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Progress in the experimental observation of thiamin diphosphate-bound intermediates on enzymes and mechanistic information derived from these observations

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Glyoxylate carboligase
1-Deoxy-D-xylulose-5 phosphate synthase
Benzoylformate decarboxylase
C2α-hydroxyethylthiamin diphosphate
C2α-lactylthiamin diphosphate

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/carbene/C2-carbanion of the thiazolium ring and the C2α-carbanion/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 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 several intermediates which are stable under acidic conditions, solid-state NMR and circular dichroism detection of the states of ionization and tautomerization of the 4′-aminopyrimidine moiety of ThDP in some of the intermediates. These methods also enabled in some cases determination of the rate-limiting step in the complex series of steps. This review is an update of a review with the same title published by the authors in 2005 in this Journal. Much progress has been made in the intervening decade in the identification of the intermediates and their application to gain additional mechanistic insight.

1. Introduction

Among the coenzymes derived from water-soluble vitamins, thiamin diphosphate (ThDP) 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 key ThDP-bound covalent intermediates, which enable both their synthesis and chemical trapping (acid quench) for eventual detection, ThDP enzymes provide both an opportunity and a challenge to the enzymologist wishing to observe and to study the catalytic competence of such intermediates. In addition, due to the special

Abbreviations: ThC, thiamin; ThDP, thiamin diphosphate; YPDC, yeast pyruvate decarboxylase from *Saccharomyces cerevisiae*; PDHc, pyruvate dehydrogenase complex; OGDHc, 2-oxoglutarate dehydrogenase complex; TK, transketolase; POX, pyruvate oxidase from *Lactobacillus plantarum*; AHAS, acetohydroxyacid synthase; ALS, acetolactate synthase; GCL, glyoxylate carboligase; BFDC, benzoylformate decarboxylase; BAL, benzaldehyde lyase; PFOR, pyruvate ferredoxin oxidoreductase; DXPS, 1-deoxy-D-xylulose 5-phosphate synthase; E1p-ec, the first component of the *E. coli* PDHc; E1p-h, the first component of the human PDHc; E1o-ec, the first component of *E. coli* OGDHc; E1o-h, the first component of human OGDHc; PDA, photodiode array; Sf, stopped-flow; CD, circular dichroism; IP, the 1′,4′-iminopyrimidine tautomer of ThDP or its C2-substituted derivatives; AP, the canonical 4-aminopyrimidine tautomer of ThDP or its C2-substituted derivatives; APH^+, the N1-protonated 4-aminopyrimidinium form of ThDP or its C2-substituted derivatives; Yl, the C2-carbanion/ylide/carbene form conjugate base of ThDP; HEThDP, C2α-hydroxyethylThDP, the adduct of acetaldehyde and ThDP; HBThDP, C2α-hydroxybenzylThDP, the adduct of benzaldehyde and ThDP; LThDP, C2α-lactylThDP, the adduct of pyruvic acid and ThDP; MThDP, C2α-mandelylThDP, the adduct of benzoylformic acid and ThDP; MAP, acetylphosphonic acid monomethyl ester; MBP, benzoylphosphonic acid monomethyl ester; PMThDP, C2α-phosphonomandelylThDP, the adduct of MBP and ThDP; PLThDP, C2α-phosphonolactylThDP, the adduct of MAP and ThDP; 3-PKB, (*E*)-4-(pyridin-3-yl)-2-oxo-3-butenoic acid; AcThDP, 2-acetylThDP, the oxidized form of HEThDP; PDC, pyruvate decarboxylase; E1p, the first (ThDP-dependent) component of PDHc; E1o, the first (ThDP-dependent) component of OGDHc; -ec, *E. coli*, *Escherichia coli* enzyme; -h, human enzyme.

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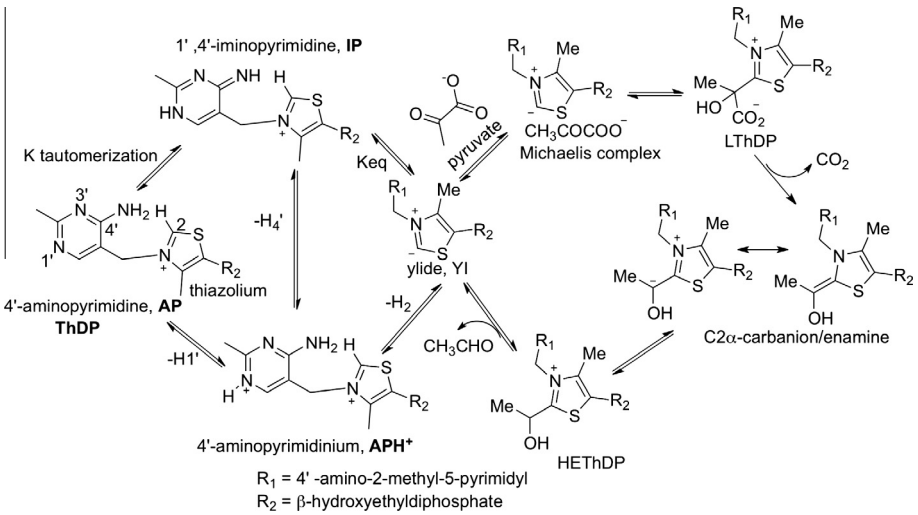
environment on the enzyme, several of these ThDP-derived intermediates give rise to circular dichroism (CD) signals, not seen in the absence of the enzymes. The authors' laboratory has during the past decades worked on 12 representatives of this large family of enzymes, starting with yeast pyruvate decarboxylase (YPDC, Scheme 1), and culminating with the ThDP-dependent first E1 components of both the human and *Escherichia coli* pyruvate dehydrogenase (E1p-h and E1p-ec) and 2-oxoglutarate (also known as α-ketoglutarate) dehydrogenase (E1o-h and E1o-ec) multienzyme complexes (Scheme 2 for the mechanism of the 2-oxoglutarate multienzyme complex). The goal of this review is to summarize data mostly from the authors' experience on these enzymes, and the authors apologize to those whose contributions have been overlooked, it was unintentional.

There are reviews appearing continuously on the enzymology of thiamin diphosphate (ThDP, the vitamin B1 coenzyme- see Fig. 1 for structures of small molecules mentioned in the review) [1–15], the purpose of this review is to summarize the current understanding of the ThDP-related intermediates on enzymes, including a fascinating, and perhaps unique aspect of thiamin enzymology, the existence of the rare 1′,4′-iminopyrimidinyl tautomeric form. This issue has been made more important by some of the X-ray crystal structure determinations of ThDP enzymes. The question raised by the finding is related to the conundrum that any plausible mechanism one can suggest for ThDP-dependent enzymes, whether they are 2-oxo acid decarboxylases or carboligases [see Schemes 1–3 for examples of a non-oxidative decarboxylase yeast pyruvate decarboxylase (YPDC; EC 4.1.1.1), an oxidative decarboxylase the 2-oxoglutarate dehydrogenase complex (OGDHc; EC 1.2.4.2), and a carboligase 1-deoxy-D-xylulose 5-phosphate synthase (DXPS; 2.2.1.7), respectively], requires some proton transfer steps. On the basis of accumulated understanding of enzyme mechanisms, such proton transfers are likely mediated by general acid–base catalysts, such as His, Asp, Glu, perhaps, Cys, Lys and Tyr, with the understanding that the enzyme active center could modulate the aqueous pK_a of these side chains, as needed.

