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Bioorganic Chemistry 33 (2005) 190–215
BIOORGANIC CHEMISTRY
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Minireview

Experimental observation of thiamin diphosphate-bound intermediates on enzymes and mechanistic information derived from these observations

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Received 21 December 2004
Available online 1 April 2005

Abstract

Thiamin diphosphate (ThDP), the vitamin B1 coenzyme, is an excellent representative of coenzymes, which carry out electrophilic catalysis by forming a covalent complex with their substrates. The function of ThDP is to greatly increase the acidity of two carbon acids by stabilizing their conjugate bases, the ylide/C2-carbanion of the thiazolium ring and the C2α-carbanion (or enamine) once the substrate binds to ThDP. In recent years, several ThDP-bound intermediates on such pathways have been characterized by both solution and solid-state (X-ray) methods. Prominent among these advances are X-ray crystallographic results identifying both oxidative and non-oxidative intermediates, rapid chemical quench followed by NMR detection of a several intermediates which are stable under acidic conditions, and circular dichroism detection of the 1′,4′-imino tautomer of ThDP in some of the intermediates. Some of these methods also enable the investigator to determine the rate-limiting step in the complex series of steps.
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doi:10.1016/j.bioorg.2005.02.001

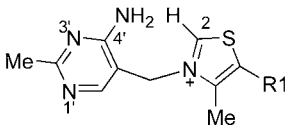
Keywords: Thiamin diphosphate; Ylide/C2-carbanion; C2α-carbanion or enamine; Circular dichroism; 1′,4′-imino tautomer; Pyruvate dehydrogenase complex; Yeast pyruvate decarboxylase; Benzoylformate decarboxylase; C2α-hydroxyethylthiamin diphosphate; C2α-lactylthiamin diphosphate

1. Introduction

Among the coenzymes derived from water-soluble vitamins, thiamin diphosphate (ThDP[1]) and pyridoxal phosphate are frequently used as examples of cofactors, which carry out electrophilic catalysis by forming covalent intermediates with their substrates. Pyridoxal phosphate gives rise to a variety of chromophoric intermediates with its substrates, which have been characterized over the years. Due to a fortuitous acid stability of several of the ThDP-bound covalent intermediates, which enable both their synthesis and chemical trapping (acid quench) for eventual detection, ThDP enzymes provide a wonderful opportunity and challenge to the enzymologist wishing to observe and ultimately to study the catalytic competence of such intermediates. In addition, due to the special environment on the enzyme, several of these ThDP-derived intermediates give rise to circular dichroism (CD) signals, not seen in the absence of the enzymes. During the past decades, the authors' laboratory has worked on three representatives of this large family of enzymes, yeast pyruvate decarboxylase (YPDC, Scheme 1), the pyruvate dehydrogenase multienzyme complex from *Escherichia coli*, especially its first ThDP-dependent subunit (PDHc-E1, Scheme 2), and (in a collaboration with scientists at Purdue and U. Michigan) benzoylformate decarboxylase (BFD). The goal of this review is to summarize data mostly from the authors' experience on these enzymes, and to quote relevant results on related enzymes from elsewhere. The organization will follow an order according to observations on the various putative intermediates, and at the outset the authors apologize to those whose contributions in this regard have been overlooked, it was totally unintentional. Several reviews on the general topic of ThDP enzymes are available [1–4], while a recent collected volume provides an overview of most types of enzymes mentioned in this review [5].

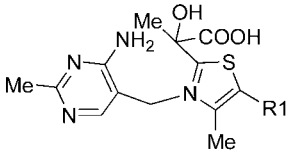
[1] *Abbreviations used:* ThDP, thiamin diphosphate; PDC, pyruvate decarboxylase; YPDC, yeast pyruvate decarboxylase; POX, pyruvate oxidase; TK, transketolase; PDHc, the pyruvate dehydrogenase multienzyme complex; PDHc-E1, the first (ThDP-dependent) subunit of PDHc; *E. coli*, *Escherichia coli*; PFOR, pyruvate:ferredoxin oxidoreductase; BFD, benzoylformate decarboxylase; BCOA, branched chain 2-oxoacid dehydrogenase complex; HEThDP, C2α-hydroxyethylthiamin diphosphate, the acetaldehyde.ThDP adduct; HBThDP, C2α-hydroxybenzylthiamin diphosphate, the benzaldehyde.ThDP adduct; LThDP, C2α-lactylthiamin diphosphate, the pyruvate.ThDP adduct; CD, circular dichroism.

Table of compounds discussed in this review

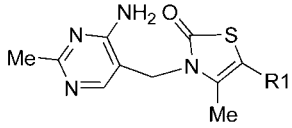


thiamin diphosphate (ThDP)

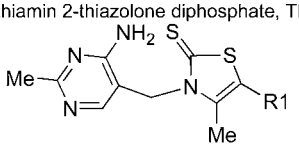
R1= β-hydroxyethyldiphosphate



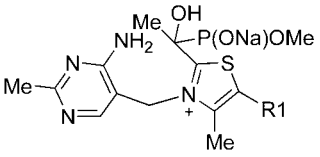
C2α-lactylThDP, LThDP



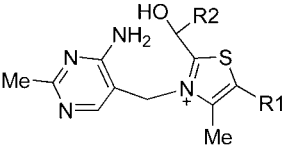
thiamin 2-thiazolone diphosphate, ThTDP



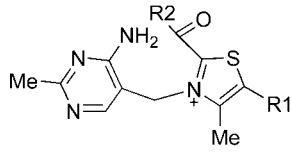
thiamin 2-thiothiazolone diphosphate, ThTTDP



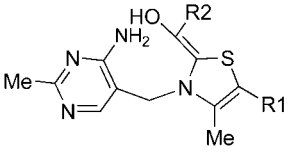
C2α-phosphonolactylThDP, PLThDP



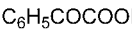
R2 = Me, C2α-hydroxyethylThDP or HEThDP
R2= C_6H_5, C2α-hydroxybenzylThDP or HBThDP



R2= Me, 2-acetylThDP
R2 in general, 2-acylThDP



enamine resonance structure of
C2α-hydroxyalkylideneThDP



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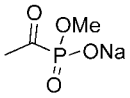
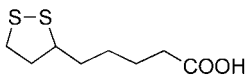


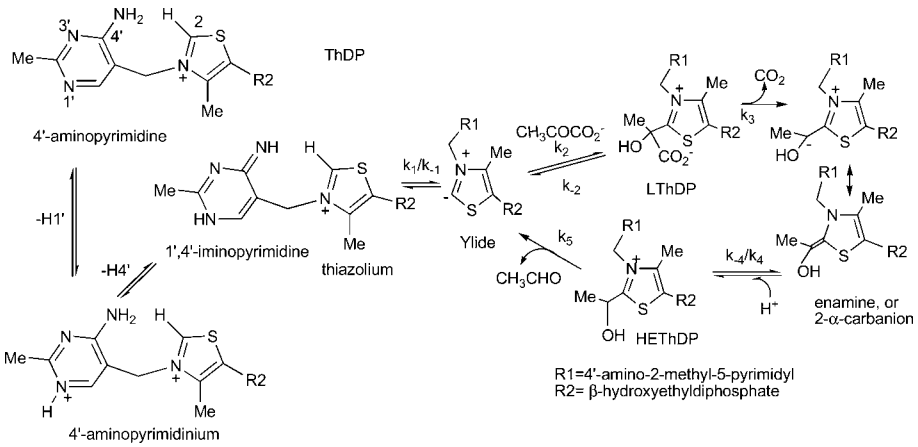
sodium methyl
acetylphosphonate



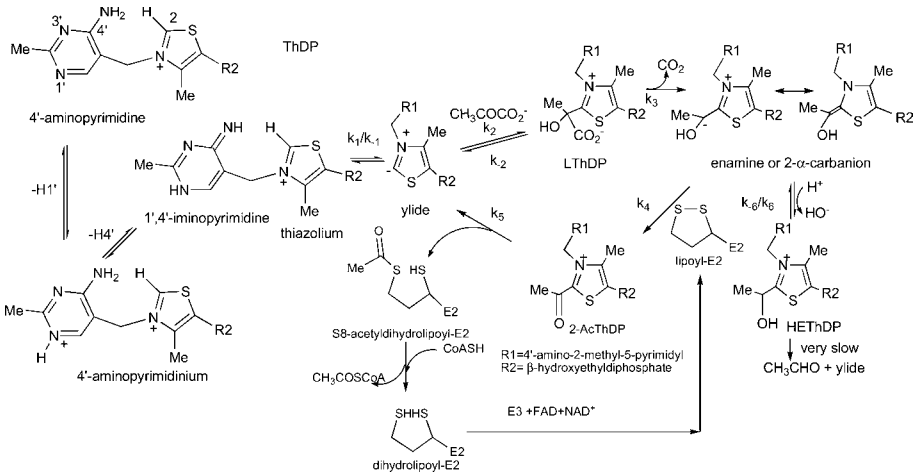
lipoic acid

2. The ylide/carbene or C2-carbanion

Dissociation of the thiazolium C2H to the conjugate base in Schemes 1 and 2 is an essential first step for all ThDP reactions. In model systems the pK_a for ionization of C2H is 17–19 [6]. So far, the only experiment to address the state of ionization at C2 of enzyme-bound ThDP was carried out using ^{13}C NMR on YPDC reconstituted



Scheme 1. Mechanism of pyruvate decarboxylase, YPDC.



