# Catalysis in Enzymatic Decarboxylations: Comparison of Selected Cofactor-Dependent and Cofactor-Independent Examples.

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ABSTRACT: This review is focused on three types of enzymes decarboxylating very different substrates: (1) thiamin diphosphate (ThDP)-dependent enzymes reacting with 2-oxo acids; (2) pyridoxal phosphate (PLP)-dependent enzymes reacting with α-amino acids; and (3) an enzyme with no known cofactors, orotidine 5′-monophosphate decarboxylase (OMPDC). Although the first two classes have been much studied for many years, during the past decade, studies of both classes have revealed novel mechanistic insight challenging accepted understanding. The enzyme OMPDC has posed a challenge to the enzymologist attempting to explain a 10^{17}-fold rate acceleration in the absence of cofactors or even metal ions. A comparison of the available evidence on the three types of decarboxylases underlines some common features and more differences. The field of decarboxylases remains an interesting and challenging one for the mechanistic enzymologist, notwithstanding the large amount of information already available.

KEYWORDS: pyridoxal 5-phosphate, thiamin diphosphate, orotidine 5′-monophosphate, catalysis, decarboxylation, circular dichroism, 2-oxo acids, α-amino acids
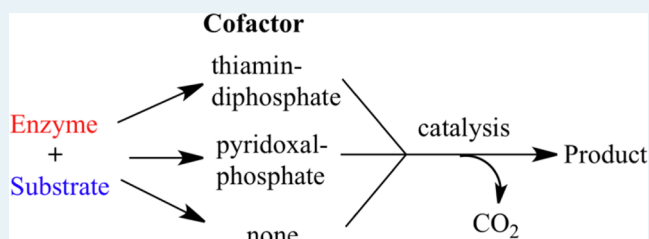
■ OVERVIEW OF THE REVIEW

Enzymatic decarboxylation mechanisms have been studied for six decades. The more classical studies dealt with decarboxylation of α-amino acids, 2-oxo acids, and 3-oxo acids, elucidating pyridoxal phosphate (PLP), thiamin diphosphate (ThDP), and either Schiff-base (lysine-dependent) or divalent metal-ion-dependent mechanisms, respectively. There have been numerous reviews of all such decarboxylations, most recently a general review in *Bioorganic Chemistry* in 2012.[1] In view of this, the authors have selected only three such enzyme families, for which much mechanistic detail has been obtained during the past decade, focusing on the mechanistic aspects of two coenzyme-dependent and one coenzyme-independent reaction: The ThDP-dependent decarboxylation of 2-oxo acids, the PLP-dependent α-decarboxylation of α-amino acids (both of them examples of electrophilic covalent catalysis), and orotidine monophosphate decarboxylase (OMPDC), an enzyme carrying out its reaction via noncovalent complexes that offers a continuing mechanistic challenge. ThDP is of special and long-standing interest to the authors and is the only one of the three enzyme types discussed that appears to have dual catalytic functions, electrophilic covalent and acid–base, the latter only recently elucidated. The plethora of methods used in these studies also demonstrates the maturity of the field.

■ INTRODUCTION TO THIAMIN DIPHOSPHATE-DEPENDENT DECARBOXYLASES

In writing this review, the authors are keenly aware of the number of other excellent reviews available in the literature[2−11] and therefore will concentrate on issues addressed for more than 30 years at Rutgers related to the coenzyme activity of ThDP and reviewed by the senior author, as well.[12−14] 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. From the large number of high-resolution X-ray structures available for the past 20 years, starting with the structures of transketolase[15] (TK), pyruvate oxidase[16] (POX) from *Lactobacillus plantarum* and pyruvate decarboxylase from the yeast *Saccharomyces cerevisae* (YPDC),[17,18] it has become clear that the 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 conserved recognition sequence GDG-X$_{26}$-NX of amino acids.[19] Chemically, the thiazolium ring is central to catalysis, as reported in seminal studies by Breslow:[20] because of its ability to form a key nucleophilic center, the C2-carbanion/ylide/carbene resonance forms. The 4′-aminopyrimidine moiety has gained more recognition as an important contributor to catalysis since the appearance of the X-ray structures showing its conserved proximity to the C2 thiazolium atom and the possibility for its participation in acid–base catalysis.[21] This issue will be a major focus of this section of the review, as it makes ThDP a truly unique and bifunctional coenzyme.