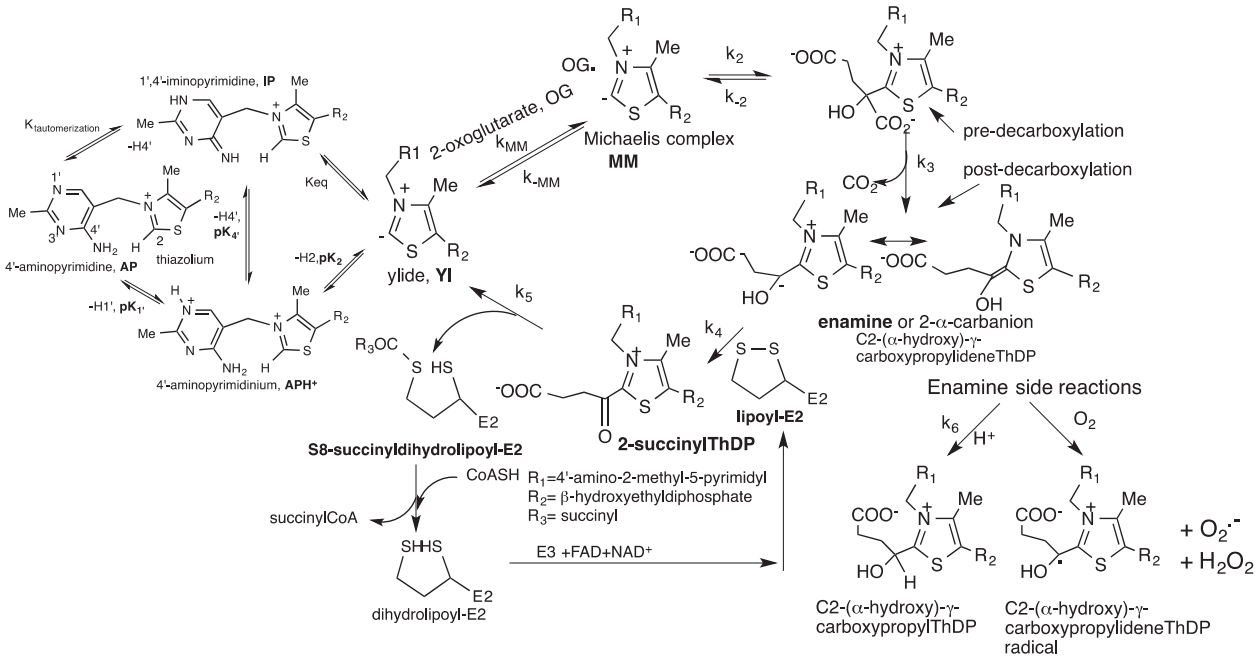
Several groups including the authors [16] spent considerable time trying to assign acid–base functions to such residues on ThDP enzymes – with limited success. More recently, McLeish and coworkers have been carrying out saturation mutagenesis experiments [17] probing the function of two active center histidine residues (His70 and His281) on benzoylformate decarboxylase (BFDC), long believed to participate in acid–base reactions [18]. Surprisingly, their results indicated that hydrophobic residues could replace the His281 with little penalty, and even with His70 there was only a 70-fold penalty on k_{cat}/K_m. In the authors' laboratory a somewhat similar behavior emerged on the E1o-ec, where of the two His residues (260 and 298) assigned (on the basis of the X-ray structure) to bind the 5-carboxyl group of the 2-oxoglutarate (OG) substrate, residue 260 could not, while residue 298 could be substituted with other amino acids, with little loss of activity according to saturation mutagenesis studies [19]. A reasonable question in the interpretation of such results is what is an appropriate contribution from His or Asp or Glu to reflect general acid–base reactivity on the enzyme? The authors prefer to use two well-explored examples which could provide benchmark values for acid–base catalysis on enzymes, although precise interpretation of these numbers is not only risky, but also depends on the particular substitution used to arrive at them [20]: (a) Serine proteases, where substitution of either His (a presumed general acid–base catalyst) or Ser (a nucleophilic catalyst) by Ala in the well-characterized Asp-His-Ser catalytic triad of subtilisin leads to ca. 2×10^6 reduction in k_{cat} [21]; and (b) Ketosteroid isomerase (EC 5.3.3.1), where substitution of the catalytic Asp38 by Asn leads to $10^{5.6}$ decrease in k_{cat} [22], while substitution of the same residue by Ala only reduced the k_{cat} by 140 [23].

Complicating this issue on ThDP enzymes is that the pH dependence of the steady state kinetic parameters does not give clear evidence for participation of such residues in the rate-limiting step(s). For example, all potential active center acid–base residues were substituted on YPDC [16], with little perturbation of the pH dependence of such plots, perhaps with the exception of the substitution at the conserved glutamate. Therefore, the 100–500-fold reduction in steady state kinetic constants could not be unequivocally attributed to acid–base function, while such numbers are certainly consistent with hydrogen bonding interactions.

The X-ray structure of two unusual ThDP-dependent enzymes was solved. The enzyme benzaldehyde lyase (BAL, EC 4.1.2.38) carries out reversible decomposition of (*R*)-benzoin to two molecules of benzaldehyde; in the reverse direction the enzyme is a carboligase. The X-ray structure of BAL contained only two acid–base residues surrounding the ThDP at the active center [24–26]: a highly conserved Glu50 within hydrogen bonding distance of the N1′ atom of the 4′-aminopyrimidine ring and a His29 residue. The residue His29 is too far from the thiazolium C2 atom to be of value in the first steps of the reaction and was suggested to have a function in removing the β-hydroxyl proton of the ThDP-bound benzoin to assist in releasing the first benzaldehyde molecule. In the authors'



Scheme 1. Mechanism of yeast pyruvate decarboxylase [81].



Scheme 2. Mechanism of OGDHc with alternative fates of the enamine [89].

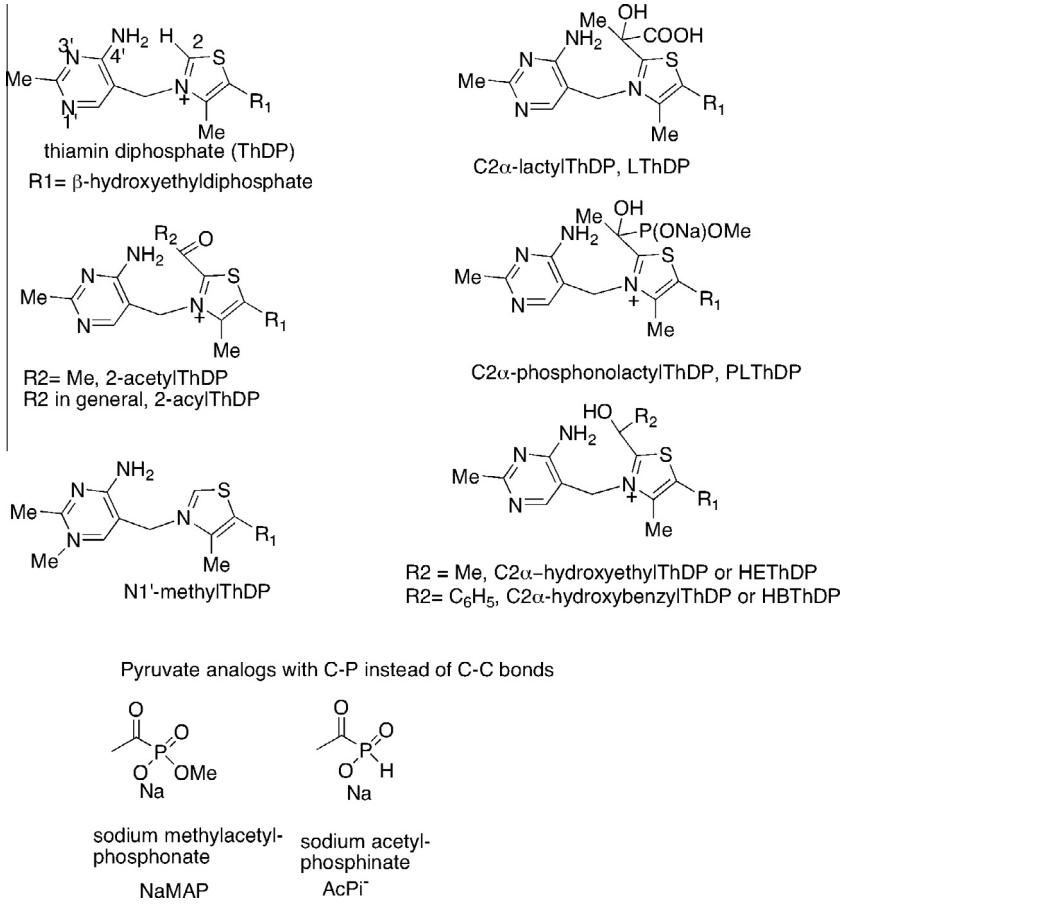
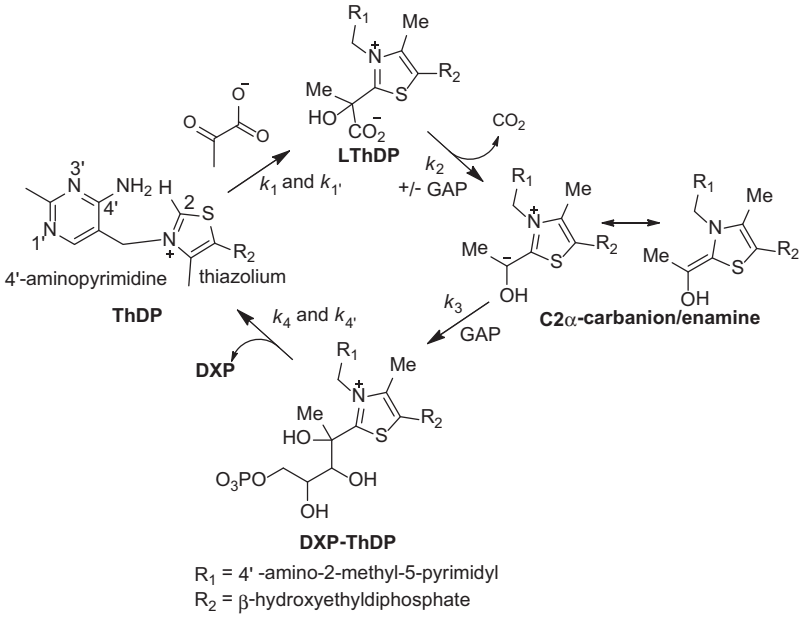