Scheme 2. Mechanism of pyruvate dehydrogenase complex-E1 subunit.

with [2-^{13}C]ThDP, and reported a ^{13}C chemical shift δ of 155 ppm, identical to that found for ThDP and thiazolium salt models. The results were interpreted to mean that C2H of the bound ThDP is mostly in the undissociated ionization state at pH 6.0 [7]. In the same year, there appeared the first report on the generation of the C2-carbanion/ylide/carbene in non-aqueous medium using KH as base from a thiazolium ring with a bulky 2,4,6-triisopropylphenyl substituent at the N3 atom. The ^{13}C chemical shift δ of this species was found to be 253 ppm [8]. Work in the authors' laboratory has shown that the species with the δ 253 ppm for the C2 atom is highly reactive with benzaldehyde and other electrophiles [9], confirming Breslow's proposals for thiamin action [10].

In a recent paper, Perham and coworkers suggested a novel pathway for information transfer between the two ThDP active centers in the E1 subunit of the PDHc from *Bacillus stearothermophilus* via a 'proton wire' created by a series of acidic groups bridging the 4′-aminopyrimidine rings of the ThDP molecules ([11]; see also a Perspective on this paper in [12]). According to their hypothesis depicted in Fig. 4 of that paper, at any instant, one half of the ThDP molecules would be in an activated, probably in the C2 conjugate base form, while the other half would be undissociated [11].

3. Observation of the enzyme–substrate/analog non-covalent (Michaelis) complex

3.1. X-ray crystallography

The crystal structures of branched-chain 2-oxoacid dehydrogenase from *Pseudomonas putida* [23] and *Thermus thermophilus* HB8 [27] in complex with their substrate analogs were reported. Earlier, the crystal structure of pyruvate:ferredoxin oxidoreductase (PFOR) in complex with its substrate pyruvate was also reported [29]. On the basis of these structures, interaction of the substrate with the protein environment prior to the catalytic reaction was suggested. The carboxylate oxygen of pyruvate (in PFOR) or of the substrate analogs in both branched-chain 2-oxoacid dehydrogenase complexes is directly hydrogen bonded to the N4′ group of ThDP. For branched-chain 2-oxoacid dehydrogenase from *Thermus thermophilus* HB8 the substrate analog is located less than 3.6 Å from the thiazolium ring of ThDP, and the Cα atom of the substrate analog forms a van der Waals contact with the C2 atom of ThDP [27]. The binding of substrate analogue to this enzyme is accompanied by an open-closed conformational transition, which leads to complete protection of the substrate analog from solvent [27].

In the structure of (*R*)-mandelate (a competitive inhibitor) with BFD there were noted several interactions that could reflect the interactions with the substrate: the carboxylate forms two hydrogen bonds with the active center residue S26, while the Cα-OH forms hydrogen bonds to the ThDP N4′ atom and a ring nitrogen of H70 [57].

3.2. Circular dichroism spectroscopy

We have explored the identity and applications of a negative CD band with maximum of 320–330 nm, which has been seen on the enzyme transketolase for many years [36,37]. Below, we report conditions under which this band could be generated on both on YPDC (see Section 5.1) and on PDHc-E1 for the first time. With the PDHc-E1, a negative CD band at 327 nm could be observed on addition of 2 mM pyruvate to the PDHc-E1–ThDP complex and was assigned to the PDHc-E1–ThDP-pyruvate Michaelis complex [38]. It appears likely that the formation of HEThDP in one of the two PDHc-E1 active centers is required before the negative band at 327 nm is generated [38]. The results clearly indicate that the

negative CD band at 320–330 nm and the positive one at 300–310 nm (see Section 5.1) pertain to different enzyme-bound species. We carried out CD experiments on many PDHc-E1 active center variants and could find no substitution that eliminated the band with the exception of the E522A and E636A variants. With the latter, there is a significant carboligase side reaction (the enamine intermediate instead of undergoing oxidation reacts with an alternative electrophile acetaldehyde or pyruvate), which produces a single enantiomer of acetolactate, giving rise to a strong negative band at 301 nm, thereby making observation of the weaker negative band at 327 nm more difficult [38,41].

An inspection of the structures of the three enzymes on which the negative band at 330 nm could be observed [transketolases (TK), YPDC, and PDHc-E1] indicated that histidine residues are the only ones common to all of them near ThDP. We therefore concluded that the negative band is a property of ThDP itself, perhaps in its interaction with an active center histidine. In an attempt to mimic the position of the electronic transition, we turned to models. UV experiments were carried out with all possible permutations of the thiazolium ring, the histidinium ion, and the 4′-aminopyrimidinium ion as electron acceptors and the 4′-aminopyrimidine (both amino and imino tautomeric forms) or histidine as electron donors. The only pair that gave a charge transfer band in the range of interest consisted of 4′-aminopyrimidine as electron donor and the thiazolium ring as electron acceptor and displayed a λ_{max} at 340 nm, tending to rule out participation of histidines in the spectral signature. Importantly, with both YPDC and PDHc-E1, we could get a strong negative CD band near 330 nm in the presence of a substrate or substrate analog [38].

We therefore assign the negative CD band at 325 nm to an 'intra-ThDP' charge transfer transition in a Michaelis-type complex of ThDP with a substrate or substrate analog.

4. Observation of enzyme–inhibitor complexes

4.1. X-ray crystallography

The crystal structure of the *Escherichia coli* PDHc-E1 subunit in its complex with thiamin 2-thiazolone diphosphate (ThTDP), a potent thiamin diphosphate (ThDP) cofactor-like inhibitor, has been determined at 2.1 Å resolution [14] and the structure was compared to the structure of the complex with ThDP presented earlier [22]. The comparison revealed some changes in the conformation of the side chains of residues M194 and K392, as well as in the V coenzyme conformation of the two complexes. The Φ_T conformational angle is reduced by 10.5° with the ThTDP substitution. Most significantly, the comparison also revealed a difference in the number and position of water molecules and their hydrogen bonding networks. While the complex with ThDP displayed only one water molecule at the active center, that with ThTDP gave evidence of three such water molecules, different in both position and bonding pattern from that found with ThDP. These water molecules lead to a substantial increase in hydrogen bonding network in the vicinity of the ThTDP binding sites

and may account for the much tighter (1000-fold) binding of ThTDP than ThDP to the *Escherichia coli* PDHc-E1. The present analysis indicates that the hydrogen bonding pattern resulting from addition of ThTDP to *E. coli* PDHc-E1 subunit is not a general feature of ThDP-dependent enzymes but is rather specific to this enzyme. The structure of PDHc-E1 in its complex with the related thiamin 2-thiothiazolone diphosphate (ThTTDP, a ThDP analogue in which the C2H is replaced by C=S thereby creating a potent slow-binding inhibitor) has also been solved and the results are being summarized [15]. In contrast to the structure of the PDHc-E1–ThTDP complex, no changes were observed in the structure of yeast transketolase (TK) complexed to ThTDP compared to its complex with ThDP [88].

Also, the crystal structures of TK with the ThDP analogs with a 6′-methyl, or N1′-pyridyl, or N3′-pyridyl substitution showed no changes from the structure with ThDP [89].

