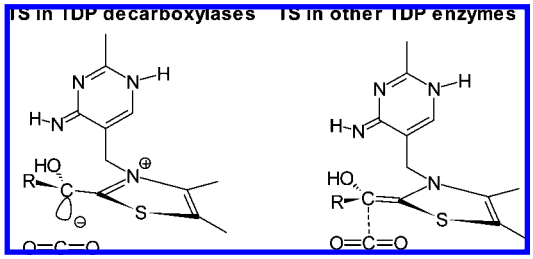
The reaction sequences and patterns of covalent on-pathway TDP intermediates correspond to those in Scheme 15 for pyruvate decarboxylase. The covalent predecarboxylation and postdecarboxylation intermediates deriving from benzoylformate and 3-indolepyruvate have been isolated at the steady state of indolepyruvate decarboxylase,[191] an enzyme with broad specificity that includes benzoylformate, *para*-substituted benzoylformates, pyruvate, and other 2-keto acids.[192] Depending on the substrate employed, the predecarboxylation intermediates are either 2-mandelyl-TDP (MTDP, substrate BF) or 2-(3-indolyl-(3)-lactyl)-TDP (IndLTDP, substrate Ipyr). Decarboxylation of these gives 2-(1-hydroxybenzyl)-TDP (HBnTDP, substrate BF), or 2-(1-hydroxy-2-indolyl-(3)-ethyl)-ThDP (IndHEThDP, substrate Ipyr), respectively. Owing to the low pK_a of the intermediates' substrate-derived carboxylic acid ($\approx$ 0.2), both MTDP and IndLTDP decarboxylate off the enzyme at pH 0.75 with $t_{1/2} \approx 40$ min at 30 °C[191] in agreement with results of studies on chemically synthesized materials.[123]

Steady-state kinetics and NMR analysis of wild-type IPDC and active site variants with BF, Ipyr, and pyruvate as substrates indicates that there is a common stereochemical course for TDP-dependent decarboxylases (Scheme 35).[191] The kinetic data in conjunction with the known structures of PDC,[54] IPDC,[188] PhPDC,[190] and BFD[100] suggest the predecarboxylation intermediates (TDP conjugates of ketoacids) are all of the (*S*)-configuration at C2α. Protonation of the central carbanion/enamine will give the (*R*)-form in a retention mechanism (the change in nomenclature is irrelevant). As noted elsewhere in this review, mandelylthiamin loses CO_2 and readily undergoes fragmentation, while MTDP in enzymes does not fragment.[191] This is consistent with the proposed role of protonation or oxidation in trapping the carbanion/enamine ahead of fragmentation.[121,169] MTDP and HBnTDP can be also detected at the steady-state of BFD wild-type and variants (K. Tittmann, unpublished).

3.1.3.6. Substituent Effects in TDP Decarboxylases and Their Interpretations. IPDC converts *para*-substituted benzoylformates to the corresponding aldehydes (Scheme 37).[192]

Analysis of the steady-state kinetic parameters reveals that electronic rather than steric effects of the *para*-substituents

Scheme 38



determine the enzymatic reaction rate.[192] A plot of log ($k_{cat}^{para\text{-X-BF}}/k_{cat}^{BF}$) versus the substituent constant σ_p[193] result in two independent linear plots with opposite sign (positive for electron-donating substituents, negative for electron-withdrawing substituents). The authors reasoned that the different electronic characteristics of electron-donating and electron-withdrawing substituents will have opposite effects on various transition states.[192]

NMR-based analysis of covalent TDP intermediates (*p*-X-MTDP and *p*-X-HBnTDP) that are formed during conversion of different *para*-substituted benzoylformates could confirm this hypothesis and give valuable insights into the electronic nature of the transition states of decarboxylation and of carbonyl addition/elimination in the course of substrate binding and product release.[191] When an electron-donating group replaces H at the *para* position, the decarboxylation of the tetrahedral *para*-X-MTDP intermediate becomes almost completely rate-determining. Electron-withdrawing substituents lead to selectively impaired substrate binding and product release. The large destabilizing effect of electron-donating substituents upon the decarboxylation step is consistent with carbanion-like transition state development at C2α. A single step generating a delocalized enamine directly requires multiple changes of hybridization along with C−C bond breaking in the transition state, a process that has an inherently higher barrier according to Guthrie's no-barrier theory.[3]

This observation and the immediate mechanistic implications for TDP decarboxylases are intruiging because model studies on lactyl-thiazolium salts and LTDP[83,152] were interpreted to favor a more neutral transition state of decarboxylation (resembling the uncharged enamine/CO_2 pair) with considerable delocalization of the electron pair at Cα. This is consistent with the large decrease in the kinetic barrier that is observed in less polar solvents and which is proposed to be a consequence of the stronger destabilization of the dipolar reactant state. On the other hand, on-pathway protonation of C2α, a reaction uniquely catalyzed by decarboxylases could be facilitated when a pyramidal form at C2α is retained for postdecarboxylation intermediates in the active site environment with a very short lifetime (Scheme 38).

In enzymes that do not require protonation at C2α to give the final product that is released from TDP, trapping of the anion by electron transfer or electrophilic addition will replace protonation. In all cases, the planar structure is entirely compatible with the products that are formed.

As noted earlier, recent results suggest that separation of CO_2 is facilitated by deactivation of the carbanion by protonation, electrophilic attack, or electron transfer.[122]

The substrate binding and product release steps are also sensitive to the *para* substituent, but the dependence is opposite to that observed for the decarboxylation reaction. For carbonyl addition–elimination, electron-withdrawing substituents (*p*-Br, *p*-NO_2) slow the reaction, whereas electron-donating substituents (*p*-OCH_3) have no effect. The transition state of product release presumably involves a carbocation/carbanion pair as sketched below (Scheme 39). Thus, electron-donating groups would stabilize the positive charge of the carbocation.

Alternatively, electron withdrawing-substituents can stabilize the post-decarboxylation intermediate inductively since addition of *p*-NO_2−BF to the enzyme generates a long-lived enamine (Scheme 40).[155]

3.1.4. Acid Quench-NMR Studies in Transketolase

The approach has been applied to the detection of covalent carbohydrate-TDP conjugates isolated from transketolase during catalysis.[154,194,195] The chemical shifts of the C6′-H singlets of the different covalent intermediates (see Scheme 16) allow an unambiguous assignment (δ ppm: C6′-H: X5P-TDP 7.35, F6P-TDP 7.34, DHETDP 7.31, TDP 8.01) and quantitative determination of the relative concentrations of the intermediates (Figure 6).

When only a donor ketose such as xylulose 5-phosphate (X5P) or fructose 6-phosphate (F6P) is present, catalysis of transketolase is restricted to the donor half-reaction (reversible covalent binding of the sugar to C2 of TDP, and cleavage of the resultant adduct to the DHETDP enamine, see Scheme 16).

The NMR-based analysis of covalent intermediates of the donor-half-reaction revealed that under equilibrium conditions, the initial X5P-TDP and F6P-TDP intermediates are highly populated on the enzyme.[154,195] Surprisingly, the DHETDP intermediate was not detectable (donor X5P) or barely detectable (donor F6P). This suggests that the reverse reaction, carboligation of the 3-carbon or 4-carbon aldose to the enamine, is significantly faster than the forward reaction (cleavage of X5P-TDP or F6P-TDP). Kinetic NMR experiments could test whether the donor-TDP intermediates are indeed in a rapid equilibrium with DHETDP or are kinetically stabilized on the enzyme.[195]

The DHETDP intermediate is very short-lived in the steady-state if both a donor ketose and an acceptor aldose are available.[195] Only unreacted TDP and the covalent donor-TDP derivative can be isolated. This implies that formation of the initial covalent intermediate is rate-determining. The minimal levels of DHETDP during turnover work against side reactions of the highly reactive enamine, such as oxygenation[196,197] or protonation of Cα and subsequent elimination of glycolaldehyde.

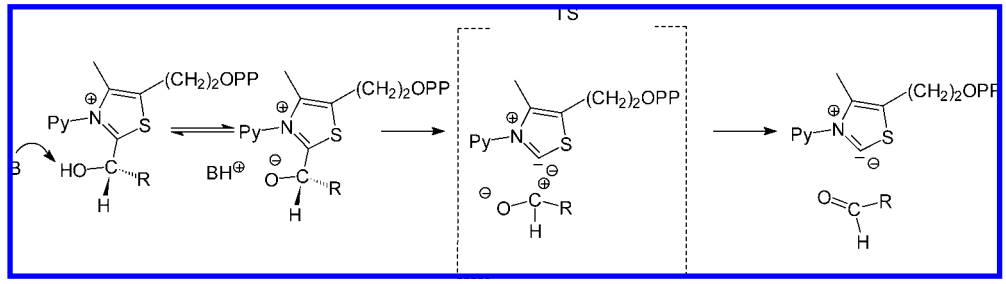
In contrast to the apparent stabilization of X5P-TDP and F6P-TDP conjugates on the enzymes under equilibrium conditions, the unnatural substrate β-hydroxypyruvate (HPA, Scheme 16) is rapidly and almost completely converted to CO_2 and the DHETDP enamine intermediate. This is because decarboxylation of the transient heavily favors the forward reaction.[154,194,195]

3.2. Direct Spectroscopic Observation of the Enamine Intermediate

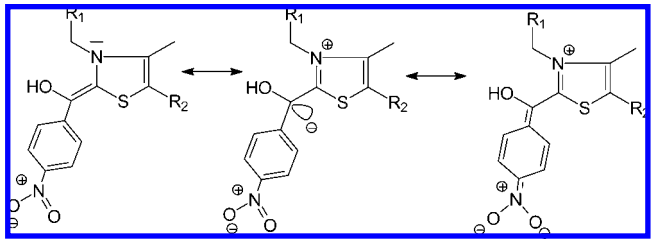
A carbanion/enamine is a central intermediate in all reactions catalyzed by TDP enzymes (Scheme 41).

Where the intermediate is a true enamine, it is a conjugated system. This will give rise to a UV–vis signal with a λ_{max} that depends on the conjugation between the thiazole and

Scheme 39



Scheme 40



the substrate substituent. The first direct spectroscopic observation of an enamine stabilized on a TDP enzyme was reported by Jordan and co-workers who observed that addition of the conjugate acid (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoate to PDC produced a visible absorbance at 440 nm. [198] As further evidence of the assignment, nonenzymic thiazolium compounds were prepared that generated similar spectral signals.[199,200]

Independent direct spectroscopic evidence has been provided for the formation of enamine intermediates from substrate analogues: *p*-NO_2−BF in BFD ($\lambda_{max}^{enamine} \approx 400$ nm),[155] and 3-indolepyruvate in IPDC ($\lambda_{max}^{enamine} \approx 380$ nm).[191] The absorption bands of 2-α-hydroxyalkyl-TDP enamines (HETDP in pyruvate-processing enzymes, DHETDP in transketolase) are blue-shifted when compared to bands from conjugated or aromatic substrates, appearing at around 295–300 nm. [59] The formation and decay of the DHETDP enamine at the active site of transketolase can be directly monitored by stopped-flow kinetics at 300 nm[194,195] (Scheme 42).