Fig. 1. Structure of small molecules mentioned in the review.

view, this enzyme provides the clearest interpretation of the pH dependence of the steady-state kinetic parameters of any ThDP enzymes to date. There is a pK_a = 5.3 at the acidic side of either the k_{cat}–pH or k_{cat}/K_m–pH profile, almost certainly corresponding to the highly conserved glutamate residue [27]. With this information in hand, the pH dependence of kinetic parameters on YPDC could be reexamined, suggesting that the conserved glutamate affected that behavior similarly. The second case reported even



Scheme 3. Mechanism of 1-deoxy-D-xylulose 5-phosphate synthase.

greater surprises: the enzyme glyoxylate carboligase (GCL; EC 4.1.1.47) carries out a carboligation reaction after decarboxylation of the first molecule of glyoxal to the enamine intermediate (Fig. 2). This enzyme is not only devoid of acid–base groups at its active center within hydrogen bonding distance of ThDP, *it is also lacking the highly conserved Glu* (Fig. 3). In its place there is a hydrophobic residue [28].

These two case studies suggest that our understanding of ThDP enzymes is not nearly as complete as many in the field have assumed (and continue to assume), and certainly suggest that the ThDP cofactor has a truly dramatic impact on the reaction pathway. With these results, the coenzyme and its chemical reactivity needed to be scrutinized from a newer vantage point.

2. Early evidence pointing to a catalytic function for the 4′-aminopyrimidine ring

The chemistry and enzymology of ThDP is intimately dependent on three chemical moieties comprising the coenzyme: a thiazolium ring, a 4-aminopyrimidine ring and the diphosphate side chain (Fig. 1). From the large number of high-resolution X-ray structures available for more than 20 years, starting with the structures of

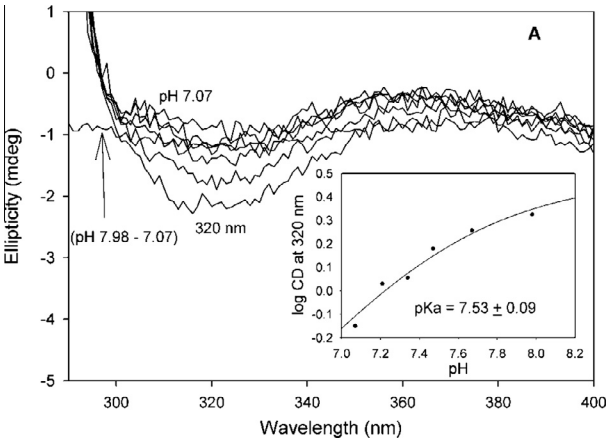


Fig. 3. pH titration of the AP form of ThDP on DXPS by CD, implicating the pK_a of the APH^+ form.

transketolase [29] (TK), pyruvate oxidase [30] (POX) from *Lactobacillus plantarum* and pyruvate decarboxylase from the yeast *Saccharomyces cerevisae* (YPDC) [31,32], it has become clear that the

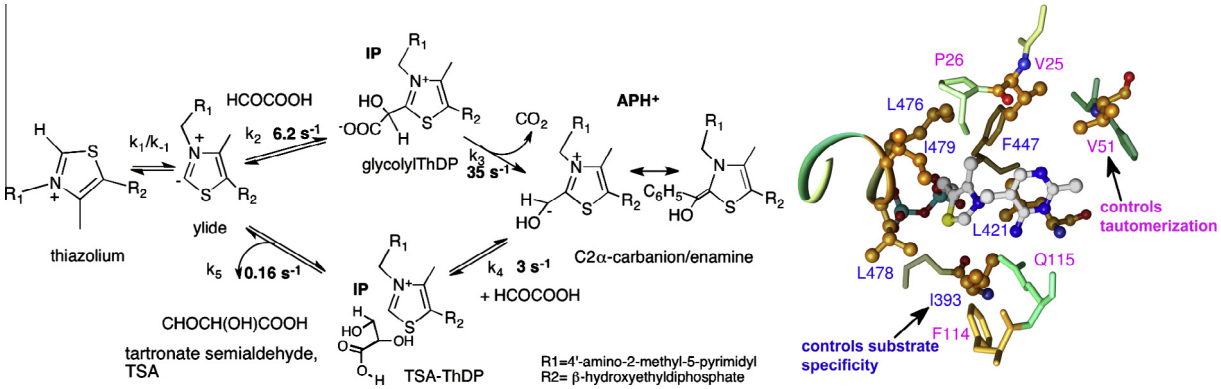


Fig. 2. Structure and mechanism of glyoxylate carboligase. Left. k_1 is formation of glycolyl ThDP, the pre-decarboxylation intermediate, positive CD band at 302 nm; k_2 is decarboxylation to enamine; k_3 is carboligation with glyoxylate to provide TSA–ThDP, the post-decarboxylation intermediate, positive CD band near 300 nm, the values represents the average from three independent experiments; k_4 is release of TSA product from ThDP.GCL. k_1, k_3 and k_4 determined at 6 °C, k_2 at 15 °C. Right. Structure of the active center of GCL demonstrating the hydrophobic environment of the thiazolium ring of ThDP (RCSB structure 2PAN) [65].

diphosphate serves to bind the cofactor to the protein. This is achieved via electrostatic bonds of the α and β phosphoryl group negative charges with the required Mg^{2+} or Ca^{2+}, the divalent metal serving as an anchor in a highly tailored environment with a universally conserved GDG recognition site and the diphosphate–Mg^{2+} binding motif consisting of GDG-X-$_{26}$-NN sequence of amino acids suggested by the Perham group [33]. As shown in a series of seminal papers by Breslow, the thiazolium ring is central to catalysis [34], due to its ability to form a key nucleophilic center the C2 atom, the C2-carbanion/ylide or carbene – depending on one's viewpoint regarding the relative importance of the resonance contributions. The demonstration that the thiazolium C2H can undergo exchange with D_2O, and that thiazolium salts *per se*, even in the absence of the 4′-aminopyrimidine ring can induce benzoin condensations in a manner analogous to the cyanide ion catalyzed benzoin condensation, led to the proposal of the pathway involving thiazolium-bound covalent intermediates as also drawn in Schemes 1–3 and Fig. 2-left. The carbene had been generated by Arduengo and coworkers under strongly basic conditions (35), but only in 2013 was the carbene reported from X-ray studies by Tittmann and collaborators (36). Does the 4′-aminopyrimidine participate in catalysis? It had been estimated that the protein environment of YPDC provides a catalytic rate acceleration of 10^{12}–10^{13} [37]. Is this simply a result of juxtaposition of amino acid side chains to provide the general acid–base catalysis, or an enzymatic solvent effect [11,12], and does it include a contribution from the special properties of ThDP when enzyme bound?