4.2. Circular dichroism spectroscopy

An important initial finding for the interpretation of all subsequent CD results was the observation that addition of ThTTDP to PDHc-E1, produced a positive CD band centered at 330 nm (Fig. 1; [13]). This compound, unlike ThDP, does have a UV band (but no CD band) at 319 nm in the absence of enzyme, hence the CD band at 330 nm could be assigned to the enzyme-bound form of the inhibitor. Since there is no chiral center in the inhibitor, this experiment clearly indicated that the enforced V conformation of the inhibitor (defines the relative arrangement of the two aromatic rings with respect to each other), and the attendant induced chirality, is sufficient to produce the CD signal. Structures of the complex between the oxygen analog thiamin 2-thiazolone diphosphate (C=O in place of C2-H) and PDHc-E1 [14] and of the complex of thiamin 2-thiothiazolone diphosphate with PDHc-E1 [15]

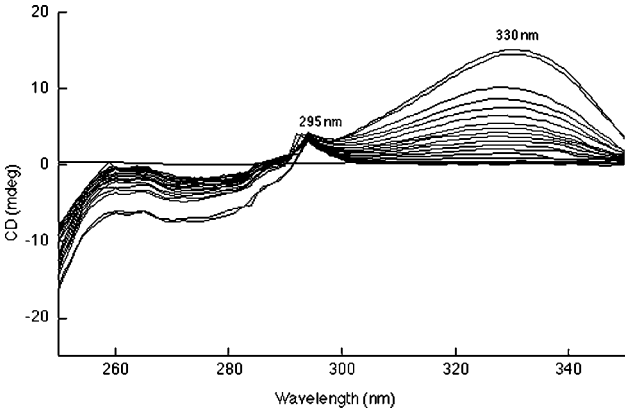


Fig. 1. Circular dichroism spectra of parental PDHc-E1 titrated with thiamin 2-thiothiazolone diphosphate [13]. The PDHc-E1 (9.0 μM) in 10 mM KH_2PO_4 buffer (pH 7.0) was titrated with thiamin 2-thiothiazolone diphosphate at concentrations ranging from 0.49 to 50 μM and 1 mM $MgCl_2$.

affirm that the V conformation is retained in these complexes. The results also suggest that any ThDP intermediate, even with no chiral centers, when bound in the V conformation on the enzyme, and if endowed with the appropriate chromophore, will give rise to a CD signal.

5. The substrate–thiamin diphosphate covalent adducts

The first ThDP-bound covalent intermediate for the decarboxylation of 2-oxoacids results from nucleophilic attack by the ylide at the carbonyl carbon of the substrate and produces 2-[1-carboxy-1-hydroxy]ethylthiamin diphosphate (commonly called C2α-lactylthiamin diphosphate or LThDP in Schemes 1 and 2). This intermediate is highly reactive (especially in its zwitterionic form) and its chemistry was well established by Kluger et al. [42]. Interestingly, in Kluger and Smyth' [43] early experiments, they did not observe decarboxylation of synthetic LThDP by wild-type pyruvate decarboxylase and suggested that a 'closed transition state' was the reason for the observation. With recently developed tools, the intermediacy of LThDP has been re-examined.

5.1. Observation via circular dichroism of a stabilized 1′,4′-iminopyrimidine tautomer of thiamin diphosphate

5.1.1. Background and assignment of UV and CD bands

One of the unanticipated features of the structure of ThDP enzymes [11,14–30] is the highly conserved V coenzyme conformation bringing to within 3.5 Å or less the N4′ and C2 atoms, tempting others and us to suggest that there is intramolecular proton transfer between these two atoms (Scheme 1). It was also suggested that for such short distances, it could be difficult to fit a proton at C2 and two protons at N4′ simultaneously, and further suggesting that the 4′-aminopyrimidine ring cycles between the 4′-amino and 1′,4′-imino tautomeric forms during the reaction sequence. This 1′,4′-iminoThDP is stabilized by three highly conserved hydrogen bonds to N1′, N3′, and N4′$H_{3'}$ (denoting the proton bonded to N4′ on the N3′ side of the pyrimidine ring; see Fig. 2 for the active center structure of PDHc-E1). Since the 1960s, Schellenberger [2] advocated the notion that the 4′-aminopyrimidine ring of ThDP has a catalytic role, while our group subsequently had suggested the participation of the 4′-aminopyrimidine ring via the N1′-protonation mechanism to influence the acid–base properties of the amino group [31–33].

In a series of papers, results from this laboratory have supported the idea that this 1′,4′-iminoThDP gives rise a UV band in the 300–310 nm region, and importantly, there is also a positive CD band in the same region, also reporting on this tautomer of ThDP. We first suspected the presence of the 1′,4′-iminoThDP from the results of rapid-scan stopped-flow experiments, where mixing the slow E477Q active center variant of YPDC with pyruvate in the presence of pyruvamide (a substrate activator surrogate) (Fig. 3, [34]) produced such a band in the UV spectrum.

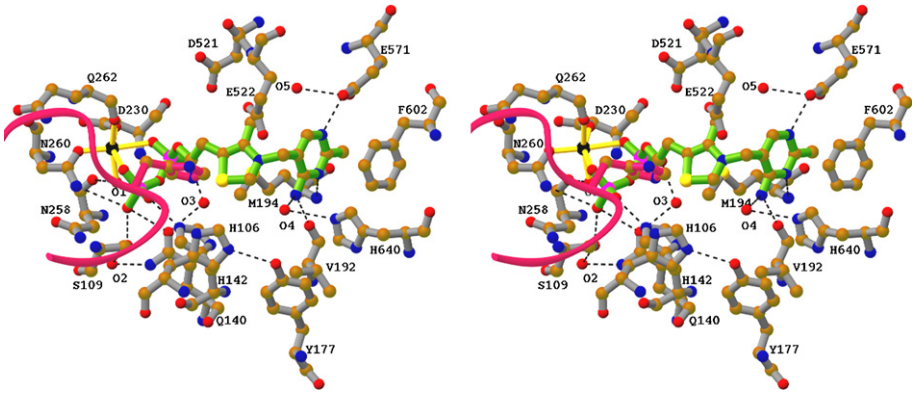


Fig. 2. PDHc-E1 active center residues [22].

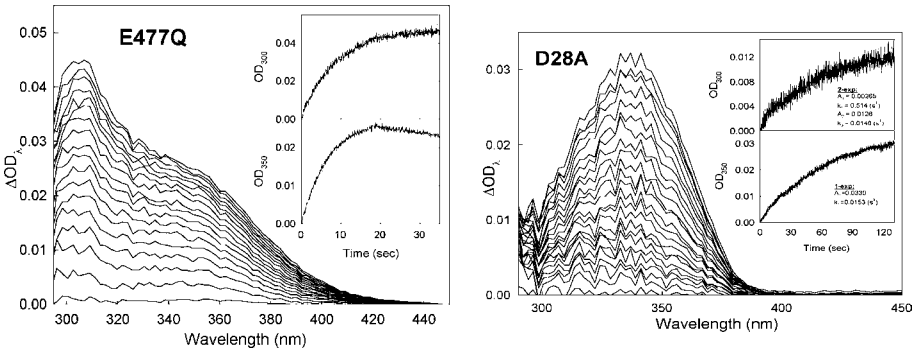


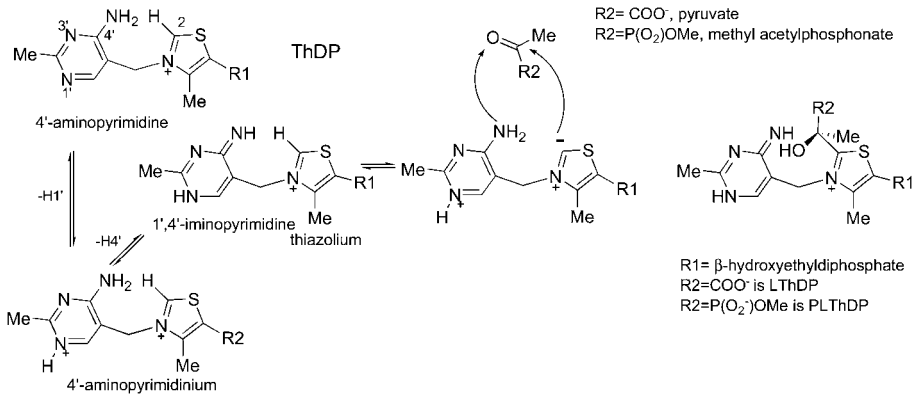
Fig. 3. Rapid-scan stopped-flow spectra on mixing pyruvate with the E477Q or D28A YPDC variants in the presence of 50 mM pyruvamide, a substrate activator surrogate at pH 6.0 [34].