Scheme 1. Mechanism of Yeast Pyruvate Decarboxylase YPDC

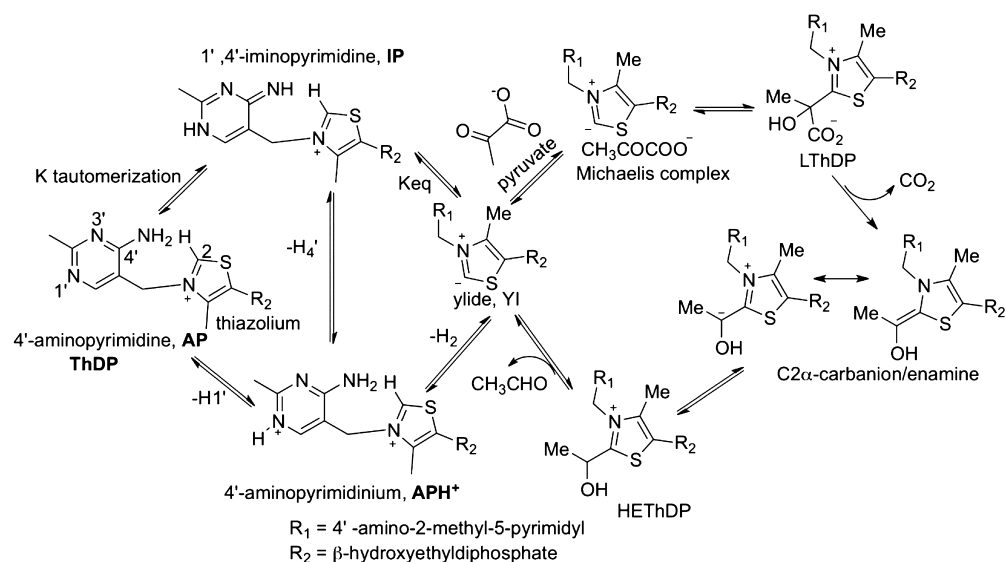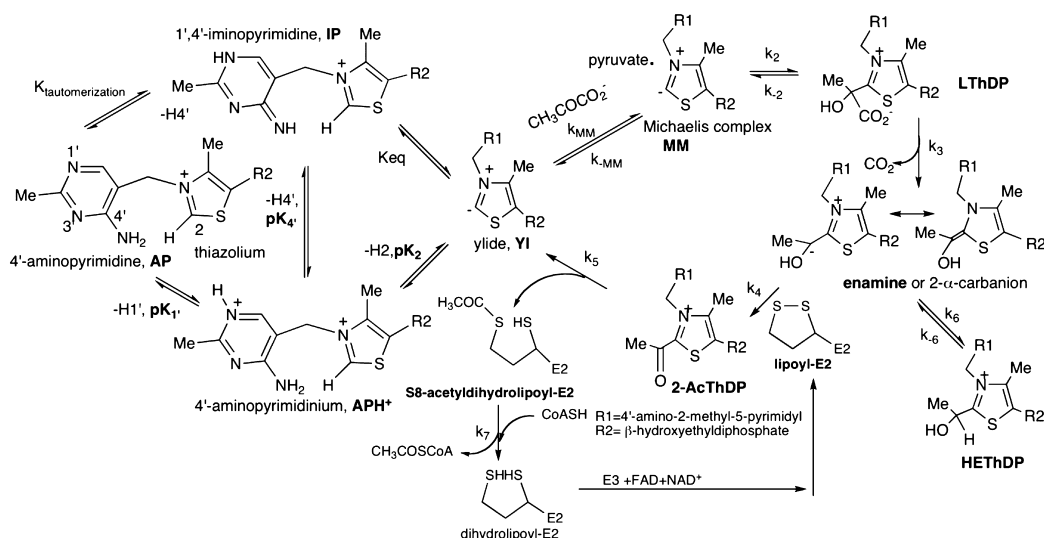R₁ = 4'-amino-2-methyl-5-pyrimidyl
R₂ = β-hydroxyethyldiphosphate

Scheme 2. Mechanism of *E. coli* Pyruvate Dehydrogenase Complex with Role of ThDP on E1ec

Table 1. Detection of ThDP-Related Intermediates

prior to substrate addition	with substrate present
AP form of ThDP (−CD at 320−330 nm)	Michaelis complex (−CD at 325−335 nm)
IP form of ThDP (+CD at 300−314 nm)	Predecarboxylation intermediate (CD +300−314 nm)[a]
APH⁺ form of ThDP (no CD signature)	enamine/C2α carbanion (290−295 nm for aliphatic, ∼380 nm for aromatic substrate)
-C2-carbanion/ylide/carbene (no CD signal)	HEThDP
	2-acetylThDP
	C2α-HEThDP radical

[a]With chromophoric substrate ∼400 nm.