Starting in the 1960s, Schellenberger and Hübner, and their coworkers examined the role of the 4′-aminopyrimidine ring [7]. Most notably, they undertook *de novo* synthesis of thiamin diphosphate analogues replacing each of the three nitrogen atoms of the 4′-aminopyrimidine ring in turn. They then tested each of these deaza analogues for coenzyme activity on a number of enzymes. The results clearly indicated that the N1′ atom and the N4′-amino group are absolutely required, the N3′ atom less so. On the basis of application of this powerful probe to a number of ThDP enzymes, their group made the totally reasonable suggestion that the 4′-aminopyrimidine ring has a catalytic role; not only does it serve as an anchor to hold the coenzyme in place. What was not obvious is how the exocyclic amino group could participate in acid–base chemistry. A mechanism for activating the 4′-amino group was suggested from authors′ laboratory with a synthetic model in which the mobile proton at the N1′ position (the principal site of first protonation of the 4′-aminopyrimidine) was replaced by a methyl group, creating N1′-methylthiaminium and N1′-methylpyrimidinium salts, then showing that the positive charge installed at the N1′ position converted the amino group to a weak acid with pK_a of nearly 12–12.5 in aqueous solution [38]. This implicitly raised the possibility of the existence of the 1′,4′-iminopyrimidine tautomer for the first time, not suggested before, and was important since among protonation sites in aminopyridines and aminopyrimidines, such as the nucleic bases, ring nitrogen protonation is preferred over protonation of the exocyclic amino group. The hypothesis suggesting the 4′-aminopyrimidine moiety as an important contributor to catalysis, and the possibility for its participation in acid–base catalysis [38] has gained wider acceptance since the observation of some common features in the X-ray structures of ThDP enzymes: (a) Strong hydrogen bonds from the protein to both the N1′ atom (via a conserved Glu with the exception of the enzyme GCL so far), and to the N4′-amino nitrogen atom on the side of the N3′ atom of the ring; (b) An unusual V coenzyme conformation (describing the disposition of the 4′-aminopyrimidine and thiazolium rings with respect to the bridging methylene group [39]) rarely observed in model ThDP structures and predicted to be in a high energy region in van der Waals conformational maps [40]; and (c) A surprisingly short <3.5 Å distance between the 4′-aminopyrimidine amino nitrogen atom and the thiazolium C2 atom, suggesting the possibility if intramolecular proton transfer between the two sites, perhaps mediated by a water molecule.

3. Detection of intermediates on ThDP enzymes

There are now available different methods to monitor the kinetic fate of each covalent ThDP-substrate intermediate along the catalytic cycle of various ThDP enzymes represented by examples in the Schemes [11,12,14]. Tittmann and Hübner proposed a powerful method for determination of rate constants for individual steps (henceforth the TH method, to be described later) [41]. Circular dichroism (CD), both steady state and time-resolved, affords the possibility of monitoring the time course of intermediate formation and depletion directly on the enzymes to the ms time scale. Below is presented an update of the observation of these intermediates and a brief summary is given of the presence of various ThDP intermediates on the enzymes, including the state of ionization and tautomerization of the 4′-aminopyrimidine ring on these intermediates. Understanding these issues is important to monitor proton movements during catalysis.

It is convenient to review ThDP-related and ThDP-bound intermediates, as being present pre-, or post-substrate (or substrate analogue) binding.

3.1. ThDP-related intermediates prior to substrate addition

While the TH method could identify several of the covalent ThDP-bound substrates and products on the pathway, the tautomeric forms and ionization states of the 4′-aminopyrimidine ring along the reaction pathway and under the reaction conditions remained to be elucidated. Additionally, as will be detailed below, the TH method depends on identification of intermediates after they are released from the enzymes.

3.1.1. The 4′-aminopyrimidine (AP) form of ThDP

The signature for the AP form is a negative CD band centered near 320–330 nm [53]. While long observed on the enzyme transketolase (TK) [42], this CD band was believed to result from a charge transfer transition between ThDP and an amino acid side chain on TK, although early reports attributed it to the ThDP itself. Studies by the authors' group on YPDC and E1p-ec and their variants, as well as chemical model studies, suggested that, in fact, this UV/CD band is due to a charge transfer transition between the 4′-aminopyrimidine ring as donor and the thiazolium ring as acceptor [43,44]. The band has been observed on several ThDP enzymes, and, the ability to observe it depends more on the pH of measurement, and to a significant extent on the enzyme environment.

3.1.2. The 1′,4′-iminopyrimidine (IP) form of ThDP [27,43–46]

The notion that the 4′-aminopyrimidine could exist in the 1′.4′-iminopyrimidine tautomeric form was suggested earlier by models attempting to mimic the reactivity of such a tautomer. In the N1′-methylpyrimidinium the pK_a of the exocyclic amine is reduced to ca. 12–12.5 [38,47], offering rationalization for the presence of conserved glutamate as a catalyst for the amino ⇌ imino tautomeric equilibration.

The first evidence for a spectroscopic signature for the IP tautomer was found by Sergienko on the slow E477Q YPDC variant [45]. In a series of studies, Zhang [45] and later Baykal [47] showed that an appropriate chemical model for the 1′,4′-iminopyrimidine will give rise to a UV absorption in the 300–310 nm range. Serendipitously, the ^{15}N chemical shifts of the three species on the left hand side of Schemes 1 and 2, the two neutral and one positively

charged forms of the 4′-aminopyrimidine are quite distinct [47] (early ^{15}N NMR experiments were reported on this issue by Roberts and coworkers [48]).

The recognition that the CD bands corresponding to the AP and IP forms have different phases, enables simultaneous observation of the two tautomeric forms notwithstanding the proximity of the bands to each other, and also makes CD the method of choice for such studies. The signature for this IP species is a positive CD band centered near 300–314 nm and is well illustrated on the first component of the human pyruvate dehydrogenase complex (E1p-h), where both the IP and AP tautomeric forms can be observed simultaneously [46].

3.1.3. The N1′-protonated conjugate acid (APH$^+$) form of ThDP

To the authors' knowledge, no electronic absorption characteristic of the APH$^+$ form or the ylide/carbene (Yl) has yet been proposed. In view of this, and in view of the large size of ThDP enzymes (at least 120 kDa mass), the authors' group collaborated with Prof. T. Polenova's group at the U. of Delaware and carried out solid-state NMR studies seeking information on both the Yl and APH$^+$ forms [49,50]. For these experiments, specifically ^{15}N and ^{13}C labeled ThDP had to be synthesized *de novo*. With [N4′–^{15}N]ThDP, [C2-^{13}C]ThDP and [C2,C6′-$^{13}C_2$]ThDP in hand, they were added to the apo-forms of YPDC, E1p-ec and E1o-ec, identifying on each of the three enzymes the APH$^+$ ionization state, and importantly confirming interpretation of the pH titrations on the AP and IP forms. The method did yield other important conclusions, but it is more difficult to use than others at our disposal. Yet, for issues such as the presence of the APH$^+$ form, there appears to be no other option. Preliminary studies with [N4′–^{15}N]ThDP indicated that the amino nitrogen atom is in an environment in-between that of the IP and APH$^+$ forms in the presence of a substrate analogue, suggesting that even at −20 °C, the ^{15}N nucleus is still in the fast-exchange regime. On the other hand, the vast differences in the ^{15}N chemical shifts of the IP, AP and APH$^+$ forms, and given that interpretation of the solid state ^{15}N NMR spectra depends exclusively on the chemical shift, the method has tremendous promise as a complement to X-ray structure of proteins [49–51].