To understand the origins of the UV and CD observations, we generated a model for the observed electronic transition. Treatment of N1-methyl-4-amino-pyrimidinium or N1-methyl-N4-*n*-butylpyrimidinium salts with either NaOH in water, or DBU in Me_2SO, revealed formation of an intermediate with λ_{max} of 300–307 nm, and this intermediate was converted back to the starting salt on acidification. Proton NMR chemical shifts are consistent with the intermediate representing the 1-methyl-4-imino tautomer [35]. More recently, Baykal in this laboratory further refined the model studies and determined both the ^{1}H and ^{15}N chemical shifts for the imino tautomer (paper in preparation). These model studies confirm that the observations on the enzyme at the same wavelength pertain to the 1′,4′-iminopyrimidine tautomer of ThDP.

5.1.2. Phosphonolactylthiamin diphosphate with PDHc-E1

We recently reported CD results for the complex formed between PDHc-E1 and phosphonolactylthiamin diphosphate (PLThDP), a stable analog of LThDP, the

covalent adduct between substrate pyruvate and ThDP (Scheme 3, [44]). Addition of racemic PLThDP to PDHc-E1 produced significant changes in the 300–350 nm region of the CD spectra, which on subtraction of the spectrum of the PDHc-E1, revealed a positive peak centered at 305 nm (Fig. 4). A plot of the ellipticity at 305 nm versus PLThDP concentration displayed saturation enabling us to calculate a $K_{d,PLThDP} = 1.37 \pm 0.29\ \mu M$. The development of the full CD amplitude at 305 nm was time-dependent. Approximately one-half of the full amplitude was achieved immediately, while the second half of the amplitude was achieved with a first-order rate constant of $0.070 \pm 0.026\ min^{-1}$, indicating fast saturation by PLThDP of one of the two active centers/dimer of PDHc-E1, followed by slower binding of PLThDP at the second active site. In view of the non-cleavable C2α—P



Scheme 3. Mechanism of formation of LThDP and PLThDP.

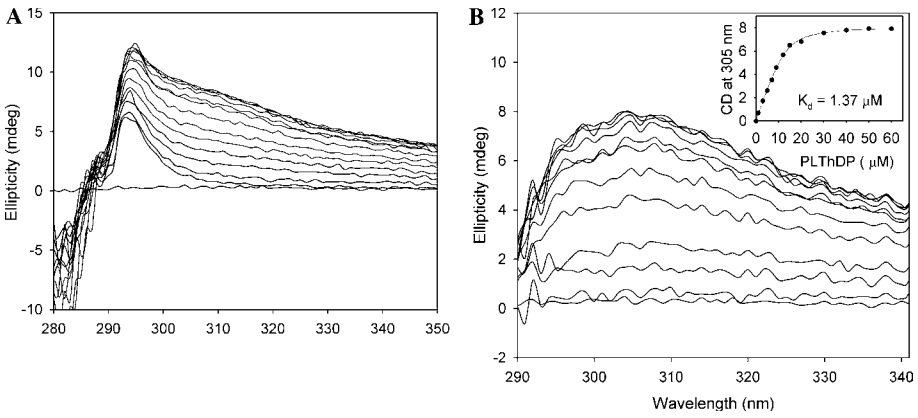


Fig. 4. (A) Titration of PDHc-E1 with phosphonolactylthiamin diphosphate [44]. (B) Difference spectra resulting from subtraction of the spectrum of PDHc-E1. (Inset) Variation of the CD signal at 305 nm with inhibitor concentration.

bond in PLThDP replacing the C2α—C bond in LThDP, we could rule out formation of the enamine as the source of the signal. The experiments provide the clearest spectroscopic evidence to date for the existence of the 1′,4′-imino tautomer of PLThDP in the V conformation at the active centers of PDHc-E1.

With the H407A (an amino acid residue important in the reductive acetylation of the PDHc-E2 [45]), and E571A PDHc-E1 substitutions (the highly conserved glutamate present in all ThDP-dependent enzymes and located within hydrogen-bonding distance of the N1′ atom of ThDP), no CD signal could be detected at 305 nm under the experimental conditions, indicating that these amino acids are required for stabilizing the 1′,4′-iminoThDP [44].

The CD spectra of the E636A and E636Q variants (amino acid residue located in the active center cleft leading to the ThDP binding site) on addition of PLThDP were similar to that of parental PDHc-E1, indicating formation of the 1′,4′-imino tautomer of PLThDP on the enzyme. However, the amplitude of the CD signal was only one-half of that with parental PDHc-E1, suggesting half-of-the-sites-reactivity [41].

In yet to be published work, the positive CD band at 305 nm has now also observed on PDHc-E1 and its variants in the presence of sodium methyl acetylphosphonate (NaMAP), an analog of pyruvate, indicating the synthesis of 1′,4′–imino tautomer of PLThDP from NaMAP and ThDP in the active centers of PDHc-E1 (Scheme 3) as was reported by Kluger and Pike [46].

5.1.3. Formation of 1′,4′-imino PLThDP by yeast pyruvate decarboxylase on addition of sodium methyl acetylphosphonate

Since it is difficult to release ThDP from YPDC, we generated the 1′,4′-imino PLThDP on the YPDC by addition of NaMAP to the holo-enzyme, with the expectation that, in analogy with the PDHc results in the previous paragraph, YPDC would catalyze formation of PLThDP from ThDP and NaMAP. We reported recently [38], that addition of NaMAP to the E91D YPDC variant produced major changes in the CD spectrum in the 300–400 nm spectral region. The difference CD spectra displayed two bands: a negative one at 330 nm and a positive one at 300 nm (Fig. 5). The negative band at 330 nm suggests the formation of YPDC-ThDP-NaMAP Michaelis complex in one-half of the active centers. The positive band at 300 nm clearly signals the formation of the 1′,4′-imino tautomer of PLThDP in the second half of the active centers of YPDC with $S_{0.5,\mathrm{NaMAP}} = 5.56 \pm 0.61$ mM and the Hill coefficient $n_{\mathrm{H}} = 1.48 \pm 0.23$. Similar spectra were observed on addition of NaMAP to either the wild-type YPDC or to its E51D variant (glutamate 51 in YPDC is analogous to glutamate 571 in PDHc-E1). Since YPDC is a homotetramer with the suggested mechanism of ‘‘alternating active site in a functional dimer’’ [47], these findings provide excellent support for half-of-the-sites type behavior.

Using CD detection, we next asked whether and which active center residues of YPDC were involved in the synthesis of PLThDP from ThDP and NaMAP, with the hypothesis that substitution of such a residue would not allow us to observe the CD signal mirroring the presence of the 1′,4′-imino PLThDP, and would, in turn also implicate this residue in the synthesis of LThDP from ThDP and pyruvate. With the E51A/E91D doubly substituted YPDC variant no signal at 300 nm, and only a

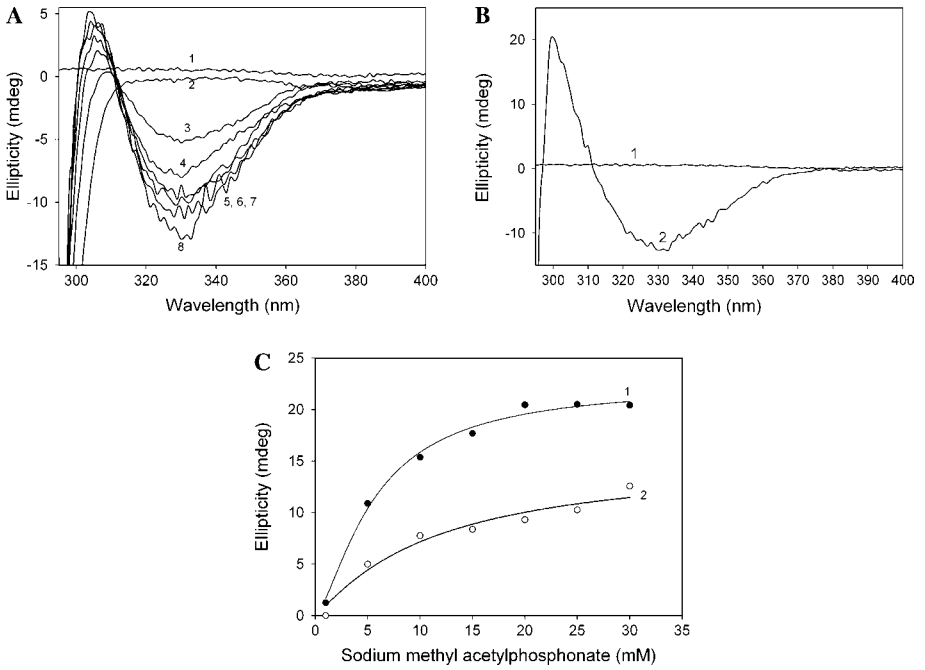


Fig. 5. Formation of phosphonolactylthiamin diphosphate from sodium methyl acetylphosphonate (NaMAP) and the E91D YPDC variant [38]. (A) Spectra in presence of increasing concentration of NaMAP; (B) difference spectra on addition of 30 mM NaMAP; (C) dependence of ellipticity at 300 nm (curve 1) and at 330 nm (curve 2) on the concentration of NaMAP.