The equilibrium for a protonic state within an enzyme is related to equilibria within the bound state (consider the cases of citrate syntase and mandelate racemase where much higher apparent pK_a values are overcome). The ability of TDP enzymes to stabilize carbanion/enamine intermediates at the active site requires an environment that promotes the ionization of Cα-H for HBnTDP whose pK_a is 15.4 in water.[201]

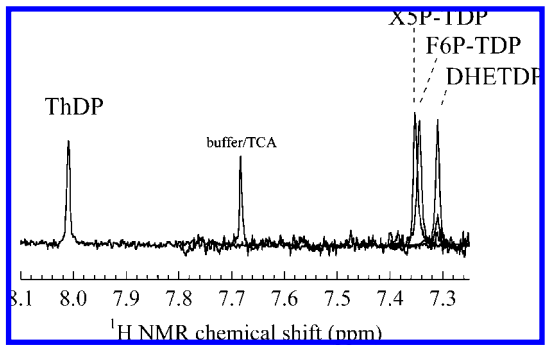


Figure 6. ^{1}H NMR-based (C6′-H fingerprint region) detection of covalent carbohydrate-derived TDP-conjugates in TK at 0.2 M acid.

When apo-PDC is exposed to exogenous racemic HBnTDP at pH 6.0, the compound binds to the active site and partitions between releasing benzaldehyde and forming the enamine.[201] The apparent pK_a suppression by >9 units indicates that the catalytic power of TDP enzymes resides not only in the chemical nature of the cofactor itself, but is also by the active site environment provided by the protein.

Jordan proposed that the lower pK_a might rather be induced by a low dielectric constant in the active site since catalytic groups cannot affect this thermodynamic parameter.[201] Reconstitution of *apo*-PDC with the cofactor analogue thiochrome diphosphate, whose fluorescence emission maximum is highly sensitive to the polarity of its environment, led to an estimate of the dielectric constant of the TDP binding site of PDC of 13–15. The authors reasoned that the low polarity of the active site could sufficiently account for the observed 10^9-fold rate acceleration of decarboxylation of the intermediate on the enzyme. However, this presents a problem for desolvation of the highly water-soluble substrate. Ionization of TDP itself normally is considered as proceeding via the dipolar ylide, which would be highly disfavored in a nonpolar environment. While desolvation after addition of TDP to the substrate could account the energy needed for such a process.[78] Alternatives involving coupled equilibria with groups on the protein or cofactor[53] can also account for such an apparent perturbation.

3.3. Spectroscopic Detection, Electronic and X-ray Structures of Radical Intermediates in TDP Enzymes

There are three major classes of enzyme within the TDP enzyme superfamily that catalyze oxidation of the central enamine intermediate. In some cases these involve radical TDP intermediates. These include 2-keto acid:ferredoxin oxidoreductases, 2-keto acid oxidases, and 2-keto acid dehydrogenase multienzyme complexes.

3.3.1. Pyruvate:Ferredoxin Oxidoreductase

Pyruvate:ferredoxin oxidoreductase (PFOR)[202] shuttles two electrons from the oxidative decarboxylation of pyruvate to a ferredoxin via a chain of Fe_4S_4 clusters (Figure 7)[203] to produce acetyl-CoA.

Addition of pyruvate to PFOR results in the formation of a stable TDP-derived radical intermediate that produces a signal in the continuous wave X-band EPR spectrum centered at $g = 2.006$.[202] The use of ^{14}C-labeled substrate analogues provided compelling evidence that the radical is a postdecarboxylation TDP intermediate (attributed to an AcTDP radical generated upon $1e^-$ oxidation of the enamine, Scheme 43), because radioactivity is tightly bound to the enzyme

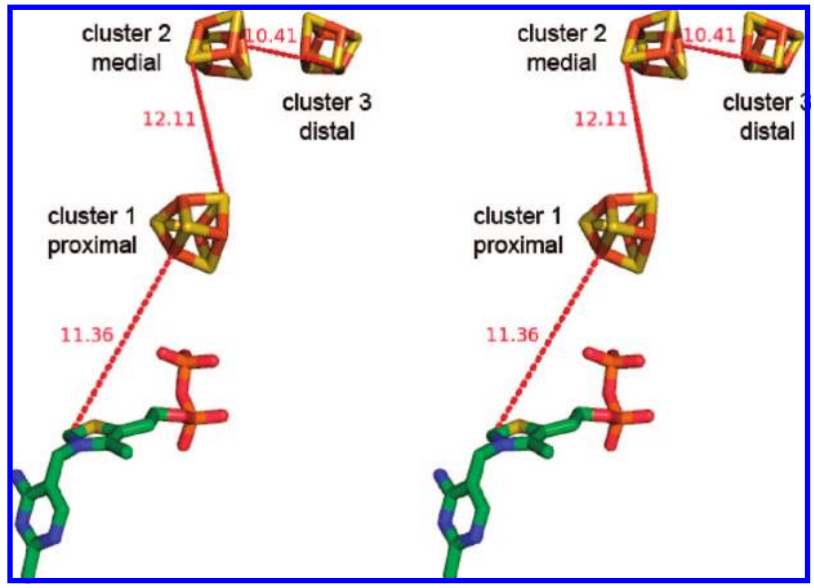
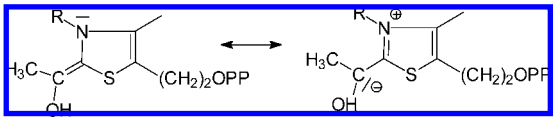


Figure 7. Relative orientation of cofactors in PFOR in stereoview.

Scheme 41



when $^{14}C3$-pyruvate ($^{14}CH_3COCO_2^-$) is added, whereas no enzyme-bound radioactivity was detectable from ^{14}C-pyruvate ($CH_3CO^{14}CO_2^-$).[204]

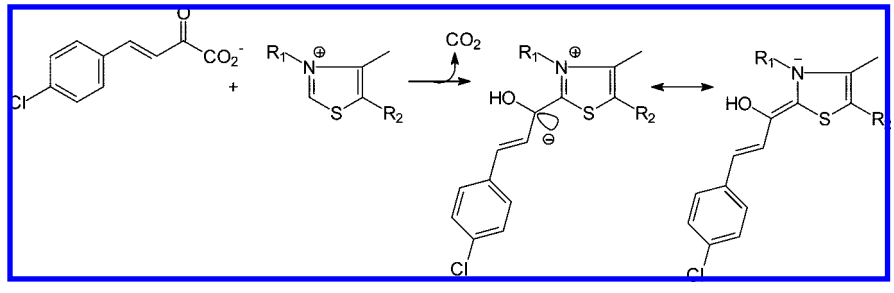
It was shown that the unpaired spin of the radical is at least partially distributed over the pyruvate-derived atoms as the coupling pattern and hyperfine splitting of the EPR signal were clearly dependent on the number of protons attached to C-3 of pyruvate.[205] Later, Ragsdale reported that upon reaction with pyruvate-d_3 ($CD_3COCO_2^-$) the EPR signal of the TDP radical in PFOR becomes narrower and almost featureless. (An unpaired electron will show different coupling with protons with $I = ½$ compared to deuterons, which have $I = 1$ and a smaller nuclear moment.) He concluded that the unpaired spin is in substantial electronic interaction with the substrate methyl, presumably through hyperconjugation.[206] A rigorous spectroscopic examination and concomitant spectral simulation of the HETDP-radical in PFOR employing different isotopologs (^2H,^{13}C, ^{15}N) of the substrate and the cofactor TDP by X-band and D-band EPR undertaken by Reed and Ragsdale provided the electronic structure of the radical intermediate.[207] The g-values and $^{14}N/^{15}N$ hyperfine-splitting were indicative of a planar π-type hydroxyethylidene-TDP radical in which there is considerable delocalization of the unpaired spin onto the thiazolium moiety (Figure 8).

Furthermore, the ^1H-hyperfine splitting of the substrate-derived methyl and the ^{13}C-hyperfine splittings of TDP's C2 as well as that of the carbonyl carbon of pyruvate suggested an intermediate state between a Cα O-protonated and O-deprotonated forms, most likely resembling a structure with an intramolecular hydrogen-bonding interaction of C2α-OH, presumably with the N4′ of the aminopyrimidine (Scheme 44).

In striking contrast to those results and mechanistic conclusions, the X-ray structure of the free radical intermediate trapped in the active site of PFOR from *Desulfovibrio africanus* (Figure 9) has been interpreted to correspond to a σ/n-type AcTDP radical in which the thiazolium ring is markedly bent, indicating a loss of any aromaticity.[208] The structure also suggests that the thiazole might be stabilized in an unusual tautomeric form in which a proton from the 4-methyl group is labilized and attached to the sp^3-hybridized C5. In addition, the C2−Cα bond was found to be exceptionally long (1.86 Å) and prompted the authors to propose that the unpaired spin resides mostly at the atoms of the acetyl moiety or at TDP's C2 rather than being delocalized over the thiazole ring.[208]

The differences in the proposed electronic structures of the TDP radical in PFOR in solution[207] and in the crystalline state[208] indicate that the structure is still uncertain. Frey has noted[209] that if the thiazole is present as a tautomer with a double bond to the exocyclic carbon[208] conducting the experiments in D_2O will result in the incorporation of D at position C4. Also, an important caveat with respect to the X-ray structural studies is that the crystallization and substrate soaking were carried out at pH 9.0, conditions where the

Scheme 42



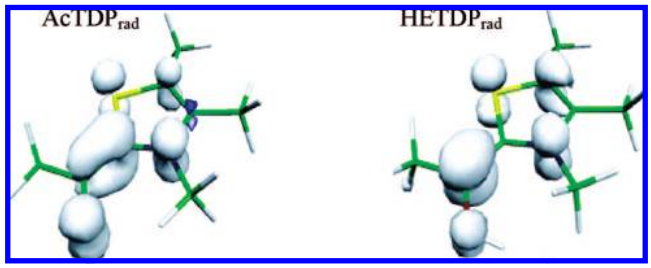
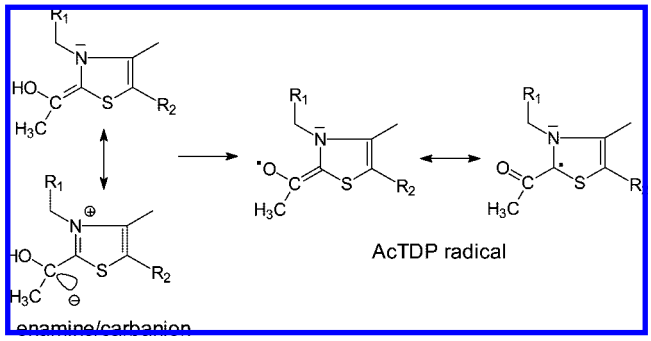


Figure 8. Isosurface plots of the calculated spin densities of AcTDP and HETDP radicals in PFOR (reprinted with permission from ref 207. Copyright American Chemical Society).