3.1.4. Determination of the pK_a for the enzyme-bound APH$^+$ form [27]

As the pH is lowered in Fig. 3, the amplitude of the band for the AP form diminishes and titrates with an apparent pK_a = 7.53 for the (AP+IP)/[APH$^+$] equilibrium on DXPS [27]. This pK_a in water for ThDP is 4.85 [48], while on the enzymes it ranges from 5.6 to 7.5 (Table 1, Refs. [27,52]). It was concluded from data in Table 1, that the pK_a for the APH$^+$ coincides with the pH of optimum activity for each enzyme, indicating that all three forms IP, AP and APH$^+$ must be readily accessible during the catalytic cycle. The pK_a elevation on the enzymes could be rationalized by the presence of the highly conserved glutamate near the N1′ position of ThDP (residue E571 on E1p-ec), that would tend to make the 4′-aminopyrimidine ring more basic. The tautomeric equilibrium constant $K_{tautomer}$, in conjunction with the pK_as led to novel insight regarding ThDP catalysis, best viewed by the thermodynamic box for enzymes that are not substrate activated (left hand side of Schemes 1 and 2), such as E1p-h [46] and pyruvate oxidase from *L. plantarum* (POX) [53]. For these enzymes, both the IP and AP forms could be monitored over a wide pH range, providing both pK_a and $K_{tautomer}$ within reasonable error limits. The equilibria shown in the left hand side of Schemes 1 and 2 are valid prior to addition of substrate and lead to the following tantalizing conclusions: (a) On POX and E1 p-h, p$K_{1'}$ and p$K_{4'}$ have similar magnitudes; the enzymes shifted the p$K_{4'}$ from 12 in water [38] to 5.6 and 7.0, respectively! [27]. (b). With a known forward rate constant from APH$^+$ to the ylide (Yl) of ~50 s^{-1} determined for E1p-h [54], and assuming a diffusion-controlled reverse protonation rate constant of 10^{10} s^{-1} M^{-1} (giving a pK_2 of 8.3 on E1p-h compared to an estimate in water of 17–19 [55]), one could next speculate about the right triangle in Schemes 1 and 2. The most important conclusion is that the proton-transfer equilibrium constant for [IP]/[Yl] is 10^1–10^2 on E1p-h [27,46]. These thermodynamic parameters are the first estimates on any ThDP enzyme and should be generally applicable to such enzymes; the results also suggested conditions under which a significant fraction of the thiazolium ring may be in the Yl form [53]. The solid state NMR results also inform that when the AP form is observable by CD, at pH values near and below the pK_a there is indeed present as well the APH$^+$ form.

Table 1
pK_a of enzyme-bound APH$^+$.

Enzyme	pK_a ([AP] + [IP])/(APH$^+$)
BAL	7.42 ± 0.02
BFDC	7.54 ± 0.11
POX	5.56 ± 0.03
E1p-h	7.07 ± 0.07
E1o-ec	7.2 ± 0.01
GCL V51D	6.1 ± 0.02
DXP synthase	7.5 ± 0.09

3.1.5. The C2-carbanion/ylide/carbene

According to the Breslow findings, proton loss at the thiazolium C2 position is required to initiate the catalytic cycle [34]. In 1997 there were two reports with significant implications regarding this issue: (i) Arduengo and colleagues showed that the conjugate bases of imidazolium and indeed of thiazolium salts could be generated and their structure could be studied by NMR methods [35]. Some of these carbenes have in the intervening years been used in organometallic reactions, including olefin metathesis. Arduengo and coworkers showed that the ^{13}C chemical shift of the C2 resonance shifted from 157 to 253 ppm on conversion of their model thiazolium compound to its conjugate base, thereby providing the all important guide for future attempts to observe the ylide. (ii) Solution NMR studies on YPDC reconstituted with [C2-^{13}C]ThDP suggested that the thiazolium ring C2H of bound ThDP is in its undissociated state both in the absence and in the presence of the substrate activator surrogate pyruvamide, i.e., no evidence was found for the presence of the conjugate base in the activated or unactivated forms of the enzyme [56]; (iii) Recent X-ray evidence on pyruvate oxidase revealed a thiamin structure that was interpreted to be consistent with the carbene ionization state [36]. All of these results are intriguing and certainly should inspire further studies on these issues, in view of the large size of the enzymes for solution studies, difficulty in some cases with exchange of unlabeled and labeled ThDP, and the need for *de novo* synthesized labeled ThDP.

A different approach has been developed by Leeper and coworkers over the past decade where the N3 atom of the thiazolium ring of ThDP was replaced by a neutral ring, initially a carbon atom leading to a thiophene ring leading to 3-deazathiamin diphosphate. By virtue of the neutral ring in place of the positively charged thiazolium ring, the authors suggested that this would serve as an electrostatic mimic of the carbene [57,58]. Later the thiazolium ring was replaced by a triazole ring in yet a different mimic of a neutral 5-membered ring [59].

3.2. Thiamin-bound intermediates with substrate or substrate analogue present

3.2.1. The Michaelis Menten complex (MM)

Earliest detection of an MM complex in the authors' laboratory was on addition of a substrate analogue methyl acetylphosphonate (MAP) and acetylphosphinate (AcP) to several ThDP enzymes

[44,60]. An example is shown with acetylphosphinate added to YPDC (Fig. 4) leading to a negative CD band at ca. 325–335 nm, very reminiscent of the band observed for the AP form. In this example, addition of ThDP alone (curve 1) did not display the AP form, the negative CD band only appeared after addition of substrate analogue, hence the band must pertain to a MM type complex [60,61]. Similar results were also seen when low concentrations of pyruvate were added to E1p-ec [44].

A clear example of the formation of the MM complex with substrate itself was apparent from the appearance of a negative CD band centered near 330–335 nm when pyruvate was added to the low reactivity inner active center loop E1p-ec variants [60,61]. Important support for the claim that the MM was indeed being detected was provided by kinetic measurements: both stopped-flow photodiode array (PDA) spectra in the absorption mode, and stopped-flow CD spectra at the appropriate wavelength, showed formation of the absorbance/CD band attributed to MM formation, within the dead-time of the stopped-flow instruments (<1 ms), as expected of a non-covalent MM [60,61].

The 3-deazaThDP synthesized by the Leeper group enabled observation of the MM complex with substrates in a E1p from *Geobacillus stearothermophilus* [57] and in indolepyruvate decarboxylase [58] and the residues important in maintaining the MM complex.

3.2.2. The covalent substrate-ThDP pre-decarboxylation complex (LThDP and analogues)

3.2.2.1. Observation of pre-decarboxylation intermediate derived from aliphatic 2-oxo acids. The TH method enabled observation of LThDP directly under steady state conditions where decarboxylation to the enamine was slowed down. Three clear examples emerged.

On *L. plantarum* POX, there was significant concentration of LThDP found in the active centers [41] after acidification and precipitation of protein.

The enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXPS), the first enzyme in the non-mevalonate pathway of isoprenoid biosynthesis, carries out carboligation of pyruvate as acetyl donor subsequent to ThDP-catalyzed decarboxylation with D-glyceraldehyde 3-phosphate (D-GAP) as acceptor. CD experiments, complemented with the TH method to assist the CD assignments, revealed that formation of LThDP is rate-limiting overall in the sequence of reactions carried out by DXPS [63,64]. However, LThDP is remarkably stable on the enzyme until the acceptor D-GAP is added. Addition of D-GAP increased the rate constant of decarboxylation by 600-fold. This is a particularly interesting case

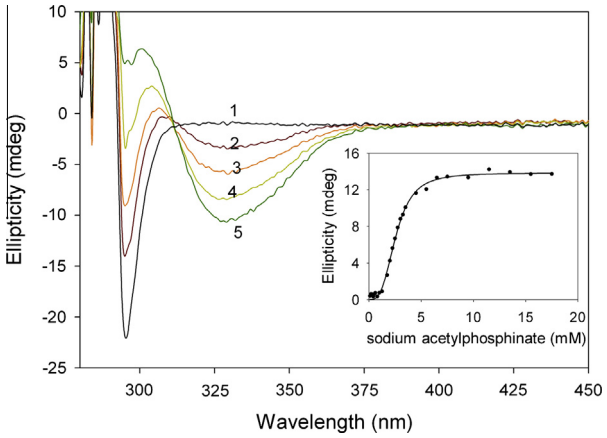


Fig. 4. Near-UV CD spectra of YPDC. YPDC on its own (spectrum 1) and in the presence of acetyl- phosphinate (2–5). In evidence is 1′,4′-iminophosphino-lactyl-ThDP at 302 nm and Michaelis complex at 328 nm. Inset: concentration dependence for 1′,4′-iminophosphino-lactyl-ThDP formation.

mechanistically, related to glyoxylase carboligase (GCL), where ThDP-catalyzed decarboxylation of the donor substrate is coupled to carboligation with the acceptor substrate [65].

Formation of the pre-decarboxylation complex on the V51D GCL variant was detected on addition of glyoxylate, according to the IP form of the adduct. This substitution is an attempt to recapitulate the role of the missing conserved glutamate in this enzyme [65].