The goal of this review is to touch on themes concerned with observation and characterization of enzyme-bound intermediates comprising both noncovalent and covalent complexes of ThDP. Recent studies of the mechanism have provided ever-greater insight, as detailed by recent reviews both by the authors' group and by Kluger and Tittmann.[22]

Detection of ThDP-Related Intermediates and Their Kinetic Fates, And the Information Gained from Such Data. The presentation of thiamin-related and thiamin-bound intermediates represent pre-, or postsubstrate (or substrate

analogue) binding, an important distinction needed with the recent identification of several forms of ThDP on the enzymes.

ThDP-Related Intermediates Prior to Substrate Addition. During the past decade, the authors' laboratory with collaborators (whose enzymes enable generalization of the findings) established the presence of various tautomeric and ionization states of ThDP (Schemes 1,2). The first high-resolution X-ray structures of ThDP enzymes,[15−18,21] clarified two issues for the first time: (i) The conformation of the bound ThDP (defining the dihedral angles formed by the thiazolium

and 4′-aminopyrimidine rings with respect to the bridging methylene group) was quite different (so-called V) from the conformation found for both free coenzyme (F) and coenzyme with a C2 substituent (S).[23−25] (ii) This V conformation brings the N4′ and C2 atoms to within 3.5 Å of each other, consistent with participation of the 4′-aminopyrimidine ring in catalysis, as suggested by two groups over the years.[8,26−28] A need arose to identify spectral signatures for various tautomeric and ionization states of the 4′-aminopyrimidine ring of ThDP (Table 1).

The Canonical 4′-Aminopyrimidine (AP) Form of ThDP. The signature for this species is a negative circular dichroism (CD) band centered near 320−330 nm, as illustrated with the enzyme benzaldehyde lyase (BAL). Although this CD band had been observed on the enzyme transketolase (TK),[29] it was believed to be the result of a charge transfer transition between ThDP and an amino acid side chain on TK. A number of studies on YPDC and E1ec and their variants, as well as chemical model studies in the authors' laboratory, suggest that this UV/CD band is due to a charge transfer transition between the 4′-aminopyrimidine ring as donor and the thiazolium ring as acceptor. The band has been observed on 10 ThDP enzymes, and its observation depends both on the pH and on the specific enzyme environment.

The 1′,4′-Iminopyrimidine (IP) Form of ThDP.[30−34] The notion that the 4′-aminopyrimidine could exist in its 1′,4′-iminopyrimidine tautomeric form was suggested by models attempting to mimic the reactivity of such a tautomer. The $N1'$-methyl analogue of both the 4-aminopyrimidine ring and of thiamin was synthesized and gave evidence of two relevant points: (i) In this $N1'$-methylpyrimidinium, the pK_a of the exocyclic amine was reduced to ca. 12−12.5,[26,35] offering a rationale for the presence of a conserved glutamate as a catalyst for the amino ⇆ imino tautomerization, and (2) with the positive charge on the ring, the amino protons undergo differential exchange rates, and the exchange is buffer-catalyzed.[28] A spectroscopic signature for the 1′,4′-iminopyrimidylThDP tautomer was first identified on the slow E477Q variant of YPDC,[30] prompting generation of chemical models by Zhang[30] then Baykal,[35] showing that a synthetic chemical model would give rise to a UV absorption in the 300−310 nm range. It bode well for future NMR studies that the ^{15}N chemical shifts of the three species on the left-hand side of Schemes 1 and 2, the AP, IP, and APH⁺ (two neutral and one positively charged) forms, are quite distinct (consistent with early results for the AP and APH⁺).[36]

The CD bands corresponding to the AP and IP forms have different phases, enabling observation of both bands simultaneously, making the CD method useful. The signature for the IP form is a positive CD band centered near 300−314 nm, and as seen in Figure 1, on E1h, both the IP and AP tautomeric forms are seen simultaneously (Figure 1). The electronic absorption characteristics of the APH⁺ and the ylide (Yl) forms are yet to be established.

1′H,4′-Aminopyrimidinium (APH⁺) Form. The presence of the protonated 4′-aminopyrimidinium ion APH⁺ received positive confirmation by solid state NMR measurements (T. Polenova at Univ. of Delaware) on three enzymes: YPDC and the E1 components of both the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes from *Escherichia coli*, on the basis of the characteristic ^{13}C and ^{15}N chemical shifts. The need for solid state NMR is due to the large molecular mass of all ThDP enzymes (at least 120 kDa).[37]
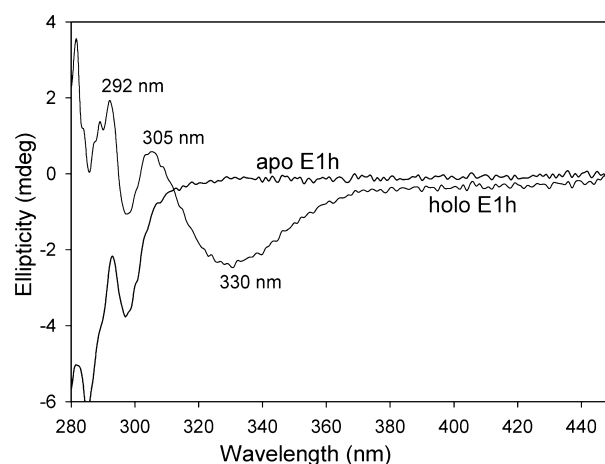