Scheme 43



thiazole is known to undergo ring-opening and rearrangements.[210] Most desirably, a rigorous EPR spectroscopic characterization and simulation of the HETDP/AcTDP radical in the crystalline phase using different substrate and cofactor isotopologs, together with structural analysis from a sample at neutrality, could provide more definitive evidence on the hypothesis of a σ/n-type AcTDP radical in PFOR.

There remains another problem in the chemistry of PFOR. It is established that only one electron is transferred from the enamine to one neighboring Fe_4S_4 cluster.[204] This is corroborated by detecting a spin-coupling interaction of the HETDP radical with one Fe_4S_4 cluster. This was later identified to be from the medial, second cluster,[132,206] while the second electron is insulated at TDP until the substrate CoA enters the catalytic stage.[211] It is not clear why only one Fe_4S_4 cluster remains reduced in the absence of CoA. Ragsdale sugegsts that utilization of binding energy of CoA to stabilize the transition state is unlikely to account for the 10^5-fold rate enhancement of transfer of the second electron: addition of the CoA analogue desulfo-CoA has binding energy that differs only by a few kJ/mol from that of CoA but does not result in the achievement of the rate enhancement observed for CoA.[211,212]

Two alternative modes of action can explain the special role of CoA in electron transfer (Scheme 45). CoA itself could transfer an electron to an FeS cluster, yielding a CoA thiyl radical that could collapse with the HETDP radical in a biradical mechanism. This is consistent with the observation that binding of CoA in the absence of pyruvate reduces one FeS cluster. However, the suspected CoA thiyl radical could not be detected.[206] On the other hand, CoA could attack the HETDP radical (as an AcTDP-type radical) as a nucleophile, forming a transient CoA-AcTDP radical anion. This will have a lower redox potential compared to the more positively charged HETDP ↔ AcTDP radical[207] and increase the driving force for transfer of the second electron.[211,212]

3.3.2. Pyruvate Oxidases

A common reaction of all TDP-dependent pyruvate oxidases (POX) is the transfer of two electrons resulting from the oxidation of the HETDP enamine to a neighboring FAD cofactor. In *Lactobacillae*, such as *L. plantarum* or *L. casei*, POX converts pyruvate in the presence of oxygen and inorganic phosphate to acetyl phosphate, CO_2 and H_2O_2, the latter resulting from the reoxidation of the two electron-reduced flavin by oxygen.[185,213,214]

$$CH_3\text{-}CO\text{-}CO_2^- + H_2PO_4^- + O_2 + H^+ \rightarrow CO_2 + H_2O_2 + CH_3\text{-}CO\text{-}OPO_3H^-$$

In the acetate-producing POX from *E. coli* (*Ec*POX), the reduced flavin is unreactive toward oxygen, directly shuttling both electrons from the HETDP enamine to the membrane-bound carrier ubiquinone 8 (Q_8).[215–218]

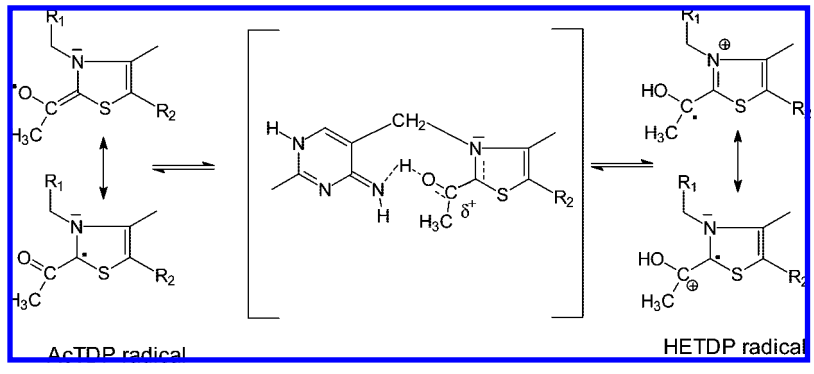
$$CH_3\text{-}CO\text{-}CO_2^- + OH^- \rightarrow CO_2 + CH_3\text{-}CO_2^- + \text{TDP-}Ec\text{POX-FADH}_2$$

$$\text{TDP-}Ec\text{POX-FADH}_2 + Q_8 \rightarrow \text{TDP-POX-FAD} + Q_8H_2$$

In the X-ray structure of *Lp*POX,[219,220] the two cofactors TDP and FAD are held in close proximity, with the dimethylbenzene moiety of the flavin's reactive isoalloxazine ring pointing directly toward the thiazole of TDP (Figure 10).

The structural architecture of the active site rules out a direct carbanion mechanism involving a transient covalent Cα-HETDP−N_5-FAD adduct or a hydride transfer from HETDP to the pteridine of the isoalloxazine. In line with this assumption, the FAD analogue 5-deaza-5-carba-FAD (5d-FAD), a good hydride acceptor, is not reduced in the course of catalysis, although it binds to the enzyme with affinity similar to that of FAD. [221] The HETDP enamine is readily formed in the 5d-FAD reconstituted enzyme,[222]

Scheme 44



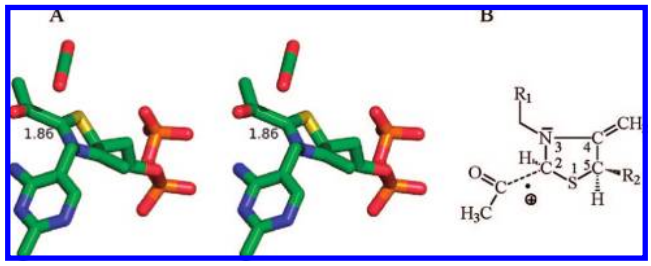


Figure 9. X-ray structure (A, stereoview) and postulated chemical structure (B) of AcTDP radical in PFOR.[208]

suggesting that a stepwise electron transfer in *Lp*POX with a transient HETDP (or AcTDP) - FAD semiquinone (FAD_{sq}) produces a radical pair. Even so, FAD radicals, which would give rise to typical visible absorbance (the blue FAD semiquinone absorbs at $\lambda_{max} \approx 500–600$ nm), could not be detected at the steady-state of *Lp*POX[223] or in the course of the reductive half-reaction[159] (Scheme 46) under anaerobic conditions where the flavin is reduced in a single turnover and will not be reoxidized.

This observation could not rule out a stepwise transfer of the two reducing equivalents because a kinetic stabilization of the putative biradical intermediate ($HETDP_{rad}$-FAD_{sq}), and hence the ability to detect it, will critically depend on the rate constants of its formation and decay. If indeed two electrons are transferred in a stepwise manner from the HETDP enamine to FAD in *Lp*POX, the lack of observation of radical intermediates implies that the transfer of the second electron is markedly faster than that of the first.[223]

The kinetic analysis of the reductive half-reaction was done with a sample that contained phosphate, which is not only a substrate of *Lp*POX, but also stabilizes the enzyme thermodynamically. As seen in the reaction scheme, phosphate could have no immediate role in the reductive half-reaction, and would enter after electron transfer and formation of AcTDP. However, when *Lp*POX was allowed to react with pyruvate *in the absence of phosphate* and oxygen under single turnover conditions, the time-resolved absorbance spectra indicated the transient formation of a kinetically stabilized FAD_{sq} radical intermediate with a lifetime of a few seconds that is not seen in the presence of phosphate (Figure 11).[159]

Stopped-flow kinetics and quenched-flow NMR revealed that phosphate has a unique effect on the transfer of the second electron, with at least a 10^2-10^3-fold rate enhancement.[159] The rates of all preceding steps of catalysis (substrate binding, decarboxylation of LTDP, and transfer of the first electron) are virtually independent of phosphate.[159] As well, an examination of the two kinetic phases (radical formation, radical decay) observed in the absence of phosphate according to Marcus theory[224] suggests that the radical decay is a true electron transfer reaction, whereas the formation of the $HETDP_{rad}$-FAD_{sq} radical pair is gated by preceding adiabatic reaction steps (formation and decarboxylation of LTDP).[159] These findings pose a question about the specific role of phosphate for facilitating transfer of the second electron from the HETDP radical to the FAD semiquinone. Several viable mechanisms could account for the observed rate enhancement of electron transfer in the presence of phosphate.

Phosphate could have an effect on the redox potential of the flavin to change the driving force of the reaction, and/or on the ability of *Lp*POX to stabilize FAD radicals thermodynamically. Redox potentiometric studies revealed, however, that both the midpoint potential ($E_m^{FAD} \approx -65$ mV) and the thermodynamic stabilization of FAD_{sq} on the enzyme ($\approx$ 55–60%) do not depend on phosphate.[159] Binding of phosphate to the enzyme could also induce a conformational change to bring TDP and FAD closer together. In initial experiments, fluorescence resonance energy transfer (FRET) experiments on POX reconstituted with the fluorescent TDP analogue N3′-pyridyl-TDP (FRET donor) and FAD (FRET acceptor) indicated that a putative structural rearrangement is an unlikely scenario as similar FRET efficiencies were observed in the presence and absence of phosphate.[159] Later, X-ray crystallographic snapshots of *Lp*POX at different catalytic stages[76] corroborated the FRET results.

Alternatively, the phosphate could bind in the active site between TDP and FAD in a way that its orbitals could serve in through-bond electron transfers, which are more favorable than through-space jumps or via solvent molecules. This mode of action is implausible for *Lp*POX because phosphate should then facilitate transfer of both electrons that arise from oxidation of the enamine, which is clearly not the case.

An X-ray structural study on *Lp*POX has provided evidence that phosphate indeed binds to the enzyme before electron transfer, at the HETDP enamine stage (Figure 12), but not in a position that would permit its orbitals to be involved in intercofactor electron transfer. Instead, it may attack the AcTDP radical (formed in the next step) to give a transient phosphate-AcTDP anion radical (Scheme 47)[76,159] as suggested for CoA in case of PFOR.

The negative charge of the resulting phospho-AcTDP radical anion adduct would certainly make it a low potential intermediate. This would increase the intrinsic driving force of the electron transfer reaction. In order to achieve the 10^2-10^3-fold rate acceleration, the potential of the radical adduct would have to be 330 mV lower than that of the AcTDP radical. This is based on the assumption that a change of the intrinsic driving force solely accounts for the change of the ET rate and all other Marcus parameters remain unaltered.