weak one at 330 nm was apparent in the presence of 35 mM concentration of NaMAP, indicating that this variant did not form the 1′,4′-imino tautomer of PLThDP, similarly to that found with the E571A variant of PDHc-E1 (the residues E571 in PDHc-E1 and E51 in YPDC are in analogous positions). Of the D28A, H114F, H115F, and E477Q active center YPDC variants (see Fig. 6; all four of these residues are implicated in transition state stabilization [48]), the signal at 300 nm was in evidence only with the D28A variant but not with the H114F, H115F, and E477Q variants. We concluded that the residues H114, H115, and E477 are all involved in some step(s) leading to synthesis of PLThDP, and by inference, of LThDP from pyruvate and ThDP. However, the D28A variant did not display a strong negative signal at 330 nm, related to the YPDC-ThDP–NaMAP Michaelis complex, indicating that the D28A substitution perhaps affects communication between active centers (data not published).

5.1.4. Intermediates of YPDC detected by stopped-flow spectrometry

Rapid-scan stopped-flow experiments gave evidence for the formation of two bands with maxima at 300 and 350 nm on addition of pyruvate to the slow E477Q YPDC variant that had been activated by 50 mM pyruvamide (Fig. 3).

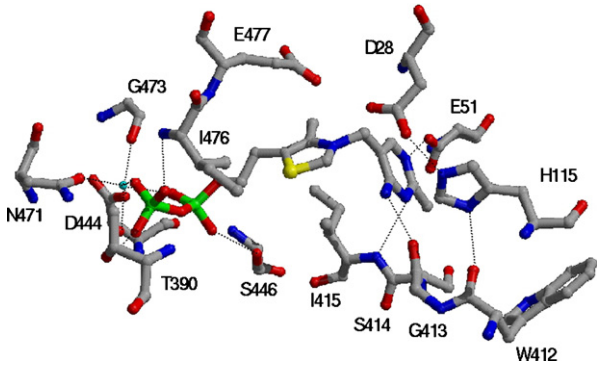


Fig. 6. Active center residues on YPDC [17].

Time-resolution indicated that the two bands corresponded to different species. The longer wavelength absorbance also developed with the slow D28A variant, but neither band was observed with the wild-type YPDC [34]. At the time of the experiments, we had not assigned the UV bands, we now believe that they pertain to the 1′, 4′-imino tautomer of perhaps LThDP, and the Michaelis complex, respectively. We note that addition of NaMAP results in the formation of stable LThDP analog, whereas, the rapid-scan stopped-flow experiments detect only intermediates which accumulate on this time scale, i.e., depend on their relative rates of formation and depletion.

In summary, on YPDC and PDHc-E1, we assign the positive CD band at 300–310 nm to the 1′,4′-iminoThDP and the negative one at 325 nm to an 'intra-ThDP'charge transfer transition in a Michaelis-type complex of ThDP with a substrate or substrate analog.

We also point out that while a similar negative CD band at 330 nm observed on TK had earlier been attributed to binding of ThDP [36,37], two recent publications on TK claimed that both that negative band and the positive one at 300–310 nm (which we reported on enzymes) pertain to the 1′,4′-iminoThDP on the enzymes [39,40]. Our results are not consistent with those claims. Nor were we successful in producing a UV band appropriate to this 1′,4′-imino tautomer using conditions reported in those papers, rather we rely on methods outlined above.

5.2. ^{1}H NMR detection of the intermediates subsequent to acid quench

The group of Hübner and Tittmann in Halle recognized that the C6′ proton resonance of the various ThDP-bound intermediates in Schemes 1 and 2 have sufficiently distinct chemical shifts to enable quantification of the relative amounts of the various species subsequent to acid quench of a mixture of pyruvate, ThDP, and pyruvate decarboxylase [49]. The experiment is carried out in a chemical quench instrument. In fact, resonances corresponding to both LThDP and HEThDP could be observed under these conditions [49], confirming that both are on the pathway.

Because the experimental protocol calls for acid-quench, the concentration of HEThDP measured most likely corresponds to the sum of the enamine and HEThDP concentrations on the enzyme in Schemes 1 and 2. A kinetic scheme was derived to determine the forward rate constants for all steps, hence the rate-limiting step could be deduced under various experimental conditions. This is a very powerful approach with many potential applications to ThDP enzymes and already applied to several such enzymes [49,50].

5.3. Reconstitution of apo-enzymes with LThDP and stable analogs

We explored the potential to reconstitute ThDP enzymes with synthetic racemic LThDP and its analogs. First, it was shown that the E91D YPDC variant could form a stable apo-enzyme, which could be reconstituted with virtually any ThDP derivative, including LThDP [51]. Later, it was found that LThDP is partitioned on YPDC, to pyruvate in the reverse direction, and to the enamine and acetaldehyde in the forward direction (both species quantified independently) [52]. This evidence strongly supports the intermediacy of LThDP as a distinct chemical entity on pyruvate decarboxylases. Such experiments were also carried out on PDHc-E1, leading to similar conclusions, i.e., under steady-state conditions, both YPDC and PDHc-E1 could decarboxylate LThDP [52]. With the PDHc-E1, there was also a single-turnover experiment carried out, in which the enamine was trapped by the external oxidizing agent, 2,6-dichlorophenolindophenol. Under these conditions, a rate constant of 0.4 s^{-1} was measured; however, this value clearly represents a lower limit, since the steps include binding of LThDP, its decarboxylation and, finally, oxidation of the enamine for detection purposes. It was concluded that true kinetic competence would be difficult to prove, but that the conformational change that LThDP must undergo to assume the V conformation at the active site, could indeed limit the measured rate [52].

At the same time, in a model study, it was shown that by simply lowering the dielectric constant of the organic solvent used, the first-order rate constant for decarboxylation of a C2-α-lactyl-3,4,5-trimethylthiazolium salt, as a model devoid of the 4′-aminopyrimidine ring, could be raised to 53 s^{-1}, a value very similar to the turnover number of YPDC and PDHc-E1 [52]. As shown by Tittmann et al. [49] the rate constant for decarboxylation could exceed this value significantly on the enzymes, nevertheless, the model studies suggest that the enzymes need to provide only modest additional catalysis for the decarboxylation step, other than a low polarity environment. We reached similar conclusions regarding the HEThDP intermediate below (Section 6).

5.4. Crystallographic evidence for a LThDP analog on PDHc-E1

The authors' crystallographer collaborators have succeeded in co-crystallizing the phosphonolactyl analog of LThDP (PLThDP) with PDHc-E1 from *E. coli* [53]. This is the first structure of an analog of the substrate–ThDP covalent complex visualized on a ThDP enzyme and it underlines the importance of attempts to obtain further

such structures, since the structure reveals major changes in the mobility of a variety of loops, as well as changes in the positions of some key active center residues, on forming this key intermediate analog.