Figure 1. CD spectra of E1h in the absence (apo E1h) and presence (holo E1h) of ThDP. The positive CD band at 305 nm is assigned to IP, and the negative at 330 nm, to the AP form of ThDP.

Determination of the pK_a for the Enzyme-Bound APH⁺ Form.[34] As the pH is lowered, the amplitude of the band for the AP form diminishes and titrates with an apparent pK_a of 7.42 for the ([AP] + [IP])/[APH⁺] equilibrium on BAL. This pK_a in water for ThDP is 4.85,[36] whereas on the enzymes, it ranges from 5.6 to 7.5 (Table 2).[34] It was concluded from data

Table 2. The pKa of Enzyme-Bound APH⁺ on ThDP Enzymes

enzyme	pK_a for the ([AP] + [IP])/(APH⁺)
BAL	7.42 ± 0.02
BFDC	7.54 ± 0.11
POX	5.56 ± 0.03
E1h	7.07 ± 0.07
E1o	7.2 ± 0.01
GCL V51D	6.1 ± 0.02
DXP synthase	7.5 ± 0.09

in Table 2, 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 E1ec), which would tend to make the 4′-aminopyrimidine ring more basic. The tautomeric equilibrium constant $K_{tautomer}$, in conjunction with the pK_a's, 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 1,2), such as E1h and pyruvate oxidase from *Lactobacillus plantarum* (POX). 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 Schemes 1 and 2 are valid prior to addition of substrate and lead to the following conclusions: (a) On POX and E1h, $pK_{1'}$ and $pK_{4'}$ have similar magnitudes; the enzymes shifted the $pK_{4'}$ from 12 in water[26] to 5.6 and 7.0, respectively! (See left triangle in Schemes 1 and 2.) (b) With a known forward rate constant from APH⁺ to the ylide (Yl) of ∼50 s⁻¹ determined for E1h[38] and assuming a diffusion-controlled reverse protonation rate constant of 10¹⁰ s⁻¹ M⁻¹ (giving a pK_2 of 8.3 on E1h compared with an estimate in water of 17−19[39]), one could next speculate

about the right triangle in Schemes 1 and 2. The most interesting prediction is that the proton-transfer equilibrium constant for [IP]/[Yl] is 10^1–10^2 on E1h, providing the first thermodynamic estimates for possible observation of the ylide.

The C2-Carbanion/Ylide/Carbene. According to Breslow's findings, proton loss at the thiazolium C2 position is required to initiate the catalytic cycle. In 1997, there were two reports with significant implications regarding this issue: (i) A ^{1}H NMR-based method was presented for measuring the rate of H/D exchange at the C2 position of bound ThDP, providing the rate constant for the dissociation of the C2H to the ylide.[40] (ii) Arduengo and colleagues showed that the conjugate bases of imidazolium and, indeed, of thiazolium salts could be generated and their structure evaluated by NMR methods.[41] 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 ylide.[41]

Thiamin-Bound Intermediates with Substrate or Substrate Analogue Present. What makes detailed mechanistic studies of ThDP enzymes feasible is a powerful array of methods to monitor the kinetic fate of each intermediate along the catalytic cycle of all ThDP enzymes. An important method was developed for determination of the rate constants for individual steps by Tittmann and Hübner ("TH method").[42] The TH method takes advantage of the known acid stability of the intermediates in Schemes 1 and 2, so that using either rapid quench or manual quench methods, one can "freeze" the intermediates under acidic conditions while also precipitating the enzyme. The chemical shifts of the C6′H resonances of each intermediate are sufficiently distinct from each other and from that of the unsubstituted ThDP, making ^{1}H NMR an efficient method for evaluation of the relative concentration of the intermediates under steady state conditions, which in turn enables calculation of rate constants for individual reaction steps. The authors' group demonstrated that substitutions of the protein distant from the active site could have a dramatic influence, not only changing the rate, but even changing the rate-limiting step. Further developments of this method were needed to address these issues on complex systems, such as with the multienzyme complexes. The ThDP-bound intermediates have been synthesized, and their chemistry has been established both by the authors' group and others.[13,14,42–45]