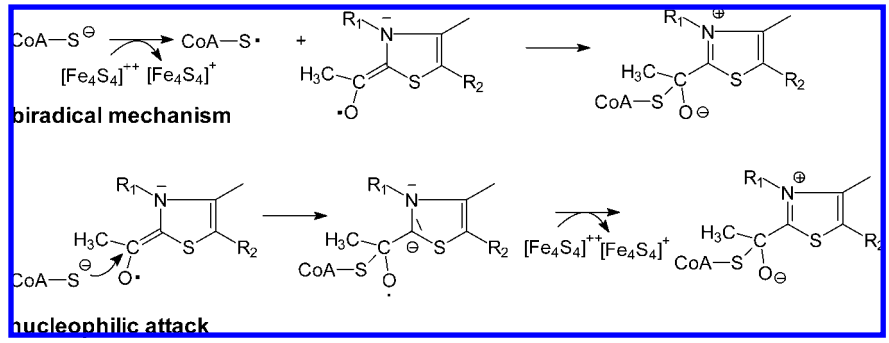
In an alternative mechanism (Scheme 48), phosphate might add to the O-protonated HETDP cation radical. The radical adduct would then undergo homolytic fragmentation to the *C*2 ylide and the O-protonated acetylphosphate radical. Transfer of the second electron generates O-protonated acetylphosphate, which then loses its proton to complete the reaction.

EPR spectroscopic studies on *Lp*POX (K. Tittmann, S. Ragsdale, G. Reed, unpublished)[225] show a strong coupling between the TDP-based radical and the FAD_{sq}. When the AcTDP-HETDP radical is generated on the enzyme in the absence of FAD_{sq} with different substrate isotopologs the calculated electronic structure of the thiamin radical is virtually identical to that of $HETDP_{rad}$ in PFOR. That is, a π-type radical in an intermediate state between an O-protonated (HETDP cation radical) and conjugate base form (AcTDP radical). In *Ec*POX, no evidence for the kinetic stabilization of TDP-based radicals has been obtained.[159]

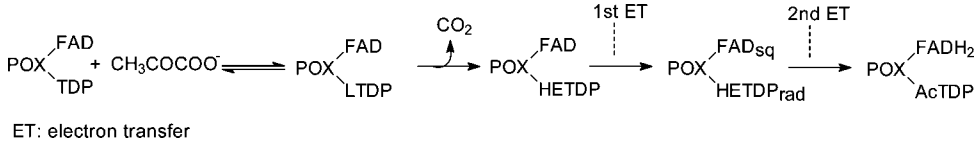
3.3.3. Pyruvate Dehydrogenase Complex

The pyruvate dehydrogenase multienzyme complex (PDHc) catalyzes the irreversible conversion of pyruvate, CoA and NAD^+ into CO_2, NADH, and acetyl-CoA, the latter serving as the main precursor for the TCA cycle and the biosynthesis of fatty acids and steroids.[226–228] The complex consists of at least three major components in multiple copies: thiamin diphosphate (TDP)-dependent pyruvate dehydrogenase (E1),

Scheme 45



Scheme 46



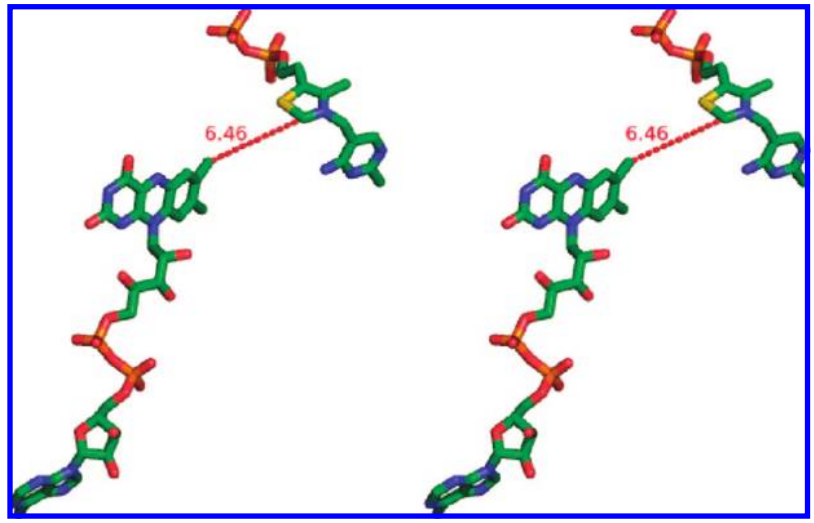


Figure 10. Relative spatial orientation of cofactors in POX (pdb code 1pox).

dihydrolipoamide transacetylase (E2) containing covalently bound lipoyl groups, flavoenzyme lipoamide dehydrogenase (E3), and as a unique component of mammalian PDHc, the E3-binding protein (E3BP).

Unlike PFOR and POX, where the HETDP enamine is oxidized by neighboring groups of the same protein component, the enamine formed in PDHc-E1 reductively acetylates a flexible lipoamide "swinging arm" of a neighboring E2 component.[229,230] X-ray structural studies suggest that the lipoyl-lysine of E2's lipoyl domain is likely to penetrate a suitably organized substrate channel of E1 and to reach the active site.[231] Yet, the underlying chemical mechanism by which the substrate channeling between E1 and E2 occurs is unknown. Reed proposed that reduction and acyl transfer are coupled reactions and occur simultaneously involving a covalent lipoamide-HETDP intermediate. On the other hand, studies of Frey provide strong evidence for formation of AcTDP, which serves as an electrophilic acceptor for attack

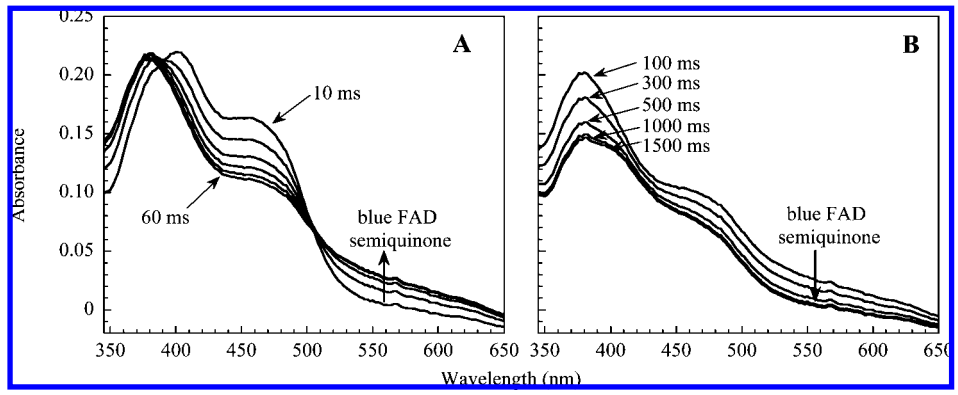


Figure 11. (A, B) Kinetic analysis of the reductive half-raction of POX in the absence of phosphate.[159]

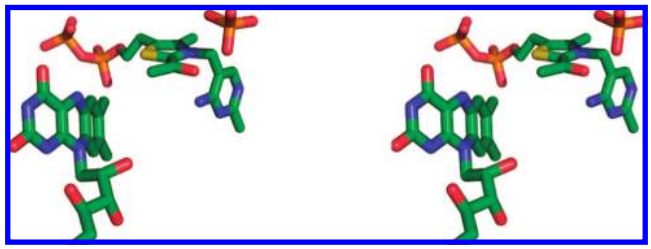


Figure 12. X-ray structure of FAD, HETDP enamine, and phosphate in POX prior to electron transfer.[76]

by dihydrolipoate (Scheme 49).[153] When AcTDP is generated on the enzyme using the substrate analogue 3-fluoropyruvate, AcTDP has been shown to be a chemically competent acetyl group donor to dihydrolipoamide.[85] There is also evidence that acid–base catalysis is required for reductive acetylation to occur.[232] This could be achieved by an active site histidine protonating the dithiolane ring.[233]

As seen in the scheme, the covalent adduct of HETDP and lipoamide would be a common intermediate invoked in both mechanisms. It is possible that AcTDP is in equilibrium with that intermediate in a carbanion mechanism. It may not necessarily be formed via pathway B and the observation of AcTDP cannot rule out the carbanion mechanism. Observation of a π-type HETDP radical at the steady state of PDHc from *E. coli* is consistent with pathway B (K. Tittmann, unpublished).[225] However, the corresponding lipoamide thiyl radical has so far escaped detection and oxygen appears to react with the HETDP enamine during formation of TDP-based radicals. While this observation needs further substantiation, it demonstrates that HETDP radicals can be stabilized on the enzyme. Therefore, an alternative mechanim for reductive acetylation involves bond formation between both radicals (Scheme 50), circumventing transient formation of the AcTDP radical.[225]

3.3.4. Acetohydroxyacid Synthase and Glyoxylate Carboligase

Both acetohydroxyacid synthase (AHAS) and glyoxylate carboligase (GCL) belong to the POX familiy and contain a flavin cofactor in addition to TDP. However, in these enzymes the flavin does not appear to be involved in catalysis and may serve a structural purpose only.[56,234–236]

AHAS isozymes from *E. coli* engage in off-pathway intracofactor electron transfer from the HETDP enamine to FAD in competition with the physiological reaction, the carboligation of the enamine with the acceptor ketoacid. [237] The analysis of time-resolved absorbance spectra under aerobic and anaerobic conditions was not indicative of kinetically stabilized radical intermediates. However, in the case of GCL there is even no reduction of the flavin.[237,238]

3.4. Different Protonation States of the TDP Aminopyrimidine in the Course of Catalysis and Spectroscopic Signatures

CD analysis of models and on different TDP enzymes suggest that the aminopyrimidine of TDP exists in three different forms: the 4′-aminopyrimidine (AP), the N1′-protonated 4′-aminopyrimidinium (APH^+), and the 1′,4′-iminopyrimidine (IP). The IP form gives rise to a positive CD spectral band centered at 300–310 nm, while a negative band at 330 nm is assigned to intramolecular charge transfer between AP and the thiazolium ring.[171,172,239–241] In some enzymes, the latter signal is detectable in the ground state, while in others it is an exclusive reporter of the Michaelis complex of negatively charged substrates and substrate analogues, respectively. The APH^+ form in enzymes has not been reported. There is evidence that all three forms of the aminopyrimidine (AP, APH^+, and IP) can interconvert on TDP enzymes. Whereas tetrahedral intermediates are very likely to exist as the IP form,[171] the central enamine/carbanion is predicted to be in the APH^+ and/or AP state[172] (see Scheme 51 below for PDC). Thus, it is conceivable that the active site of a TDP enzyme controls the internal equilibria of the aminopyrimidine so that all three forms are accessible.

Besides using the corresponding physiological substrates of a TDP enzyme, a convincing approach for the spectral assignments uses the phosphonate substrate analogue of pyruvate, methyl acetylphosphonate (MAP).[103,242] The compound forms a covalent bond with C2 of TDP that parallels that of the true substrates, but the adjacent Cα-P bond of the addition intermediate is more stable than the C−C bond of the substrate. As a result, processing stops at this intermediate, allowing for structural, kinetic, and thermodynamic analysis of a normally transitory catalytic state. The formation of the tetrahedral intermediate analogue gives rise to a circular dichroism signal attributed to the cofactor's IP form that is centered around 300 nm.