3.2.2.2. Observation of pre-decarboxylation intermediate derived from aromatic 2-oxo acids. In some favorable cases, such as with BAL, the CD band for the pre-decarboxylation intermediate (via the IP form) could be observed from the slow substrates benzoylformate or phenylpyruvic acid [66]. This is plausible since BAL, while a carboligase/lyase enzyme, also catalyzes decarboxylation of aromatic 2-oxo acids, albeit very slowly.

3.2.2.3. Observation of stable pre-decarboxylation intermediates derived from substrate analogue phosphonates and phosphinates. The initial identification of the IP form (positive CD band, 300–314 nm) resulted from formation of a stable pre-decarboxylation adduct of ThDP with (*a*) MAP or acetylphosphinate [43,44] ($CH_3C(=O)P(H)O_2Na$, AcP^- [43]), with pyruvate-specific enzymes and (*b*) the aromatic 2-oxo acid analogue methyl benzoylphosphonate (MBP) with BFDC and BAL [26,67]. With 12 enzymes tested so far, the IP form appeared on the stopped-flow time scale (either absorption or CD mode): the reaction is efficiently catalyzed by all of the enzymes. There is an important additional finding shown in Fig. 4 resulting from mixing YPDC and AcP [46]: since we are seeing evidence for coexistence of the MM and the covalent pre-decarboxylation intermediate, the results are consistent with 'alternating active site reactivity' suggested for YPDC and BFDC [68–70].

Formation of phosphonomandelylThDP (PMThDP) on BFDC from MBP and ThDP was also confirmed in solution (FT-MS) [71], and of PLThDP (from MAP.ThDP) by X-ray methods on E1p-ec [72] and on POX [73].

3.2.2.4. Observation of pre-decarboxylation adducts of ThDP with chromophoric substrate analogues. On three enzymes, YPDC, BFDC and BAL, formation of the pre-decarboxylation adduct formed with ThDP from a chromophoric substrate analogue (*E*)-2-oxo-4(pyrid-3-yl)-3-butenoic acid (3-PKB) (as well as its ortho- and para isomers) was observed.

With a stopped-flow photodiode array (SF-PDA) instrument, two transients resulted on mixing YPDC with 3-PKB: the first transient termed T_1 was assigned to the pre-decarboxylation intermediate analogous to LThDP (LThDP* with λ_{max} near 470 nm), while the second transient T_2 was assigned to the enamine (λ_{max} near 430 nm). The second transient T_2 was formed at the same rate as the rate of depletion of T_1 (Fig. 5). In a series of studies on BAL [67], BFDC [74] and YPDC [53,67], this compound provided outstanding information about the rates of formation of these two important intermediates, not readily available from other experiments. Where applicable, this method is complementary to the TH method, which could not differentiate the enamine from the HEThDP in Scheme 1.

Extensive experimental evidence supported the assignment of intermediates to the observed spectral features. For example, in the case of BAL, simultaneous formation of both the IP form at 310 nm and of the 475 nm band from (*E*)-3(pyrid-3-yl)-2-propenal (PAA, the product of decarboxylation of 3-PKB) could be observed by CD affirming that the C2α-tetrahedral pre-decarboxylation intermediate is accompanied by the IP tautomer. In contrast, no band at 300–314 nm was apparent with the enamine on any of the three enzymes studied, suggesting that the enamine is in the

APH^+ ionization state. Essentially the same absorbance resulted when 3-PKB (477 nm) or its decarboxylated product PAA (473 nm) was added to BAL or BFDC, suggesting a similar type of C2α-tetrahedral intermediate either the LThDP-like (predecarboxylation) or the HEThDP-like (post-decarboxylation, resulting from protonation of the enamine at C2α). Brandt at Brandeis succeeded in growing crystals of BFDC with both PAA and 3-PKB and solved the structure to high resolution [74]. The structure with PAA clearly indicated (a) covalent binding to ThDP as the C2α-hydroxymethyl derivative with the vinylpyridyl substituent attached to the C2α atom, (b) a tetrahedral rather than trigonal environment at that atom since the planar thiazolium and vinylpyridine rings were not coplanar with each other – as would be expected if the electron density corresponded to the enamine.

3.2.3. The first post-decarboxylation intermediate: the enamine/C2α-carbanion

3.2.3.1. Observations of enamines with aromatic and longer conjugation at C2α. According to Schemes 1 and 2, this is the only covalent ThDP-bound intermediate with a conjugated system. Electronic spectral observation of the enzyme-bound enamine derived from aliphatic substrates is difficult due to the expected λ_{max} near 290–295 nm, according to thiazolium-based models [11,12].

The enamine intermediate derived from benzoylformate (modeled with λ_{max} of 380 nm, Refs. [11,12]) could be observed directly on the enzyme BFDC at 390 nm [74]. While BFDC converts benzoylformate to benzaldehyde, the enzyme catalyzes the reaction in the reverse reaction and the enzyme, as BAL, has been used in the chemo-enzymatic synthesis of α-ketols. When reacting the benzaldehyde product with BFDC, there appeared an absorbance (and a CD band) at 390 nm, in the wavelength region predicted by models [11,12], but no CD band was evident in the 300–310 nm region [74]. Also, when (*R*)-benzoin was added to BAL, there was formed the same CD band at 390 nm indicating slow release of the first benzaldehyde, and the stability of the enamine in the forward direction [66]. These experiments provided fundamental information: (a) The 'real' enamine could be observed (due to its long λ_{max} at 390 nm) for the first time derived from benzoin or benzaldehyde; (b) The enamine was found to be in its AP or APH^+, but not in its IP form; and (c) Since it gives rise to a CD signal, the enamine is chiral on the enzyme, even though it is planar and conjugated.

With YPDC, BFDC and BAL, the enamine could be observed directly near 430 nm from 3-PKB as alternate substrate on YPDC (Fig. 5).

3.2.3.2. Observations of enamines with aliphatic and unconjugated side chains at C2α. The enamine has also been detected indirectly using the TH method [41] on the E1p-ec. The method takes advantage of the known stability of the ThDP-bound covalent intermediates in Schemes 1 and 2 under acidic conditions, so that using either rapid quench or manual quench methods one can 'freeze' the intermediates under acidic conditions while also precipitating the enzyme. Under acidic conditions (all substituted ThDPs in the APH^+ form), the ^1H chemical shifts of the C6′H resonances of each intermediate are sufficiently distinct from each other, and from that of the unsubstituted ThDP, enabling quantification of the relative concentration of the intermediates under steady state conditions. The only intermediate that does not lend itself to direct detection by the method is the enamine, which is converted to C2α-hydroxyalkylThDP derivatives under acid quench. In the absence of the E2 component, or an external oxidizing agent, the enamine is apparently long lived, and since HEThDP is not on the pathway, we assume that its presence is a result of the acid quench of the enamine. The method is demonstrated with a comparison of results obtained with E1p-ec and the entire PDHc-ec complex (Fig. 6), where the synthetic [C2,C6′-$^{13}C_2$]ThDP was used enabling measurement of the rate of enamine formation via the HEThDP [75]. The labeled ThDP allowed observation of only those protons directly attached to ^{13}C nuclei, simplifying analysis in this otherwise busy aromatic region, especially for the complex, in which there are additional aromatic moieties (FAD, NADH, coenzyme A). The accumulation of the enamine/HEThDP, but not of LThDP, suggests that decarboxylation is faster than LThDP formation. Also, for this particular variant, assembly to the complex appears to accelerate the rate by a modest factor.

On addition of 2-oxoglutarate to the E1o-h and E1o-ec, a new absorbance/positive CD signal was observed near 350 nm and attributed to the enamine resulting from decarboxylation as in Fig. 7 [79,89]. Given that the λ_{max} of the enamine derived from C2α-hydroxyalkyl thiazolium salts is expected near 290–295 nm [76], several steady state and time-resolved experiments were carried out to confirm the assignment. Given that the same spectral signature was not seen with 2-oxovalerate or 2-oxoisovalerate (or on E1p-ec or E1p-h with a variety of aliphatic 2-oxo acids), it was deduced that the unusual stability of the enamine, as well as its very much red-shifted λ_{max} are the result of some interaction between the C2α-C or the C2α-OH with the δ-carboxylate of the substrate, making this observation unique for 2-oxoglutarate.