The Michaelis Menten Complex (MC). The earliest detection of an MC was on addition of a substrate analogue methyl acetylphosphonate (MAP) and acetylphosphinate to several ThDP enzymes. An example is shown with acetylphosphinate added to YPDC, leading to a negative CD band at ca. 325–335 nm, reminiscent of the band observed for the AP form. In this example, addition of ThDP alone did not display the AP form; the negative CD band appeared only after addition of substrate analogue; hence, the band must pertain to a MC.[46] Similar results were also seen when low concentrations of pyruvate were added to E1ec.[32]

Clear formation of the MC also resulted when adding pyruvate to the "inner loop" E1ec variants.[46] Support for the claim that the MC was, indeed, being detected is provided by kinetic measurements: both stopped-flow photodiode array (PDA) spectra in the absorption mode and stopped-flow CD spectra showed formation of the bands attributed to MC formation within the dead time of the instruments (<1 ms), as expected of a noncovalent MC (see below).

The Covalent Substrate-ThDP Predecarboxylation Complex (LThDP and Analogues); Observation of the Inter-mediate Analogues Derived from Substrate Analogue Phosphonates and Phosphinates. Initial observation of the IP form (positive CD band, 300–314 nm) resulted from formation of a stable predecarboxylation adduct of ThDP with (a) MAP or acetylphosphinate[31,32] (CH$_3$C(C=O)P(H)-O$_2$Na),[34] and (b) the aromatic 2-oxoacid analogue methyl benzoylphosphonate (MBP).[47,48] With 10 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. An important additional finding results from mixing YPDC and acetylphosphinate:[34] evidence for coex-istence of the MC and the covalent predecarboxylation intermediate, consistent with "alternating active site reactivity" suggested by the authors' group earlier for YPDC and BFDC.[49–51]

The product phosphonomandelylThDP (PMThDP) formed on BFDC from MBP and ThDP was also confirmed (FT-MS) in solution,[48] and of PLThDP (from MAP·ThDP) by X-ray methods on E1ec[52] and on POX.[53] The X-ray structures of the PMThDP adduct on BAL and BFDC have also been determined to high resolution and are informative,[47,48] providing proof of configuration at the C2α position, not accessible with the pyruvate analogs (CH$_3$ and OH could not be differentiated at current resolution).[52,53]

In addition, all LThDP-like structures displayed some distortion where the C2–C2α bond is found noncoplanar with the thiazolium ring (unlike in the structure of PLThDP in the absence of enzyme), although the Dunathan hypothesis (according to which the C2α–P bond should be essentially perpendicular to the thiazolium plane) is adhered to.[47,48,52,53]

In some favorable cases, the CD band for the true predecarboxylation intermediate (via the IP form) could be observed from the slow substrates. This was accomplished when BAL was reacted with benzoylformate or phenylpyruvic acid,[54] slow alternate substrates for this enzyme. LThDP was also observed by cryo-crystallographic methods on POX.[53] More recently, formation of surprisingly stable LThDP was observed on the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXP synthase condenses the enamine derived from pyruvate with the acceptor glyceraldehyde-3-phosphate, GAP) in the absence of GAP.[60]

Observation of LThDP Analogues from Chromophoric Substrate Analogs. On three enzymes, YPDC, BFDC, and BAL, formation of the predecarboxylation 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 also observed.

With a stopped-flow photodiode array (SF-PDA) instrument, two transients resulted on mixing YPDC with 3-PKB: the first transient formed (T$_1$) was assigned to the predecarboxylation intermediate analogous to LThDP (LThDP* with λ_{max} near 470 nm), and the second transient (T$_2$) was assigned to the enamine (λ_{max} near 430 nm). T$_2$ was formed at the same rate as the rate of depletion of T$_1$. This compound provided outstanding information about the rates of formation of these two important intermediates on YPDC,[55–57] BFDC,[58] and BAL,[54] not available from any other source. On YPDC, evidence was obtained whether an active center residue (a) had a role pre-, or postdecarboxylation, (b) was involved in active center communication, (c) led to the alternating active site behavior,[55,56] and about kinetic properties and consequences of a mobile loop near the active center.[57] Where applicable, this method is complementary to the TH method, which could not

differentiate the enamine from the HEThDP in Scheme 1; these species are at protolytic equilibrium, and the acid quench converts the enamine to HEThDP.