4. Structural Studies on Covalent TDP Intermediates on Enzymes

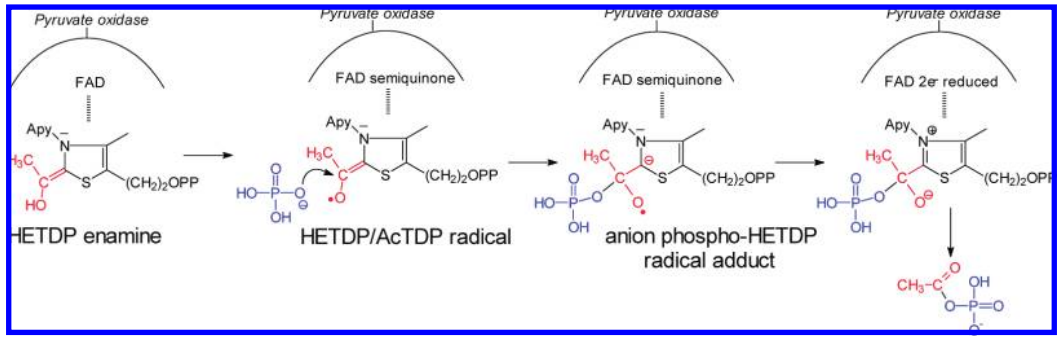
The large size of TDP enzymes (≥120 kDa) has restricted high-resolution structural studies almost exclusively to X-ray crystallography. The structures of yeast transketolase,[55,243] pyruvate oxidase from *L. plantarum*,[219,220] pyruvate decarboxylase from yeast[54] and *Z. mobilis*, pyruvate dehydrogenase E1 component from *E. coli*,[244] *H. sapiens*[245] and *Bacillus stearothermophilus*,[179] pyruvate:ferredoxin oxidoreductase from *Desulfovibrio africanus*,[203] benzoylformate decarboxylase,[100] benzaldehyde lyase,[246] 1-deoxy-D-xylulose 5-phosphate synthase,[247] N2-(2-carboxyethyl)arginine synthase,[248] oxalyl-CoA decarboxylase,[249] branched-chain keto acid dehydrogenase E1 from various organisms,[231,250] phenylpyruvate decarboxylase from *Azospirillum brasilense*,[190] indolepyruvate decarboxylase from *E. cloaceae*,[188] acetohydroxyacid/acetolactate synthase from yeast,[236] *Klebsiella pneumonia*[251] and *Arabidopsis thaliana*[252] and branched-chain keto acid decarboxylase KdcA from *Lactococcis lactis* were solved,[253] while other structures are in progress.

In contrast to protein structures in the resting state, there is only scarce structural information available for those with covalent TDP intermediates at the active site. However, in the recent years, several key covalent intermediates were structurally characterized by cryocrystallography. In pyruvate oxidase and transketolase, multiple intermediates were trapped in the active site and characterized. The structure of the AcTDP radical in PFOR[208] has been already discussed in the section on radical TDP intermediates (Figure 13).

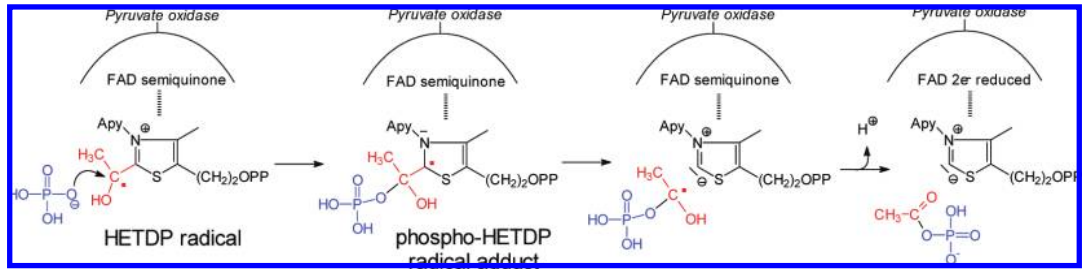
4.1. Structures of Covalent TDP Intermediates in Pyruvate Oxidase

A systematic NMR-based analysis of the intermediate distribution of pyruvate oxidase wildtype and several variants revealed that the initially formed tetrahedral predecarboxy-

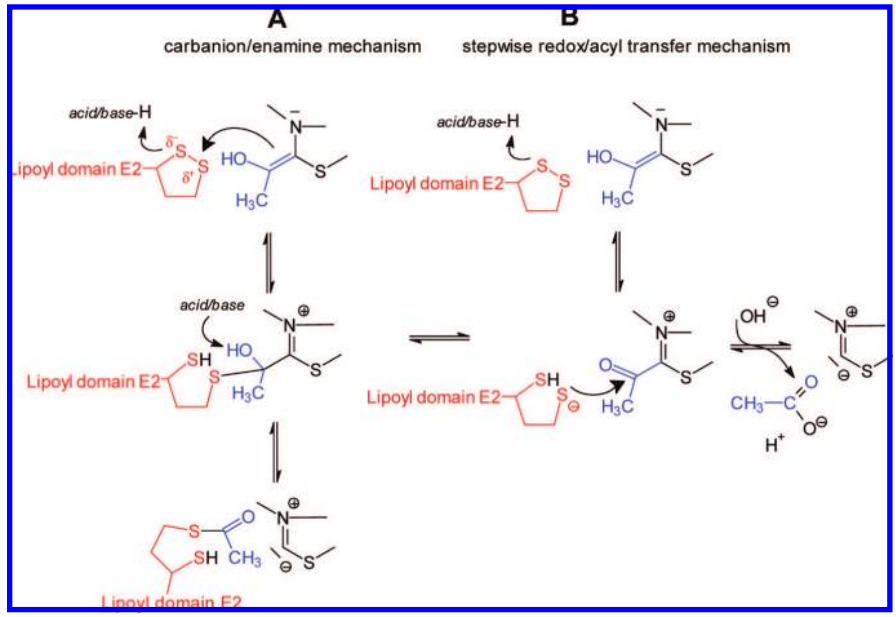
Scheme 47



Scheme 48



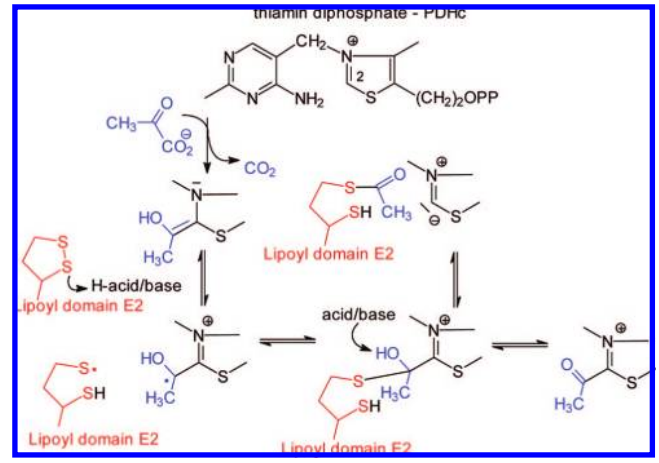
Scheme 49



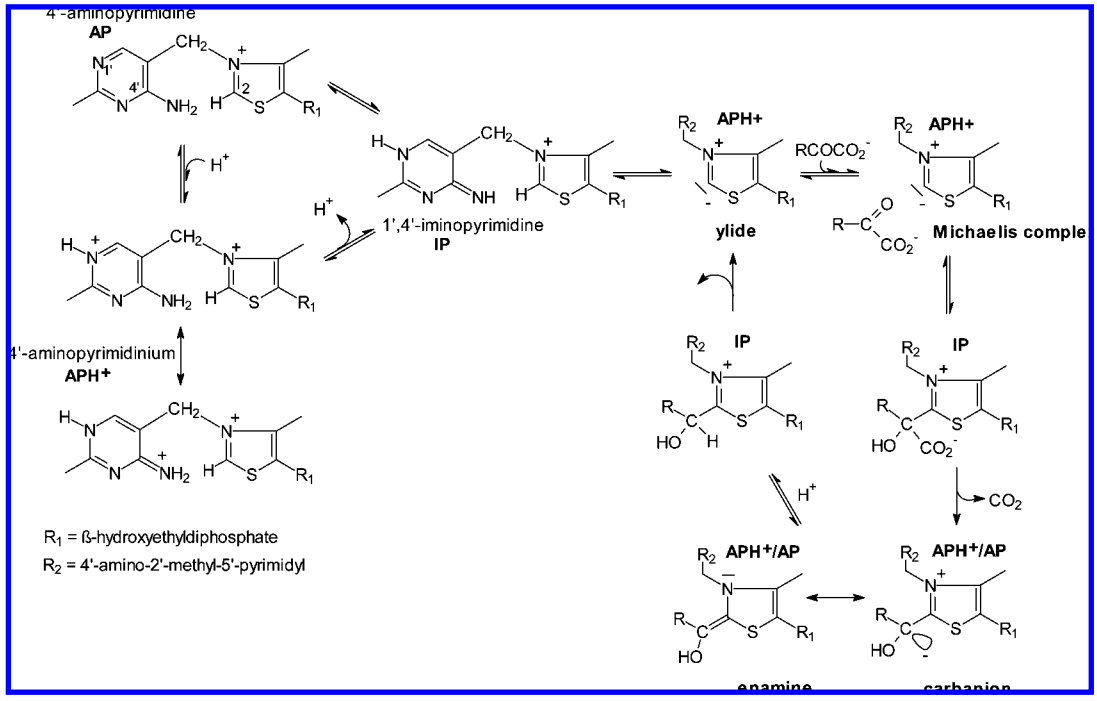
lation intermediate, LTDP, accumulates at the steady-state of POX variant Phe479Trp with high occupancy (≥90%).[76] In all other TDP enzymes examined so far, LTDP is a low-populated and short-lived intermediate.[154,156,158,159,191] Hence, this POX variant constitutes an important example for a reliable structural characterization of the LTDP intermediate at the active site of a TDP enzyme. A high-resolution X-ray crystallographic analysis of POX Phe479Trp at steady-state revealed LTDP to be formed at the active site as the (*S*)-enantiomer (Figure 14),[76] a configuration that had been predicted for related pyruvate-processing enzymes on the basis of NMR studies.[154,191]

The LTDP intermediate forms multiple interactions with the aminopyrimidine part of the cofactor, active site side chains, and the backbone in a three-point binding motif (carboxylate pocket, substrate carbonyl/hydroxyl pocket, methyl pocket). The carboxylate leaving-group forms hy-