5.5. Studies with conjugated pyruvate analogs

Many years ago, we had reported that when using conjugated pyruvate analogs, the enamine intermediate could be observed by electronic absorption spectroscopy in the visible range [54,55]. Our earlier studies exploited compounds with the structure $C_6H_5CH{=}CHCOCOOH$ (substituting styrene for the methyl group of pyruvate and leading to the conjugation in the enamine in Schemes 1 and 2) with a variety of substituents mostly *para*- and *meta*- to the exocyclic double bond of the phenyl ring. These compounds allowed us to observe the enamine near 440 nm. Time-resolution via stopped-flow showed that activation of the enzyme by the substrate activator surrogate pyruvamide increased the rate of enamine formation, essentially to a rate observed with pyruvate. This approach was revisited more recently on the enzyme benzoylformate decarboxylase (BFD). Given the fact that the substrate for BFD is already aromatic, it was reasonable to ask whether or not rapid mixing of this enzyme with an aromatic analog would generate intermediates in the visible spectrum. Remarkably, on mixing BFD with *p*-nitrobenzoylformic acid, two absorbances were seen, at 410 nm and at 620 nm. The absorbance at 620 nm displayed a rapid rise followed by a slower decrease, while the one at 410 nm rose at a rate paralleling the rate of disappearance of the 620 nm absorption (Fig. 7). On the basis of this kinetic behavior, and model studies, the band at 410 nm was assigned to the enamine (see below), while the very broad one centered at 620 nm was assigned to a charge transfer band, probably representing the intermediate formed between

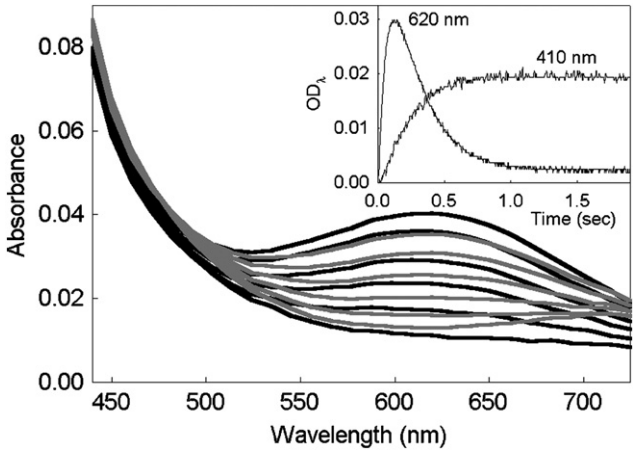


Fig. 7. Addition of *p*-nitrobenzoylformic acid to benzoylformate decarboxylase on a rapid-scan stopped-flow spectrometer with insets of the time-course at 410 and 620 nm [56].

p-nitrobenzoylformic acid and ThDP (C2-α-*p*-nitromandelylThDP, corresponding to LThDP in Scheme 1). It is difficult to envision any other 'stable' intermediates on these pathways. The dependence of both observed relaxations on substrate concentration suggested that the active centers of BFD interact in the presence of this alternate substrate, and we suggested 'alternating active site reactivity' in a functional dimer [56,57].

More recently, a group of different conjugated substrate analogs was tested with YPDC [58] and BFD: pyridylCH=CHCOCOOH, where the nitrogen is at the *ortho*, *meta* or *para* positions. The results with the *meta* derivative are particularly useful. When mixed with YPDC or BFD on the stopped-flow, there result two new absorbances, similarly to the findings with BFD in the previous paragraph, one near 420 nm, the other broader one near 490 nm. Again, the absorbance at 490 nm rapidly rises and diminishes while the one at 420 nm rises with the same rate constant as the absorbance at 490 nm disappears. We suggest that the shorter wavelength absorbance corresponds to the enamine, the longer one to a charge transfer band, a property of the adduct formed between the alternate substrate and ThDP on the enzyme.

It is important to point out that the observation of the time-course of two key intermediates on the pathway (two relaxations) and their concentration dependence, provide detailed mechanistic information regarding specific rate constants for a number of key steps in the mechanism [56–58].

6. C2α-Hydroxyethylidene thiamin diphosphate- the enamine or second carbanion, and C2α-hydroxyethylthiamin diphosphate, the 'product'–ThDP complex

These two intermediates are at equilibrium with each other as a conjugate acid/base pair both on and off the enzymes, hence it is convenient to discuss them together.

6.1. C2α-Hydroxyethylidene thiamin diphosphate- the 'enamine'

The enamine intermediate could properly be called a central intermediate given its likely presence on all ThDP-dependent catalytic pathways. The authors' group has spent the past 20 years elucidating the properties of this intermediate in the absence and in the presence of ThDP enzymes. These are very important comparisons if we wish to understand the contribution of the protein to catalysis of individual steps.

In model compounds, most of our work centered on the generation of the enamine by addition of a non-nucleophilic base in an aprotic solvent to compounds related to C2α-methoxybenzyl-thiazolium or C2α-methoxyethylthiazolium salts. It was found that under these conditions the enamine could be sufficiently stable for spectroscopic characterization. The most important finding from these studies regarding structure is that the enamine is indeed planar, favoring the enamine over the carbanion resonance contribution according to both NMR and electronic spectroscopic studies. The UV spectra provided for the first time a $\lambda_{max} = 290$–295 nm

for the enamine derived from pyruvate (relevant to YPDC and PDHc-E1) and 380 nm for the enamine derived from benzoylformic acid, consistent with conjugation and planarity [59,60]. These observations were very useful for subsequent mechanistic studies.

6.1.1. Direct observation of the enamine from conjugated substrate analogs

As discussed in the previous section, exposing YPDC or BFD to conjugated 2-oxo acids ($XC_6H_4CH{=}CHCOCOOH$ and $XC_6H_4COCOOH$, where X is *o*-, *m*- or *p*- from the 2-oxo acid substituent), enabled us to observe the visible spectrum of the enamine intermediate by stopped-flow methods. It could be demonstrated that decarboxylation is quite fast for these alternate substrates ([55,56] and Fig. 7). With several types of such compounds, we could identify an intermediate preceding the enamine, presumably corresponding to an LThDP-like intermediate from the charge transfer band formed on the enzymes. This and earlier results, including model studies from the authors' laboratory, strongly suggest that the structure of the enamine is dominated by the planar uncharged resonance contribution.

6.1.2. Indirect observation of the enamine by oxidative trapping

Over the decades, there has been considerable interest in measuring the rate of the reaction of the E1 subunit of the PDHc and related complexes. While the quantification of carbon dioxide release provides the most direct method to test for decarboxylation, it is a difficult experiment to implement in a continuous assay format. Alternatively, oxidation of the enamine by a chromophoric reagent has been used in continuous assays, first the change from Fe^{3+} to Fe^{2+} [61], later 2,6-dichlorophenolindophenol with a λ_{max} of 600 nm for the oxidized form [62]. In our laboratory, we have developed a method based on disulfides mimicking the action of lipoic acid on the PDHc. In particular, the combination of 4,4′-dithiodipyridine and its reduced form 4-thiopyridine provide a chromophoric alternative, since on trapping of the enamine, its oxidation and release from ThDP on the enzyme, 4-thiopyridylamide of acetic acid is formed with λ_{max} of 382 nm [63–65]. All of these methods have their drawbacks, perhaps the most severe one is that access to the active site varies from enzyme to enzyme, and in our experience at least, when comparing the E1-specific activity of a series of variants, these methods do not provide a reliable measure, but they do inform whether or not decarboxylation has indeed taken place at all.

6.1.3. Observation of the enamine on enzymes by X-ray methods

On transketolase from *Saccharomyces cerevisiae* Schneider and coworkers [66] solved the structure with (α,β-dihydroxyethyl)ThDP intermediate (DHEThDP) at 1.9-Å resolution. The intermediate was generated on the enzyme taking advantage of the artificial decarboxylase activity exhibited by transketolases towards the 'donor substrate' β-hydroxypyruvate (HPA), and flash-freezing this material into crystals of transketolase. According to the CD spectra of holo-transketolase, a positive CD signal developed between 300 and 310 nm on addition of HPA, which was assigned to the formation of the α-carbanion/enamine conjugate base of (α,β-dihydroxyethyl)ThDP, i.e., (α,β-dihydroxyethylidene)ThDP. On the basis of the crystal

structure of transketolase, the authors suggested a planar structure for the DHEThDP (presumably in its anionic ionization state) intermediate on the enzyme, hence the intermediate is predominantly in the enamine resonance form. No structural changes were observed in the side chains interacting with the intermediate or in the cofactor itself. The authors suggest no significant structural changes during catalysis in transketolase [66].

Subsequently, in a very comprehensive structural paper of the branched chain 2-oxo acid (BCOA) dehydrogenase (E1) from *Thermus thermophilus* HB8 [27], the following crystal structures were analyzed: the apoenzyme (E1 apo), the holoenzyme (E1 holo), E1 holo in complex with the substrate analogue 4-methylpentanoate as an ES complex model, and E1 holo in complex with 4-methyl-2-oxopentanoate, a substrate of BCOAE1, which after decarboxylation is expected to form the α-carbanion/enamine intermediate on the enzyme. According to the authors, in the crystal structure of E1 holo with 4-methyl-2-oxopentanoate, formation of the reaction intermediate does not significantly change the position of the active site residues, similarly to that observed with transketolase.

These are remarkable findings in view of the length of time needed to complete the experiment during which the enamine would have to survive on the enzyme without being protonated. The structures indeed suggest that the C2–C2α environment is planar, as expected for the enamine resonance contribution. This is important evidence since a pyramidal environment would suggest that there may be a hydrogen atom bonded to the C2α atom. In view of the basicity of the enamine (see next section), these reports of stable enamines on the enzyme are highly informative since they suggest special properties of the active site.