On BAL, Nemeria observed the simultaneous presence of both the negative CD band at 475 nm and the positive one at 312 nm on addition of PAA: the former a direct result of the tetrahedral adduct between PAA and BAL, the band at 312 nm of the IP form characteristic of C2α-tetrahedrally substituted intermediates.[58]

Reactivity of LThDP on and off the Enzymes. With the ability to synthesize the putative intermediates LThDP and HEThDP (thanks mostly to Kluger's group), a reexamination of the reactivity of LThDP on and off the enzymes could be carried out, leading to the following results: (1) In aprotic solvents,[59] decarboxylation of model C2α-lactylthiazolium and C2α-lactylthiamin salts could achieve a first-order rate constant essentially identical to the enzymic turnover numbers (∼60 s^{-1}); that is, LThDP can undergo decarboxylation at rates that are catalytically competent with the turnover numbers. A low-polarity active center may be sufficient to ensure that decarboxylation of LThDP is not rate-limiting. (2) When YPDC and E1ec were reconstituted with synthetic LThDP, both enzymes catalyzed its decarboxylation (unpublished observations at Rutgers). Rates at steady state are limited by the slow off-rate for ThDP, so a new molecule of LThDP can be bound. Pre-steady-state experiments demonstrated that the reaction rates with LThDP reconstitution are limited by factors other than catalysis, that is binding or conformational changes. (3) Rapid acid quench of certain YPDC variants reacting with pyruvate enabled direct observation of LThDP under steady state conditions.[42] It was concluded that LThDP is, indeed, on the pathway of both YPDC and E1ec.[59] As mentioned above, DXP synthase displayed the presence of a surprisingly stable LThDP. In the absence of GAP, decarboxylation of LThDP on the enzyme proceeded with $k = 0.07$ s^{-1}, whereas addition of GAP accelerated the decarboxylation rate by at least 600-fold.[60]

The First Postdecarboxylation Intermediate: The Enamine/C2α-Carbanion. According to Schemes 1 and 2, the enamine is the only conjugated covalent ThDP-bound intermediate. UV−vis observation of enzyme-bound enamine derived from aliphatic substrates is difficult because of the expected λ_{max} near 290−295 nm, according to thiazolium-based models.[61,62]

The enamine intermediate derived from benzoylformate (modeled with λ_{max} of 380 nm)[63,64] has been observed directly on the enzyme BFDC at 390 nm.[58] Although BFDC converts benzoylformate to benzaldehyde, the enzyme also catalyzes a benzoin-type condensation of benzaldehyde in the reverse reaction, similar to the reverse reaction of BAL. 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,[63,64] but no CD band was evident in the 300−310 nm region.[58] In addition, 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.[54] It was concluded that (a) the "real" enamine could be observed (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; hence, all intermediates here discussed are chiral on the enzymes, both pre and post substrate addition.

The CD measurements nicely confirm this expectation for all intermediates, both covalently bound and even noncovalent ones such as the MC. With YPDC, BFDC, and BAL, the enamine could be observed directly at 430 nm with 3-PKB as alternate substrate on YPDC.[54−58]

The Second Postdecarboxylation Intermediate, The Product−ThDP complex (HEThDP, HBThDP). Evidence on YPDC. Evidence for formation of this intermediate also will be reported in a later section using the TH method. Clear evidence was obtained for HEThDP-analog formation from reacting 3(pyrid-3-yl)-acrylaldehyde (PAA, the product of decarboxylation of 3-PKB), with BAL or BFDC.[58] An absorbance with λ_{max} = 470 nm appeared (similar to that observed with 3-PKB) and was attributed to the HEThDP analogue. X-ray results confirmed formation of the tetrahedral postdecarboxylation intermediate on addition of PAA to BFDC. Striking confirmation of HEThDP formation resulted from mixing acetaldehyde, the product of pyruvate decarboxylation, with YPDC on the SF-PDA instrument,[56] giving the characteristic absorption for IP form (λ_{max} = 310 nm), with no alternative assignment to the IP of HEThDP (Scheme 1).

Evidence on E1ec.[66,32] Although HEThDP is not usually considered to be on the reaction pathway of ThDP-dependent oxidative decarboxylases (Scheme 2), groups working with POX[67] and the PDHc's[68] have long used HEThDP as an alternate substrate. Chemical model studies from the authors' laboratory on the enamine and related intermediates[9,13,14] have unambiguously signaled that HEThDP cannot be oxidized directly by either FAD or lipoic acid as oxidizing partners. Instead, oxidation must be preceded by ionization at the C2α position to generate the enamine, which is then prone to oxidation by even molecular dioxygen from air. However, the pK_a at the C2α position is very high, estimated at ca. 17−18 for HEThDP[163] derived from pyruvic acid. Experiments on YPDC demonstrated substantial lowering of this pK_a by the enzyme, and a low effective dielectric constant at the active center of YPDC[69] was offered as explanation.