Scheme 50



Scheme 51



drogen bonds to the side chain of Glu483 as well as to the backbone amides of Gly35 and Ser36. The structure shows the carboxylate is solvated, providing clear evidence against mechanisms of catalysis that require desolvation to destabilize LTDP. The Cα-OH of LTDP is held in place by interactions with Gln122 and the 4′-amino group of the cofactor, the latter being most likely in the 1′,4′-imino tautomeric form. The substrate-derived methyl moiety is accommodated in a hydrophobic pocket consisting of Val394, Phe121, and Trp479. The scissile Cα-C(carboxylate) bond of LTDP is almost perpendicular to the cofactor's thiazolium ring plane, suggesting a stereoelectronically controlled decarboxylation reaction in a maximum overlap mechanism in which the incipient electron pair arising from decarboxylation can be delocalized onto the thiazolium ring, as predicted from studies of cofactor conjugates.[73]

The carboxylate leaving group is near a microenvironment of mainly hydrophobic nature (Ile480, Trp479, Phe122), which would stabilize the subsequent enamine: CO_2 pair. Another intriguing feature of the covalent intermediate is a slight out-of-plane distortion of the C2(thiazolium)−Cα bond

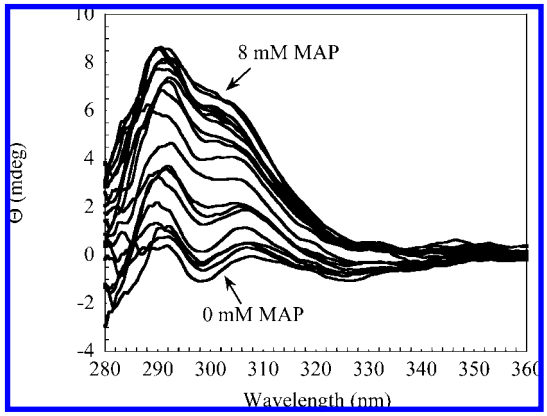


Figure 13. Titration of pyruvate analogue MAP to *Hs*PDH-E1 results in the formation of the 1′,4′-imino form of the phosphono-LTDP intermediate analogue.[156]

connecting the planar thiazolium moiety with the substrate carbonyl carbon (torsional angle C5-S1-C2-Cα ≈ 173°). This out-of-plane distortion implies that some strain is exerted on the intermediate that is eventually relieved upon decarboxylation and formation of the planar enamine (see below).[73,83,152]

NMR intermediate studies had revealed that when the FAD cofactor in POX is in a fully reduced state, pyruvate cannot transfer electrons to the flavin after binding to TDP and decarboxylation of LTDP. This generates a "stable" HETDP enamine intermediate on the enzyme. Soaking of reduced POX crystals with pyruvate and structure determination gave valuable insights into the structure of the catalytic postdecarboxylation state in POX.[76] The electron density of HETDP (see below Figure 15) clearly favors a planar, enamine-like form of HETDP in the (*E*)-configuration with sp^2-hybridization of C2α rather than a localized carbanion, which would have a pyramidal C2α hybridization.

The apparent stabilization of the enamine on the enzyme is interesting: in order to avoid protonation of Cα, the enzymic (crystallized at pH 6.0) environment lowers the pK_a by at least 11–12 units compared to water where the pK_a ≈ 17. The apparent low polarity of the active site suggests a thermodynamic stabilization of the uncharged enamine but should be considered in terms of all the equilbria at the active site.[201,254] On the other hand POX will necessarily lack the specific proton relay of TDP decarboxylases needed for protonation of C2α and subsequent aldehyde release.

The unexpected presence of phosphate close to HETDP at the active site prior to electron transfer and the mechanistic implications for a redox-coupled phosphorolysis reaction in POX have been discussed in the section dealing with radical TDP intermediates. As noted earlier, covalent binding of pyruvate to TDP gives a strained LTDP intermediate. In the course of decarboxylation, this strain is relieved, yielding a planar, unstrained intermediate (Figure 15, right panel).

When phosphate-dependent POX is reacted with pyruvate and oxygen in the absence of phosphate, catalysis does not

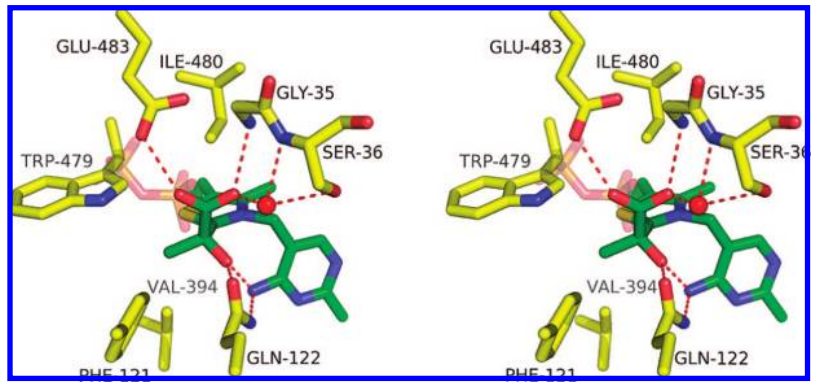


Figure 14. Structure of LTDP at the active site of *L. plantarum* POX variant Phe479Trp (stereoview).

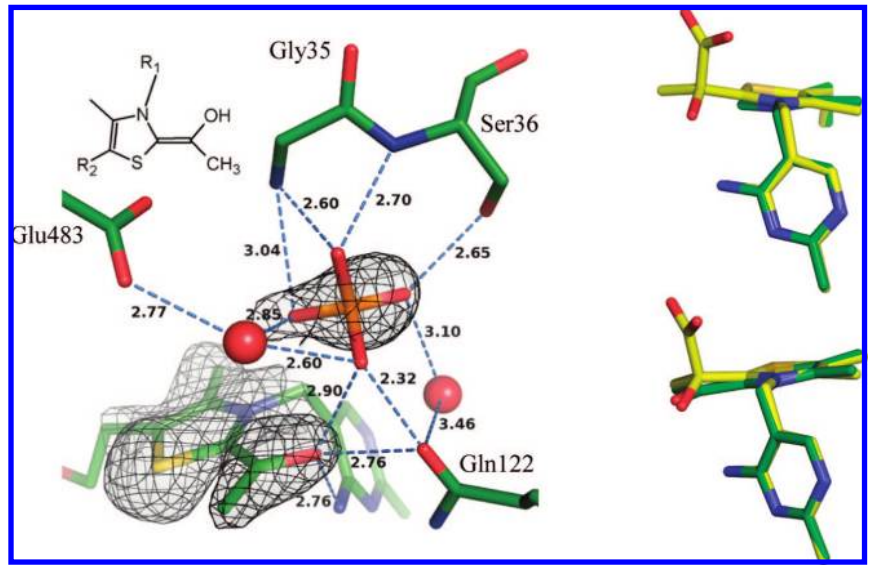


Figure 15. X-ray structure of the planar HETDP enamine in POX (left panel). Superposition of the resting state and LTDP (LTDP formation, upper right) and LTDP and HETDP (decarboxylation, lower right) in POX.

proceed past the AcTDP intermediate stage. X-ray crystallographic analysis of POX under these conditions revealed AcTDP to be in the keto form on the enzyme (Figure 16). There were no indications of the formation of an internal hydrate or a tricyclic carbinolamine form of AcTDP in POX as observed in solution.[76,87]

Remarkably, with the exception of the thiazolium part of the cofactor itself and the side chain of Gln122, there is no structural rearrangement of the active site detectable in the course of catalysis, indicating that the active site is poised for catalysis in the resting state.

4.2. Structural Studies on Covalent TDP Intermediate Analogues

Although it is most desirable to study high-resolution structures of true on-pathway TDP intermediates as in case of POX (see above), the complexity of reaction patterns of TDP enzymes involving a multitude of different in part even inherently unstable intermediates renders a reliable structural characterization of a defined single (individual) covalent intermediates in most instances virtually impossible. Therefore, cocrystallization of a certain TDP enzyme with stable intermediate analogues or soaking with substrate analogues turns out to be a promising approach in order to gather structural information for defined individual catalytic states. For TDP enzymes, several chemically synthesized intermediate and substrate analogues/transition state analogues were successfully cocrystallized with the protein (Scheme 52). These analogues include thiazolone-TDP and phosphono-LTDP, which mimic either the postdecarboxylation enamine state/transition state for decarboxylation (thiazolone)[70,255] or the predecarboxylation state (phosphono-LTDP)[71,103] of pyruvate-processing enzymes.

Recently, Leeper developed a new type of postdecarboxylation analogue, in which N3 of the cofactor's thiazole is replaced by a carbon and C2 is bonded to an enzyme-specific substrate fragment. [253] An example is 2-(1-hydroxyethyl)-3-deaza-TDP as a mimic for the conjugate acid of the HETDP enamine with a tetrahedral C2α atom (C2α is unlikely to ionize at the active site as N3 is absent and resonance stabilization is thermodynamically unfavorable). (Figure 17).

Thiamin thiazolone-DP was successfully cocrystallized with transketolase,[256] bacterial pyruvate dehydrogenase complex E1 component[257] and branched-chain keto acid dehydrogenase complex E1 component,[258] providing detailed structural insights into the slightly different organization of the active site and cofactor in the postdecarboxylation state versus the resting state. The structure of the predecarboxylation analogue phosphono-LTDP was determined both in complex with bacterial pyruvate dehydrogenase complex E1 component (cocrystallization with the chemically synthesized material)[74] and POX (generated on the enzyme upon soaking crystals with methylacetylphosphonate).[76] As exemplified