3.2.4. The second post-decarboxylation intermediate, the non-oxidative product-ThDP covalent complex (HEThDP, HBThDP)

Clear evidence was obtained for HEThDP-analogue formation from reacting 3(pyrid-3-yl)-acrylaldehyde (PAA) (the product of decarboxylation of 3-PKB), with BAL or BFDC [74]. An absorbance with λ_{max} 470 nm appeared (similar to that observed with 3-PKB in Fig. 5), and was attributed to the HEThDP analogue. X-ray results

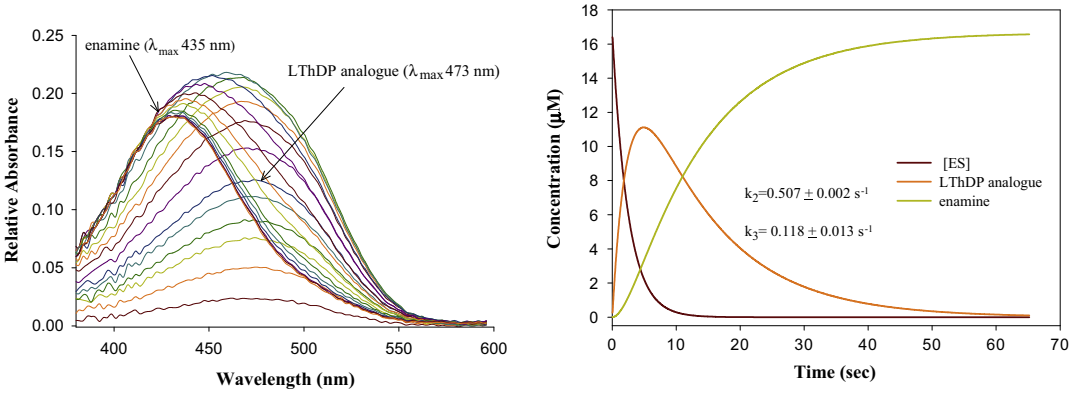


Fig. 5. Stopped-flow photodiode array spectra on mixing YPDC and 3-PKB. Left. YPDC (33.2 μM active-sites) mixed with 20 mM 3-PKB. Spectra recorded every 40 ms. Right. Time course of concentration changes of intermediates from deconvolution; $[ES]_o$ = active sites.

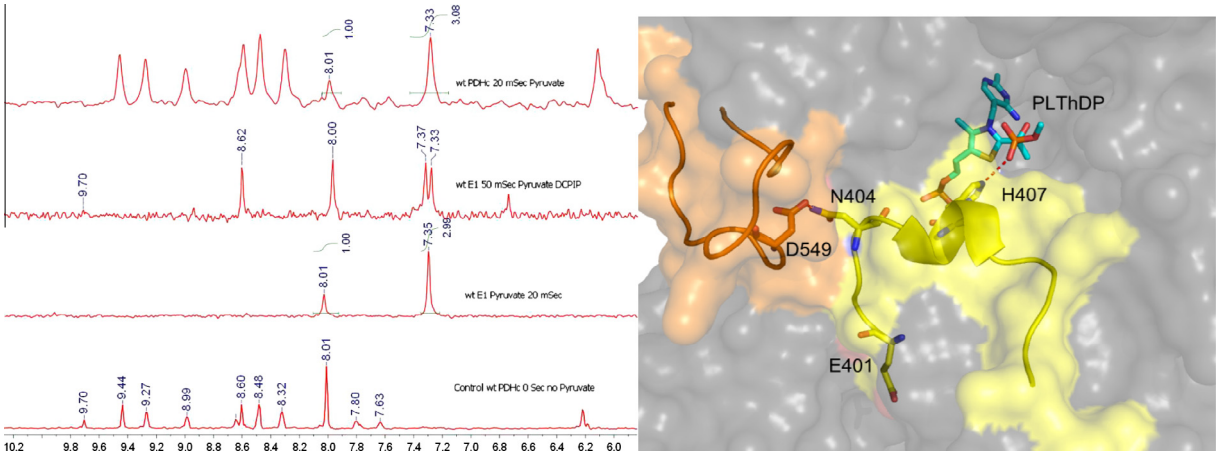


Fig. 6. Distribution of ThDP-bound covalent intermediates in reactions of E1p and PDH complex. gCHSQC NMR spectra of the supernatant after acid quench of PDHc and removal of protein from the reaction. From Top (First): 20 ms quench of the reaction of PDHc. (Second): 50 ms quench of the reaction of E1p with pyruvate and DCPIP. (Third): 20 ms quench of the reaction of E1p with pyruvate. (Fourth) control: PDHc reaction mixture quenched before addition of pyruvate. ThDP-derived peaks are marked at 9.71 ppm (C2-H), 8.62 ppm (AcThDP), 8.01 ppm (C6′-H), 7.37 ppm (AcThDP) and 7.34 ppm (HEThDP). Other peaks are due to NAD^+, CoA, DCPIP, NADH, acetyl-CoA or $DCPIPH_2$.

confirmed formation of the C2α-tetrahedral post-decarboxylation intermediate on addition of PAA to BFDC [74].

A striking confirmation of HEThDP formation resulted from mixing acetaldehyde, the product of pyruvate decarboxylation, with YPDC on the SF-PDA instrument [53], giving the characteristic absorption for the IP form, λ_{max} = 310 nm. Here, there is simply no alternative assignment than to the IP form of HEThDP (see Scheme 1).

In addition to the enamine with λ_{max} near 350 nm, mixing E1o-ec or E1o-h with 2-oxoglutarate also produces a positive CD band with λ_{max} near 300, identified by the TH method to correspond to the C2α-tetrahedral post-decarboxylation intermediate C2-(C2α-hydroxy)-γ-carboxypropylThDP, formed at a slower rate than the enamine, i.e., it is derived from the enamine (Fig. 7).

3.2.5. 2-Acetylthiamin diphosphate (2-AcThDP) and 2-succinylthiamin diphosphate

The 2-acyl thiamin diphosphates are the results of 2-electron oxidation of the enamine most commonly by the dithiolane ring of lipoic acid (covalently amidated onto a lysine side chain in the 2-oxo acid dehydrogenase multienzyme complexes); less frequently by FAD in the pyruvate oxidases – these come in two flavors, forming acetate in *E. coli* and forming acetylphosphate in *L. plantarum*; finally by NAD^+.

The mechanism of oxidation is not the topic of discussion here. The reaction in POX has been studied in detail and most recent evidence suggests that the oxidation takes place via single electron transfers with the likely intermediacy of the radical cation species delocalized onto the thiazolium ring (see next section).

Oxidation of the enamine by lipoic acid is usually referred to as the reductive acetylation of lipoyllysyl-E2. An important mechanistic issue of whether electron and group transfer take place in a single step via a tetrahedral intermediate (essentially 'cross-linking' the ThDP-bound enamine on the E1 component with the lipoyl group on E2), or stepwise (where oxidation to the 2-acetylThDP with concomitant reduction of lipoamide-E2 to dihydrolipoamide-E2 precedes acyl transfer to the S8 atom of the dihdyrolipoamideE2) was investigated by Frey and coworkers [77]. Using a number of ingenious ways to generate acetylThDP (for example, by reversing the reaction by addition acetylCoA), they provided evidence that the first option, redox followed by group transfer is the likely scenario [77]. Model studies by Pan and Jordan [78] suggested a concerted mechanism. More recent studies by Anand et al. [75] concluded that in the absence of the E2p-ec component, but with an external oxidizing agent 2,6-dichloropheno-indophenol (DCPIP), the 2-acetylThDP is an intermediate on E1p-ec, but in the presence of both E2p-ec and E3-ec, there is no evidence for the intermediate, suggesting instead that the reductive acetylation is direct with no intermediate. Similarly, the presence of DCPIP in the reaction of E1o-ec with OG also creates the 2-electron oxidation product, 2-succinylThDP [79,89].

The 2-acetylThDP was also produced from fluoropyruvate on E1p-ec [80], presumably by fluride elimination from the enamine, and revealed a new positive CD band centered at 390 nm as signature for the 2-acylThDP's derived from unconjugated 2-oxo acids [81].