Given that HEThDP is not on the direct pathway of PDHc, reversibility of the reaction from HEThDP to enamine needed to be demonstrated on E1ec. If E1ec catalyzes the exchange, this could reflect the intervention of an enzymatic "solvent effect". The E1ec catalyzed pre-steady-state rate constant for the $^2H \rightarrow {}^1H$ exchange from the C2α position of HEThDP−$^2H_4$ (steps k_6/k_{-6} in Scheme 2), as an indicator of the formation of the enamine was measured. E1ec accelerates the rate of ionization of this bond by a factor of 10^7, corresponding to 10 kcal/mol stabilization of the enamine intermediate by the enzyme.[70] This stabilization is a property of the active center per se and, with our earlier evidence reported on YPDC, suggests that such a "solvent effect" is likely a general feature of ThDP enzymes.

2-Acetylthiamin Diphosphate, the 2-Electron Oxidation Product of the Enamine. This compound is the product of oxidation of the enamine by any one of the following oxidizing agents on enzymes: the dithiolane ring of lipoic acid covalently amidated to a lysine side chain in the 2-oxoacid dehydrogenase multienzyme complexes; less frequently by FAD in the pyruvate oxidases; and finally, by NAD⁺. The POX reaction has been studied for many years, and the 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 viewed 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 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 co-workers. Using a number of ingenious ways to generate 2-acetylThDP (for example, by reversing the reaction by addition acetyl-CoA), they provided evidence that the first option, redox followed by group transfer, is the likely scenario.[45] Pan at Rutgers demonstrated in a model system that the tetrahedral intermediate can, indeed, be generated from the enamine and lipoic acid analogs, although on critical examination, this could be the obligatory intermediate with either scenario.[71] Pan showed that the cleavage of the dithiolane ring by the enamine required electrophilic catalysis. In the model system, this could be accomplished by S-methylation, whereas on the enzymes, the reaction presumably uses an acid catalyst such as a histidinium ion, probably H407 on E1ec.[72] Our group published evidence indicating that in the presence of an artificial oxidizing agent, 2,6-dichlorophenoindophenol, the enamine produced by E1p is converted to 2-acetylThDP, but in the presence of the E2p component, there is no apparent 2-acetylThDP on the way to reductive acetylation. This could signal that both pathways may be available to the enzymes.[89]

The C2α-HydroxyethylideneThDP Radical, the 1-Electron Oxidation Product of the Enamine. Early and clear evidence for a free-radical mechanism on ThDP enzymes was obtained on pyruvate—ferredoxin oxidoreductase, an enzyme that converts pyruvate to acetyl-CoA in anaerobes.[73] In addition to ThDP, the enzyme has three Fe_4S_4 clusters forming a 40–50-Å-long electron transfer chain.[74] The stability of the free radical was manifested by the fact that the crystal also displayed an electron paramagnetic resonance signal.[74] 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.[75] Subsequent detailed work on pyruvate—ferredoxin oxidoreductase clearly showed that the spin density is delocalized into the thiazolium ring, but there indeed is a significant fraction at the C2α atom.[76] The enzyme pyruvate oxidase using both ThDP and FAD as cofactors also uses a free radical mechanism.[77]

At the same time, there are ThDP enzymes with no known redox role for the bound FAD, such as glyoxylate carboligase (GCL). This enzyme carries out decarboxylative carboligation, similar to DXP synthase. Perhaps the most striking feature of glyoxylate carboligase is the replacement of the virtually universally conserved Glu residue opposite the N1' atom of ThDP by a hydrophobic residue.[78] The GCL and BAL are of particular interest since on neither enzyme is there an acid—base residue within hydrogen bonding distance of the ThDP to assist with proton transfers. There is perhaps no alternative to water carrying out the proton transfers on these two enzymes. Of course, these enzymes also provide strong support for an obligatory catalytic role of the 4'-aminopyrimidine ring of the ThDP.

Determination of Rate-Limiting Steps and Microscopic Rate Constants on ThDP Enzymes. Starting with the 1970s, both solvent and heavy-atom kinetic isotope effects

(KIE) were employed to probe the rate-limiting steps in ThDP enzymes, especially on YPDC. $^{13}C/^{12}C$ KIEs used natural abundance ^{13}C label at the pyruvate C1 atom by measuring the mass ratio of $^{45}CO_2/^{44}CO_2$ using an isotope ratio mass spectrometer.[79−81] Supported by a model study in which the decarboxylation step could be isolated,[82] the results of such studies could inform about the partitioning of the LThDP intermediate reverting to pyruvate and free coenzyme or going forward to decarboxylation to the enamine (Scheme 1). The method suggested that decarboxylation was not rate-limiting on YPDC, the forward rate constant being 4−5-fold larger than the reversion to the Michaelis complex. YPDC is subject to substrate activation (Hill coefficient ∼ 2.0). Especially, the solvent deuterium KIEs suggested that there may be a strong hydrogen bond formed during regulation and, along with heavy atom KIEs, enabled construction of a free energy diagram for the reaction pathway, including the regulatory mechanism.[83,84] Solvent deuterium KIEs were determined on some of the cysteine variants of YPDC (Cys221 is believed to be the site of the substrate activation[13,14]), again attempting to delineate the effects of substrate activation.[85,86]