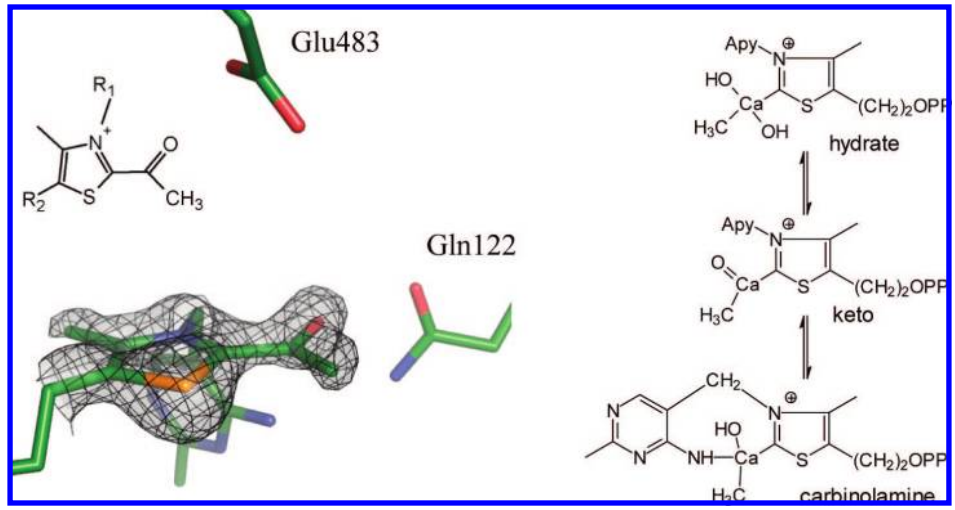
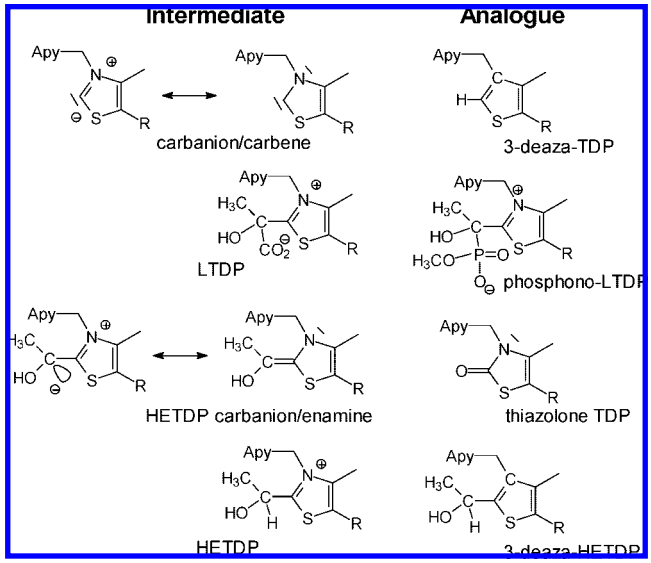


Figure 16. Structure of AcTDP keto form in POX (left panel) and chemical structures of AcTDP in models (right panel).[76]

Scheme 52. Intermediates and Corresponding Analogues Used in Crystallographic Studies on TDP Enzymes



below for POX, the structure of phosphono-LTDP is a good model for the native LTDP intermediate and exhibits similar interactions at the active site, with structural features such as stereochemistry at Cα and intramolecular strain with an out-of-plane distortion of the C2−Cα bond.[76]

All corresponding atoms in the two intermediates occupy virtually identical positions. The additional methoxy group of phosphono-LTDP points toward the exit of the active site without any steric clashes. Remarkably, the C2−Cα bond deviates from planarity by about 5–7° as also observed for LTDP in POX. In a related structural study on pyruvate dehydrogenase multienzyme complex E1, Furey and Jordan observed an even more pronounced out-of-plane distortion in phosphono-LTDP cocrystallized with the enzyme (11°).[74]

4.3. Structural Studies on Transketolase

There have been two independent studies on covalent TDP intermediates in transketolase. Schneider reported that soaking yeast TK with the artificial substrate β-hydroxypyruvate generates a long-lived DHETDP postdecarboxylation intermediate, the only report of a such an intermediate at this time.[259] According to the calculated electron density maps, the DHETDP intermediate is highly likely to adopt a planar enamine-like form in the (*E*)-configuration, similar to that subsequently reported for the HETDP enamine in POX.[76] There was no indication of structural rearrangements taking place in the course of catalysis. Later, the initial covalent complexes of TK from *E. coli* with donor ketoses xylulose 5-phosphate (see below Figure 18) and fructose 6-phosphate were characterized.[195]

As shown above, the intermediate is firmly held in place by multiple hydrogen-bonding and electrostatic interactions with active site side chains and the aminopyrimidine part of the cofactor. The sugar's scissile C2−C3 bond is directed perpendicular to the thiazolium ring plane, consistent with a maximum overlap mechanism.[73] Also, there is similar stereochemistry of intermediate formation to that of (*S*)-LTDP in POX, consistent with a common substrate binding mode in thiamin enzymes.[195]

Remarkably, the C2−C2α bond, which connects the thiazole with the substrate carbonyl, deviates from planarity by about 25–30° (the same holds true for the F6P-TDP adduct). Additional density functional theory (DFT) calculations and mutagenesis studies revealed that this out-of-plane distortion appears to be enforced by intrinsic features of the cofactor and productive interactions of the intermediate with active site groups. Both in X5P-thiamin and X5P-thiazolium models, full optimization of the structures resulted in a slight out-of-plane distortion of the C2−C2α bond although to a lesser extent (10°) than observed on the enzyme (25–30°). Hence, there should be additional factors that induce further strain in the intermediate, presumably hydrogen-bonding interactions of the sugar's OH groups with side chains. In accordance with this hypothesis, a His26Ala/His261Ala double variant, where at least three conserved hydrogen bonds cannot be formed, did not generate the initial tetrahedral intermediate.[195] DFT calculations show that the scissile C2−C3 bond is selectively weakened in the covalent X5P-TDP intermediate (in the conformation observed on the enzyme), while all remaining C−C bonds of the sugar chain exhibit the typical length of carbon−carbon single bonds (Figure 19).

With the exception of a slightly different binding of the intermediate's phosphate moiety, the structure of TK in covalent complex with F6P is very similar to that in complex with X5P.[195]

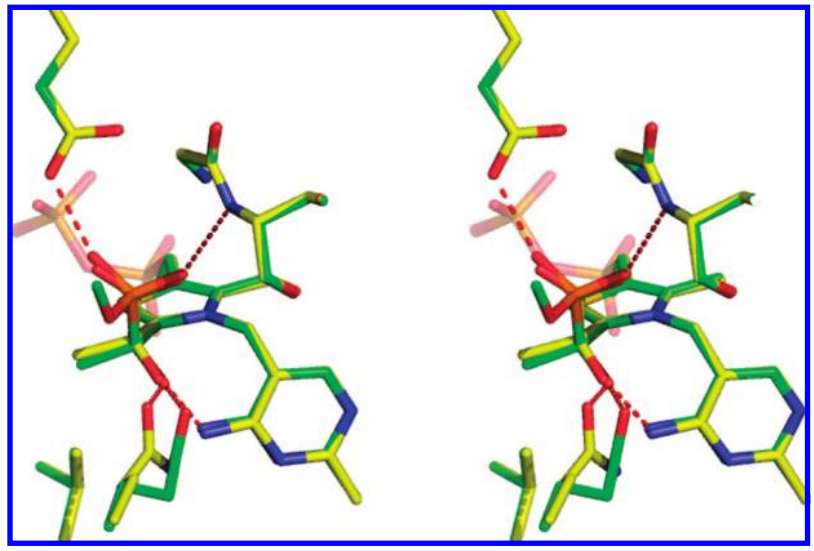


Figure 17. Superposition of POX trapped with LTDP intermediate (yellow) and with phosphono-LTDP intermediate analogue (green) in stereoview.[76]

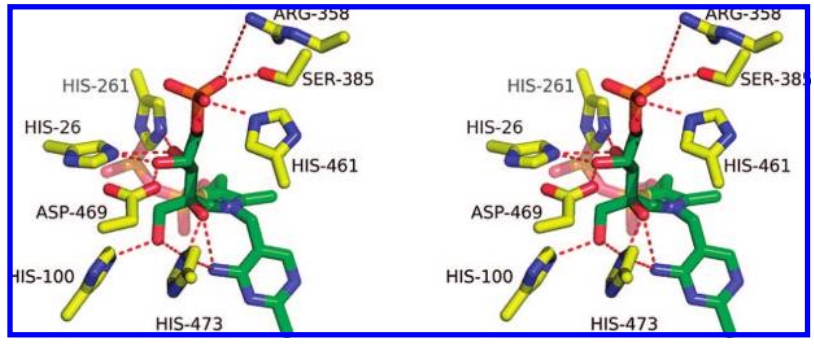


Figure 18. Structure of the X5P-TDP intermediate trapped in the active site of TK from *E. coli* (stereoview).[195]

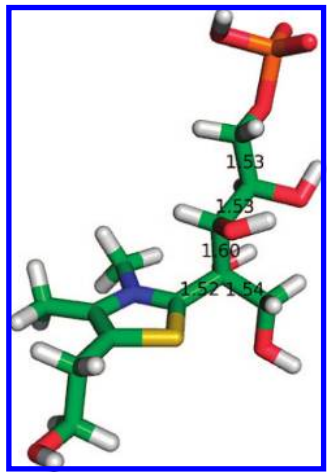


Figure 19. Partial DFT optimization of the X5P-TDP adduct (here a simplified thiazolium model) suggests a selective weakening of the scissile C2−C3 bond in the conformation of the intermediate formed at the active site.

4.3.1. Relations between Transketolases and Transaldolases/Aldolases

In cellular sugar metabolism, the enzyme superfamilies of aldolases/transaldolases and transketolases interconvert sugar–phosphates of different lengths. One major difference between the two enzyme families is that the TDP-dependent transketolases selectively cleave the C2−C3 bond of a certain carbohydrate in order to transfer a 2-carbon dihydroxyethyl unit. Aldolase/transaldolases, which form a Schiff base adduct of a lysine and the sugar substrates, initially catalyze bond fission between the adjacent C3−C4 bond followed by either release of the remaining C3 moiety or promote its transfer to another sugar substrate. What is the source of the selective bond fission in each enzyme family? In both cases, the C2 keto carbon of the ketose is attacked. As discussed above, transketolases enforce a conformation of the sugar-TDP adduct at the active site that results in a selective weakening of the C2−C3 bond of the intermediate.

A structural comparison of a lysine-fructose-bisphosphate adduct trapped in an aldolase[260] and the F6P-TDP intermediate in transketolase[195] reveals a different binding mode in the two enzymes with a different configuration at the stereocenter C2α that is the carbonyl carbon C2 of the sugar (Figure 20). If the amino nitrogen of the lysine and the C2 of TDP are compared as nucleophiles, the covalent attack onto the C2 carbon of the fructose substrate occurs from different sides in the two enzymes and results either in an extended sugar conformation (TK, yellow) or U-type bent conformation (aldolase, green).

Despite this clear indication of different binding modes in transketolases and aldolase/transaldolase, the origins of the different bond fission specificity have yet to be discovered.