While the authors on the transketolase paper [66] suggested that the positive CD band between 300 and 310 nm supports the enamine structure, we note that in models the λ_{max} for the enamine derived from pyruvic acid at 290–295 nm, and for 1′,4′-imino tautomer's λ_{max} at 300–307 nm. These are very small differences and we suspect that the enzyme environment may indeed impose a shift on them. Nonetheless, the CD signal for the 1′,4′-iminoThDP derived from PLThDP (cannot form the enamine!) is 300–305 nm, and it has a λ_{max} of 300 nm for the 1′,4′-iminoThDP derived from HEThDP, leading us to conclude that tetrahedral intermediates on ThDP enzymes exist in their 1′,4′-iminoThDP form. Therefore, reliance on the precision of the X-ray data is essential to decide between planar or non-planar environments at the C2α atom.

Regarding the studies by Nakai et al. [27], it is known that in the absence of either the E2 and E3 subunits of the keto-acid dehydrogenase complex, or of an external oxidizing agent such DCPIP, the enamine on the E1 subunit still has several options: being released as an aldehyde subsequent to protonation at C2α, or reaction with a second electrophilic substrate resulting in the 'carboligase' side products. In other words, for the enamine to survive the rigors of the crystallographic analysis, it still must avoid these potential reactions, and, protonation at C2α would be the most likely alternative to the enamine on the enzymes.

The apparent inconsistency between the results on TK and BCOA on the one hand, showing little or no protein structural changes in with the intermediates

bound, compared to the dramatic changes when PLThDP is bound to PDHc-E1, can be reconciled as follows. The PLThDP is a pre-decarboxylation intermediate analog, while those with TK and BCOA are post-decarboxylation intermediates or analogs. In addition, the most dramatic changes in the PDHc-E1–PLThDP complex compared to the PDHc-E1–ThDP complex are those involving loops not even seen in the latter (401–413 and 541–557), hence the observations on the PDHc-E1.PLThDP complex may simply reflect a much reduced mobility of these loops in the pre-decarboxylation complex.

6.2. C2α-Hydroxyethylthiamin diphosphate

6.2.1. Circular dichroism spectra of PDHc-E1 with HEThDP

We recently reported [67], that the intermediate C2-α-hydroxyethylthiamin diphosphate (HEThDP) is a substrate of PDHc-E1 after ionization of the C2α—H bond (see below). We also found that titration of PDHc-E1 with HEThDP produces changes in the 295–320 nm region of the CD spectra not seen with ThDP addition [38]. Difference CD spectra obtained on addition of HEThDP displayed a positive maximum at 300 nm, which could be assigned to the 1′,4′-imino tautomeric form of HEThDP. On the basis of these results, it was concluded that both tetrahedral intermediates LThDP and HEThDP exist in their 1′,4′-imino tautomeric form when bound to the enzyme and could be identified by the positive CD signal at 305 and 300 nm, respectively [38].

6.2.2. Intermediate partitioning experiments using apo-YPDC and HEThDP

In an attempt to assign function to active center residues, we created doubly substituted YPDC variants, E91D to help release ThDP, and a second substitution at H114, H115, D28 or E477. In results to be published, Liu and Zhang [68] demonstrated that all variants, with the exception of the D28A/E91D could catalyze acetaldehyde release from the HEThDP. This provides strong evidence that D28 is responsible for release of acetaldehyde in Scheme 1.

6.2.3. ^{1}H NMR detection of HEThDP subsequent to acid quench of a reaction mixture

As outlined before, HEThDP is commonly observed when pyruvate decarboxylating enzymes such as YPDC or PDHc-E1 are rapidly mixed with substrate, then the reaction is quenched into strong acid [49]. Interestingly, HEThDP is the intermediate most commonly and most readily observed in these experiments [49]. It should be noted that under the conditions of the acid quench, any enamine if present would be converted to HEThDP, so that the concentration of HEThDP includes both species as they are at equilibrium. In general, with some notable exceptions, apparently HEThDP release from the enzyme is the rate-limiting step. This method also identified D28 on YPDC and D27 (the corresponding position) of the *Zymomonas mobilis* pyruvate decarboxylase as being responsible for acetaldehyde release, as was also found in the previous paragraph by an independent method. In studies to be published, we found that HEThDP is indeed the most prominent product quenched when PDHc-E1 is used in the absence of any oxidizing agents [41].

6.2.4. C2α-H dissociation from HEThDP on PDHc-E1 and YPDC

We have been intrigued as to how ThDP enzymes achieve their catalytic rate accelerations, estimated at 10^{12}- to 10^{13}-fold greater than the rates achieved with thiamin models [69]. Now that we have carried out numerous site-directed mutagenesis experiments mapping out the entire active site of two examples YPDC [70] and PDHc-E1 [71], in which no single mutation ever led to more than 100- to 1000-fold diminution of k_{cat} or k_{cat}/K_m, the question is even more significant. We hypothesized that an apolar environment would assist lowering the kinetic barriers through the enamine, since an inspection of the putative mechanism in Scheme 1 shows that approaching the enamine from either direction, i.e., from LThDP or HEThDP, reduces the charge separation, and such reactions should go faster in lower effective dielectric constant media. This hypothesis was by no means original since Lienhard [72] and Kemp [73] many years ago provided model studies to support it. Our task was to try to generate support for this hypothesis on the enzymes themselves.

First, models were designed to determine the rates of the steps leading to and from the C2α-carbanion/enamine in Schemes 1 and 2: the enamine was generated by the addition of base to a C2α-hydroxybenzylthiazolium or C2α-hydroxyethylthiazolium salt [59,60], then the proton transfer rate was measured. First, the pK_a at C2α was measured in pure Me_2SO [74]. By direct observation of the enamine generated in a stopped-flow spectrophotometer, the rate constants for reversible proton dissociation at the C2α position could also be measured in water. The pK_a is between 15 and 16 for C2α-hydroxybenzylthiazolium salts [75,76], and near 15 in 32–37 mol% Me_2SO for C2α-hydroxyethylthiazolium salt (extrapolates to approximately 18 in water) [77]. There is a substantial primary deuterium kinetic isotope effect for the deprotonation reaction (4–6); the rate constant for reprotonation of the enamine/C2α carbanion by water is many orders of magnitude below diffusion control. We concluded that both YPDC and BFD assist protonation of the enamine to afford rate constants commensurate with enzymatic turnover numbers.

How do ThDP enzymes solve this high pK_a problem? The following experiments provide some answers to this riddle. When the E91D variant of apo-YPDC was exposed to C2α-hydroxybenzylThDP (in place of HEThDP in Scheme 1), this putative intermediate was partitioned on the enzyme between release of the benzaldehyde product (evidenced by regeneration of active enzyme), and dissociation of the proton at C2α to form the enamine/C2α-carbanion intermediate (evidenced by the appearance of the visible spectrum of the intermediate). While the pK_a for this dissociation is ~15.4 in water, formation of the enamine at pH 6.0 on the enzyme indicates a greater than 9 unit pK_a suppression by the enzyme environment [78]. The fluorescence emission properties of thiochrome diphosphate, a fluorescent ThDP analogue and a competitive inhibitor for YPDC, when YPDC-bound resemble that observed in 1-pentanol and 1-hexanol, suggesting an apparent dielectric constant of 13–15 for the YPDC active center. Such a low effective dielectric constant could account for much of the observed >9 unit pK_a suppression at the C2α position for ionization of HBThDP. The dramatic stabilization of this (and presumably other) zwitterionic intermediate(s) is sufficient to account for as much as a 10^9-fold rate acceleration on YPDC, providing the bulk of the rate acceleration by the protein over and above

that afforded by the coenzyme. Similar experiments have been carried out with HEThDP [67], confirming the ability of YPDC to partition this intermediate as well, to acetaldehyde in the forward and the enamine in the reverse direction (the enamine in this case has a λ_{max} near 295 nm, so that it could only be detected by indirect oxidative methods). With this intermediate, whose pK_a is even higher, perhaps 18, the pK_a suppression induced by the enzyme is even more impressive. At the same time, the enamine could also be generated from HEThDP by PDHc-E1. This result suggests that the PDHc-E1 also possess an active center that can stabilize zwitterions.