3.2.6. The C2α-hydroxyethylideneThDP cation radical and the C2(C2α-hydroxy)-γ-carboxypropylidene cation radical

Early evidence for the existence of a C2α-hydroxyethylideneThDP radical and a free-radical mechanism on ThDP enzymes was obtained on pyruvate-ferredoxin oxidoreductase (PFOR) [82–85], an enzyme that converts pyruvate to acetylCoA in anaerobes. In addition to ThDP, the enzyme has three Fe_4S_4 clusters forming a 40–50 Å long electron transfer chain. The stability of the free radical was manifested by the fact that the crystal also displayed an electron paramagnetic resonance (EPR) signal. A chemical model was generated for the electrochemical oxidation of the enamine leading to dimerization at the C2α atom, suggesting significant electron spin density at this atom. Subsequent detailed work on PFOR clearly showed that the spin density is delocalized into the thiazolium ring but there indeed is a significant fraction at the C2α atom [85].

Research on POX suggested that the enzyme proceeds via a free-radical mechanism [62]. While it is not formally a redox enzyme, the class of enzymes named acetohydroxyacid synthases (AHAS) and acetolactate synthases (ALS) also have FAD in addition to the ThDP, although the function of FAD is still somewhat uncertain since the carboligase reactions carried out by these enzymes have no immediately obvious need for an oxidizing coenzyme (There is a detailed discussion of these issues in Refs. [14,86]).

Interestingly, on both E1o-ec and E1o-h, the same thiamin-enamine-derived radical was observed by EPR spectroscopy, as were the reduced species derived from O_2, superoxide anion radical and thence hydrogen peroxide [89]. The complicated fate of the enamine on E1o components is shown in Schemes 2 and 4.

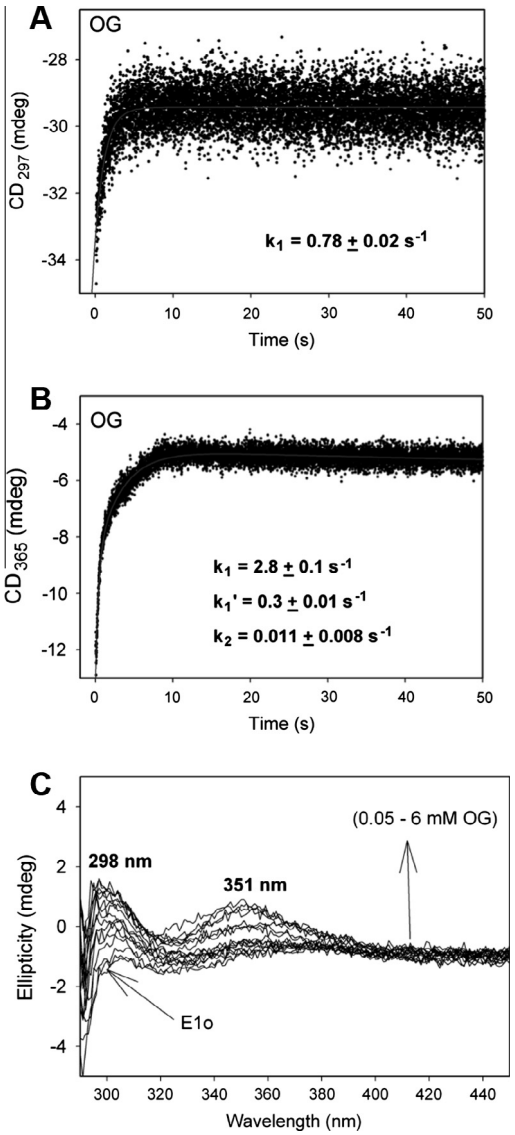
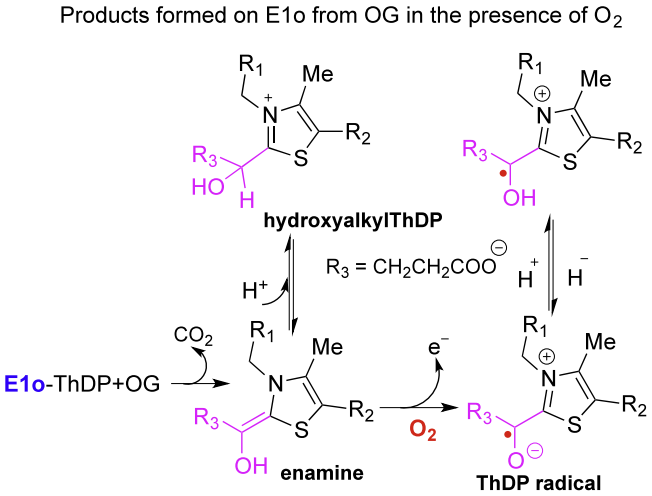


Fig. 7. Circular dichroism titration of E1o-ec by 2-oxoglutarate. Panel A, time dependence of build-up of C2-(α-hydroxy)-γ-carboxy-propylThDP intermediate. Panel B, time dependence of enamine formation on E1o-ec [79,89]. Panel C, steady state circular dichroism resulting from mixing of E1o-ec and 2-oxoglutarate.

4. Assignment of the state of ionization and tautomerization to each intermediate on the pathway

A fundamental finding from the various observations here reported is that in the intermediates with tetrahedral substitution at the C2α atom, the IP form will be the dominant one at pH values above the pK_a of the APH$^+$ form. We note that with this substitution, there are several examples now in the literature indicating that the C2–C2α bond may be out of the plane of the thiazolium ring [71,72,87]. This certainly suggests, although does not prove, that there is van der Waals repulsion between the C2α substituent and the 4′-imino nitrogen under these conditions.

With this observation and the additional observation that we did not see evidence of the IP form with either the MM complex or the enamine, we can now insert the nature of the tautomeric form on each intermediate. Most importantly, the results suggest that for several steps there are proton transfers on the reaction pathway, minimally needed to ensure the appropriate tautomeric form for the intermediate. In Table 2 is summarized the signatures



Scheme 4.

identified so far for ThDP-bound intermediates. Curiously, the mechanisms could be written invoking solely the IP and APH$^+$ forms, while the canonical AP form appears to be a bystander mechanistically. The case of PDHc-ec appears to have been subjected most extensively to such studies enabling us to assign rate constants to all principal ThDP-bound intermediate [75,88].

5. Prospects

Further examples, and alternative methods to confirm the electronic spectroscopic assignments would be desirable, although the authors believe that the spectral assignments of the IP and AP forms are on solid grounds in view of the similar observations made so far on 12 enzymes with several substrates and substrate analogues. With the solid state NMR results, the existence of the APH$^+$ form is also now confirmed on three enzymes. The report on the X-ray study on POX of the likely existence of the Yl form as a carbene in the MM intermediate constitutes a major advance in the field [36]. At the same time, in view of the limitations of the various methods, such as seeing on not seeing protons in X-ray structures even at high resolutions, and carrying out solution NMR studies on large enzymes, the authors suggest that confirmation of seminal findings by independent methods is desirable.

The observation of the 1′,4′-imino tautomeric form also raises many interesting questions, some of which are summarized below.

(1) Why is the AP form observed in some and not other enzymes?
(2) Why is the IP form observed in some enzymes, usually accompanied by the AP form?
(3) What is the energetic cost to the enzyme of stabilizing the IP form at the active center and where is that energy from?
(4) Does the simultaneous presence of the IP and AP forms on POX and E1p-h imply half of the sites reactivity?
(5) Where both the IP and AP forms are present simultaneously, is it the reflection of communication between active center ThDP's by the so-called 'proton wire' mechanism [67] via acidic residues connecting the ThDPs in adjacent active centers, or by some alternative mechanism?

Some of these questions could be addressed by experiments, while some others will have to be addressed by computational methods.

Table 2
Summary on CD detection of ThDP-related intermediates.

Prior to substrate addition	With substrate present
AP form of ThDP (negative CD at 320–330 nm)	Michaelis complex (negative CD at 325–335 nm)
IP form of ThDP (positive CD at 300–314 nm)	Pre-decarboxylation intermediate (positive CD 300–314 nm and longer λ_{max}, and NMR)
APH^+ form of ThDP (no CD signature, solid stateNMR evidence)	Post-decarboxylation intermediates
C2-carbanion/ylide/carbene (no CD signal)	Enamine/C2α carbanion (290–295 nm for aliphatic, positive CD for OG at 350 nm, ~380 nm for aromatic substrate)
	HEThDP (positive CD 300–314 nm and NMR)
	2-AcetylThDP (positive CD at 390 nm and NMR)
	C2α-HEThDP radical (no CD signal detected, EPR signal)

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