4.4. Pyramidal Carbanion or Planar Enamine?

Structural studies on postdecarboxylation intermediates in transketolase[259] and pyruvate oxidase[76] have clearly suggested a planar enamine-type conformation with consid-

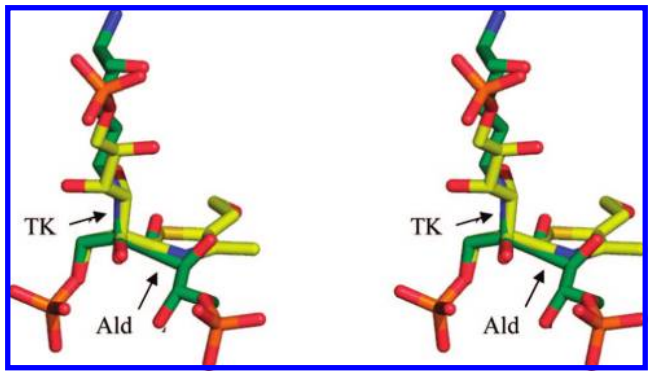


Figure 20. Superposition (stereoview) of a covalent lysine-fructose-1,6-bisphosphate adduct in an aldolase (green) and the covalent fructose 6-phosphate-TDP adduct in transketolase (yellow). For the sake of clarity, the diphosphate and aminopyrimidine part of TDP were deliberately omitted. The scissile bonds (C2−C3 in transketolase, C3−C4 in aldolase) are indicated. For superposition the spatial coordinates of 1-O, 2-O, and Cα were used.

erable conjugation of the electron pair formed in the course of decarboxylation with the π-electrons of the thiazolium. In line with these two observations, a planar postdecarboxylation intermediate was also reported for a thermophilic branched-chain keto acid dehydrogenase.[250] However, in recent studies on human branched-chain keto acid dehydrogenase[258] and a bacterial oxalyl-CoA decarboxylase,[261] evidence was presented for nonplanar postdecarboxylation intermediates. In both instances, the electron density maps suggest a nonplanar intermediate state in the crystal with a slight out-of-plane distortion of the C2−Cα bond. However, it is very difficult at the structural resolution obtained in both studies to differentiate between a Cα-protonated form (the conjugate acid of the carbanion/enamine) and the carbanion/enamine itself. It is therefore unclear whether the carbanion could be kinetically stabilized in a way that would allow a structural characterization. At this point, the protonated alternative seems the more reasonable interpretation.

4.5. Insights from Structural Studies and Unifying Concepts of Catalysis in TDP Enzymes

The crystallographic studies on covalent intermediates in TDP enzymes have revealed expected chemical features supporting the accepted mechanisms of TDP catalysis. However, they have also revealed some unpredicted properties. Covalent addition of a ketoacid or carbohydrate substrate to C2 of TDP results in a tetrahedral substrate-TDP conjugate with a strictly conserved stereochemistry at C2α of the intermediate (the (*S*)-form of LTDP serving as a prototype). The C2 carbanion of TDP attacks the substrate carbonyl in all TDP enzymes from the same side. Differences in the specificity result from enzyme-specific interactions of the protein with the different leaving groups of the covalent intermediates and with their C2α-linked substrate-specific substituent (methyl group of pyruvate, hydroxymethyl group in carbohydrates).

In a broader sense, TDP enzymes have evolved a three-center binding mode with specific interactions for the leaving group, the substrate carbonyl group and the substrate substituent. In all cases, the scissile C2α−C2β bond of the intermediate is directed almost perpendicular to the thiazolium ring plane, making conjugation of the elimination-derived incipient carbanion with the π-electrons of the thiazolium possible in terms of a stereoelectronically controlled reaction with maximum orbital overlap.

The elimination reactions of CO_2 in decarboxylating enzymes and of sugar–phosphates in TK, appear to be facilitated by strain relief as the initially formed tetrahedral intermediate, which is strained, relaxes to a planar, unstrained enamine. Whereas there is slight strain detectable in ketoacid-TDP conjugates, as in POX and PDH, it is much more pronounced in tetrahedral intermediates formed in TK with a 25–30° out-of-plane distortion of the C2-C2α bond and a selective weakening of the scissile C2α−C2β bond. Furthermore, the predecarboxylation intermediate, LTDP, is not desolvated on the enzyme strongly arguing against proposals that require desolvation as a driving force for decarboxylation.

Finally, in every case studied so far, the substrate carbonyl oxygen is in close proximity to the exocyclic 4′-amino group, suggesting a mechanism in which carbonyl addition/elimination of the substrates/product is facilitated by intramolecular acid–base catalysis that involves groups on the aminopyrimidine.

After elimination of the corresponding leaving groups, most TDP enzymes (except for decarboxylases) stabilize the true enamine. In that way, undesired off-pathway protonation of Cα in these TDP enzymes can be minimized.

4.6. The Catalytic Power of TDP Enzymes - Transition State Stabilization versus Reactant State Stabilization

As discussed in the previous chapters, TDP enzymes have evolved numerous catalytic strategies for an effective transition state stabilization of the different elementary catalytic steps. On the other hand, virtually all reactant states are stabilized thermodynamically on the enzyme with respect to the nonenzymic system evidenced most impressively for instance by the high affinity of TDP enzymes for the enamine analogue thiazolone-TDP and their ability to stabilize radical HETDP intermediates with life-times in the minutes range. This results in a significant reactant state stabilization that can be up to −30 kJ/mol in case of PDC or even more for other TDP enzymes with more complex reaction sequences.[12]

Reactant state stabilization is anticatalytic and this poses a question about the possible origins for this seemingly counterproductive property of TDP enzymes. According to Schowen,[12] there are factors that demand a reactant state stabilization because this guarantees stoichiometric binding of the cofactor to the enzyme rather than relying on availability of free TDP in solution. In addition, without reactant state stabilization, catalysis would have to be achieved by termolecular collisions (or even higher molecularity in case of multisubstrate TDP enzymes) of enzyme, TDP and substrate(s) or, alternatively, by numerous bimolecular collisions, an unlikely and inefficient prospect. In addition, some TDP enzymes are subject to regulation (e.g., PDHc and AHAS) and the underlying processes might interfere with forming catalytically relevant reaction complexes. Clearly, the catalytic power of TDP enzymes is based on a net transition state stabilization that offsets the necessary reactant state stabilization over and above. While doing so, TDP enzymes can accelerate reactions by a net factor of at least 10^{12} (correponding to 70 kJ/mol at 300 K) as in case of PDC although the potential true catalytic transition state stabilization may be - in view of the strong reactant state stabilization - even higher accounting, perhaps as high as 10^{21}.

In some enzymes, such as the acetylphosphate producing POX or PDHc with acetyl-lipoamide as a transient intermediate, these numbers could be even higher because the corresponding model reactions are so slow or thermodynamically unfavorable that they do not occur, even with heating. Thus, TDP enzymes constitute an enzyme superfamily with an exceptionally high catalytic proficiency despite the fact that the reaction sequences comprise chemically quite diverse elementary reactions that demand different catalytic strategies at different stages finally resulting in an efficient internal thermodynamic matching of all transition states (the Albery-Knowles theory[119]).

5. Why Thiamin?

The thiamin portion of TDP is unrelated to molecules in the world of proteins but carries characteristics found in nucleic acids. There are important instances where the interaction of thiamin with a nucleic acid is a key regulatory event.[262–264] The function of TDP within a protein is clearly unique, providing a reactive nucleophile in its C2-conjugate base that adds readily to carbonyl compounds that will ultimately be acyl carbanion equivalents. This is apparently the result of the ability of the C2α carbanion to become delocalized into the thiazolium functionality. The catalytic generation of such a species by decarboxylation is achieved with no other cofactor or protein side chain functional group. Thiamin is also stable and readily produced in edible plants, providing a dietary source for the core functionality.

5.1. Evolution

As we have seen, thiamin is structurally part of the world of nucleic acids and can function as a catalyst on its own, making it distinct from the proteins that now surround its TDP derivative in catalytic systems. It is clear that all proteins that utilize TDP as a cofactor have absolutely no activity in its absence and that thiamin's existence predates that of functional proteins. The presence of sulfur in the key catalytic functionality makes its presence a distinct possibility in the oldest catalytic systems.[265–267] Evolution of protein function would occur without the need for evolution of the structure of thiamin or TDP. All attempts to find functional variants on TDP have been unsuccessful.

5.2. The Chemical Basis of TDP's Catalytic Ability

The ability C2H to ionize and function as a nucleophile or leaving group with respect to carbonyl addition is the key part of its function. The pyrimidine permits a proton relay to an adjacent glutamate that allows the formation of the essential ylide. The kinds of reactions promoted by TDP enzymes involve small molecules that are held in a precise location by the formation of the covalent bonds to C2 of TDP.

5.3. Enzymes Enhance TDP's Catalytic Potential

TDP on its own has the potential to promote the reactions catalyzed by enzymes of α-ketoacids but it is ineffective. Enzymes provide a binding site for the substrate to overcome the entropy problem in a reaction of two free species. In addition, the ionization of TDP at C2 is promoted by a proton relay in the active site. Finally, the TDP conjugate will undergo reaction faster if an acceptor that competes with carbon dioxide is present. Enzymes can provide a proton, a carbonyl acceptor, or an oxidant to tame the carbanion so that carbon dioxide is able to diffuse beyond the reactive carbanion.

In our comparisons of the reactions of thiamin and its conjugates in solutions to those of the cofactor within the enzymes, we noted that pK_a values in the nonenzymic systems as the basis of calculation of equilibrium constants. However, we also emphasized that within an enzyme, equilibria involve proton transfers coupled to specific alterations within the active site, such as tautomerization of the cofactor and hydrogen bond formation, as well as conformational changes and electrostatic interactions that are coupled to the process. Our results show clearly that reactions involving TDP on a protein require consideration of the all the interacting entities. Our ability to calibrate the entire protein–cofactor-substrate catalytic system against the reactivity of the substrate−cofactor system quantitatively defines the role of the protein in catalysis in a way that cannot be approached in systems without covalent cofactor-derived intermediates. The recent observations of the enzymic intermediates from TDP by crystallography and NMR spectroscopy build upon the information from the nonenzymic intermdiates derived from thiamin and TDP in way that has not been achieved in any other system.

6. Concluding Remarks

There has been remarkable progress from key synergistic discoveries that have made thiamin and TDP enzymes fruitful areas of research based on the convergence of mechanistic and structural methods and broad interest. The discovery of TDP as a cofactor[23] and the clear elucidation of its catalytic function[61] are landmarks in both organic chemistry and biochemistry. These have led not only to the understanding of reactions, but to the development of vitamin therapy[20,21] and the synthesis of the most widely used cholesterol-lowering drug.[16] The parallel discovery of enzymic and nonenzymic reactivity in this system is unique, and more discoveries are appearing at a rapid pace. The tools of structure and reactivity analysis have matured exactly to the point where the major issues in understanding the function of TDP in proteins and the contrasting reactions of thiamin on its own have been established by experiment and analysis.

In a more specific sense, these ideas have shown that enzyme active sites provide the means for local mechanisms that routinely require preassociation, as in promoting the departure of CO_2.[122,125] These are not readily available in the absence of an enzyme. As we have noted, the observations by Jencks[128] and Schowen[129] in which the importance of having a catalyst associated with a reactant without the need for diffusion, had been limited to special cases in the study of organic reaction mechanisms. Those insights were applied for the consideration of the need of low-entropy substrates through enzymic association.[127] These can now be generalized to a wide variety of possibilities that are yet to be discovered.

7. Dedication

We dedicate this review to Professor Ronald Breslow in honor of the 50th anniversary of his demonstration of the formation of thiazolium ylides. From that discovery, he predicted the covalent intermediates derived from TDP that

would occur in enzymic catalysis. This set the stage for the fruitful study of TDP-derived intermediates and enzymes.

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