In more recent work, we examined the rate constant for ionization of the C2α-H by PDHc-E1 in HEThDP in which the hydroxyethyl group was perdeuterated at the C2α- and C2β-positions (henceforth HEThDP-d_4). Mixing this compound in H_2O with PDHc-E1, followed by acid quench on the rapid-mixing time scale, allowed us to determine the rate constant for the wash-out of a deuteron from the C2α-position, i.e., monitor the reaction:

$$\text{HEThDP-d}_4 \rightarrow \text{HEThDP-d}_3$$

The ratio of product to reactant on a pre-steady-state timescale was evaluated by FT ion cyclotron resonance mass spectrometry after purification of the reaction mixture. Since the exchange reaction has a half-life of hours in the absence of enzyme, there is no background to contend with. The rate constant for the reaction was 1.2 s^{-1}, some 10^7–10^8-times faster than the relevant model rate constants [67]. Given that this reaction is not on the pathway of PDHc-E1, it is reasonable to assume that no acid–base group evolved in the active center for this particular purpose, so that we are measuring an intrinsic property of the enzyme to ionize this weak carbon acid. The simplest explanation again is that the low effective dielectric constant helps to ionize the carbon acid. Between the experiments on YPDC and this recent one with PDHc-E1, we believe that it is reasonable to state that at least 10 kcal/mol activation energy lowering in ThDP enzymes can be attributed to the environment.

7. C2-Acylthiamin diphosphates

The ThDP-mediated enzymatic oxidative decarboxylations transfer an acyl equivalent to either water or phosphate on the one hand (in the two varieties of pyruvate oxidases known to date), or to dihydrolipoyl-E2 enzymes in the 2-oxoacid dehydrogenase multienzyme complexes, or to coenzyme A in the reaction of pyruvate:ferredoxin oxidoreductases. Whether or not the 2-acylthiamin diphosphate is a distinct intermediate is an important mechanistic issue. A concerted reaction via a tetrahedral intermediate which collapses to *S*-acyldiphydrolipoyl-E2 is one option, while a stepwise reaction in which the enamine first reacts with lipoyl-E2 to yield 2-acylThDP and dihydrolipoyl-E2, followed by acyl transfer from ThDP to dihydrolipoyl-E2 is the second option. Formation of the tetrahedral intermediate sooner or later is likely. In a series of papers, Frey's group provided strong evidence for the existence of 2-acylThDP on both pyruvate dehydrogenase (forms 2-acetylThDP) and α-ketoglutarate dehydrogenase (forms 2-succinylThDP) [79–81]. Frey's group

demonstrated that the series of reactions in Eqs. (1) and (2) is reversible, since addition of succinylcoenzyme A to the α-ketoglutarate dehydrogenase complex enabled detection of 2-succinylThDP via an E1.ThDP-catalyzed reaction. Apparently, all of these high-energy bonds are nearly isoenergetic.

$$\text{E1.2-acylThDP} + \text{dihydrolipoyl-E2} \rightarrow \textit{S}\text{-acyldihydrolipoyl-E2} \quad (1)$$

$$\textit{S}\text{-acyldihydrolipoyl-E2} + \text{coenzyme A} \rightarrow \text{acyl-coenzyme A} + \text{dihydrolipoyl-E2} \quad (2)$$

In a further exploration of this idea, Frey's group used 3-fluoropyruvate as alternate substrate. Once this compound undergoes decarboxylation on ThDP, it releases fluoride ion thereby generating the enol form of 2-acetylThDP, and the keto form could then acylate dihydrolipoyl-E2 subunit. This experiment nicely demonstrates the feasibility of acyl transfer between 2-acylThDP on E1 and the dihydrolipoyl-E2.

The authors' laboratory developed a chemical model for this reductive acetylation inspired by earlier work of Rastetter et al. [82]. It was first shown that lipoic acid, or its amide form, is incapable of reacting with the enamine derived from a C2α-methoxylthiazolium salt [83] unless the lipoyl dithiolane ring is first S-methylated, which places a positive charge on the ring. This S-methylated lipoamide not only reacted with the above enamine, but the tetrahedral adduct with the enamine could be isolated and characterized by NMR methods [84]. Most likely, this reaction is very facile, since on ring opening the sulfur in the thiolate form would render the reaction reversible, while the attached methyl group renders the reaction with the enamine irreversible. Sometimes later, we suggested that residue H407 in PDHc-E1 is the electrophile which donates a proton to most likely the S6 sulfur atom [45], and the methyl group pre-attached in our model is a surrogate for this proton electrophile. Given that the C2α-OH group was alkylated for this model, the formation of the tetrahedral intermediate may have been pre-determined. It is useful to note, however, that with flavin analogs as oxidizing agents, the reaction only worked with an unprotected C2α-OH.

It is also noteworthy that 2-acetylThDP had been observed by Tittmann et al. [49] on acid-quench of pyruvate oxidase from *Lactobacillus plantarum*.

8. A stable radical derived from C2α-hydroxyethylidenethiamin diphosphate

The enzyme pyruvate ferredoxin oxidoreductase (PFOR) carries out the typical decarboxylation of pyruvic acid by ThDP to the enamine, followed by oxidation of the enamine via Fe_4S_4 clusters, leading to acetylCoA from the acetyl equivalent and CoASH. This is so far the only ThDP enzyme known to proceed by free radical chemistry [85]. The enzyme was recently reviewed by Ragsdale [86]. Fontecilla-Camps [28] reported the first X-ray structure for a PFOR, and followed it up with a remarkable structure of the enzyme with a stable radical signal (according to ESR) [29]. The authors interpreted the results in terms of a putatitve acetyl-ThDP

radical (a one-electron oxidation product of the enamine/2α-hydroxyethylidene) with the electron spin distributed mostly around the now non-planar thiazoline ring. The ring itself may have undergone tautomerization of a proton from the C4-methyl group to C5, while the double bond between C4 and C5 had migrated to the C4–$C4_{exomethylene}$ position. Another surprising feature was the remarkably long (>1.8 Å) C2—C2α bond.

The enzyme has a series of three Fe_4S_4 clusters spanning a large distance from electron acceptor to reoxidation. There are two plausible mechanisms for this interesting reaction:

$$\text{(a) Enamine} + Fe_4S_4 \rightarrow \text{acetylthiazolium cation radical} \quad (3)$$

$$\text{2-acetylthiazolium cation radical} + Fe_4S_4 \rightarrow \text{2-acetylThDP} \quad (4)$$

$$\text{2-acetylThDP} + \text{CoASH} \rightarrow \text{acetyl-CoA} \quad (5)$$

$$\text{(b) Enamine} + Fe_4\ S_4 \rightarrow \text{acetylthiazolium cation radical} \quad (6)$$

$$\text{CoASH} + Fe_4\ S_4 \rightarrow \text{CoAS}^{\cdot}\ \text{(a thiyl radical)} \quad (7)$$

$$\text{acetylthiazolium cation radical} + \text{CoAS}^{\cdot} \rightarrow \text{acetyl-CoA} \quad (8)$$

Model studies from this laboratory obtained evidence for the existence of the thiazolium cation radical from the enamine with an alkylated oxygen. Under these conditions, the spin density appears to reside principally at the C2α position, since a symmetrical dimer derived from a pair of such putative radicals being recombined via their C2α atoms could be isolated [87].

9. Summary and prospects

During the past few years several new approaches have been proposed for the detection of ThDP-bound intermediates. As the science of biochemistry is more and more high-resolution structure based, the ideal detection and characterization of such intermediates would be by X-ray crystallography or NMR methods directly on the enzyme during the reaction sequence. While tantalizing as future prospects and goals, their realization may take some years yet. For one, the size of ThDP enzymes is such that solution NMR methods face the challenge of very broad lines. At the other end of the electromagnetic spectrum, X-ray studies would need to have much higher resolution than hitherto achieved with ThDP enzymes to enable observation of proton positions. Since many of the key intermediates have multiple resonance contributions in a conjugated system, distances and bond angles for heavy atoms only are unlikely to provide the answers regarding proton positions such as in the 1′,4′-imino tautomer, the enamine, or even the C2-carbanion/ylide. On the other hand, in the experience of the authors, equipped with appropriate model systems, electronic spectroscopic methods (both absorption and CD) can be used not

only to monitor the presence of several intermediates, such as the enamine, the charge transfer bands corresponding to LThDP analogs, and the 1′,4′-iminoThDP, but can also afford time resolution on the stopped-flow timescale. The powerful method developed by the Halle group for evaluating the concentration of ThDP-bound intermediates, and the corresponding microscopic forward rate constants [50] affords determination of rate-limiting steps under a variety of experimental conditions for many ThDP enzymes.

Acknowledgments

Research in the authors' laboratory has benefited from financial support from NIH Grants GM-50380 and GM-62330.

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