The earlier quoted TH method[42] is based on observation of covalent ThDP-bound intermediates (Schemes 1 and 2) after their release from the enzyme by acid quench of a reaction mixture resulting from rapid mixing of enzyme and substrate on a chemical quench instrument. Fortuitously, HEThDP, LThDP, and 2-acetylThDP are all (a) stable under these conditions and (b) have distinct ^1H chemical shifts for their C6'−H resonances. Integration of these resonances provides the relative steady-state distribution of intermediates, and with the turnover number of the enzyme, the forward rate constants could be calculated for the pathway (rate constants for formation of LThDP (k'_2), its decarboxylation (k'_3), and acetaldehyde release ($k_{4,5}$)).[42] The method has been applied to a number of ThDP enzymes.[22] The power of the experiment is illustrated with the YPDC loop variants.

$$ R1 = 4'\text{-amino-2-methyl-5-pyrimidyl} $$
$$ R2 = \beta\text{-hydroxyethyldiphosphate} $$

(1)
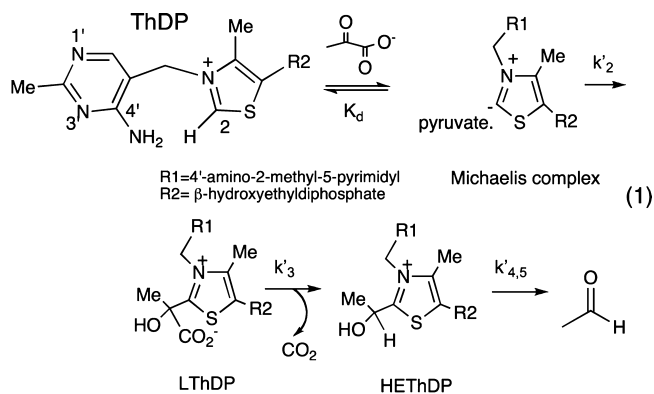
It was concluded that acetaldehyde release is rate-limiting for all of the loop variants; hence, the loop opening/closing must be impaired.[87] The method has also been applied to active center variants of YPDC,[42] along with several substitutions on the E1ec.[88] The method has some limitations: (a) It measures intermediate distribution once those are released from the enzyme. (b) It cannot differentiate the level of enamine and HEThDP, since under acid quench, the former is converted to the latter. Therefore, the HEThDP measured in the quench corresponds to the sum of the relative concentration of enamine and HEThDP. (c) Given that the method depends on

the observation of the aromatic C6′−H resonances, many aromatic cofactors would interfere with the observations.

Balakrishnan and Chakraborty[37,89] in the authors' laboratory addressed the NMR issue and extended the power of the TH method by synthesizing ThDP specifically labeled at both the C2 and C6′ positions with ^{13}C ([C2,C6′−^{13}C$_2$]ThDP). Using 1D-gradient HSQC NMR methods, only protons attached to these two ^{13}C-labeled atoms were detected. This enabled detection of C2H and C6′H in the aromatic region of the ^{1}H NMR spectrum, even in the presence of other cofactors with aromatic resonances. As an example, the PDHc complex, even after the protein is acid precipitated, leaves aromatic resonances pertinent to FAD, NAD$^+$, and coenzyme A in the supernatant, in addition to the C2H and C6′H of ThDP. A comparison of the rates of formation of HEThDP in the E1ec component and in the entire PDHc-ec[89] was accomplished by using [C2,C6′−^{13}C$_2$]ThDP and demonstrating that assembly to the complex accelerates the rate of HEThDP formation in a particularly slow E1ec variant by ca. 9-fold. Such information is not readily available from any other method.

The chromophoric conjugated substrates exploited by the authors' group provide complementary information to the TH method, since with the chromophoric substrates, the enamine is directly observed and several microscopic rate constants could be directly assessed.

To observe directly the time-course of enzyme-bound intermediates, a method was developed that is a combination of CD methods and the TH method: the time-course of intermediate formation/depletion is assessed by stopped-flow CD, by looking at the intermediates with signatures identified as above. If there is "degeneracy" in the CD assignments (an important case in point is that all ThDP-bound intermediates with tetrahedral substitution at C2α appear to exist in their IP tautomeric forms at pH values above the pK_a of the APH$^+$ form), we rely on the TH method to identify the intermediate after acid quench under identical reaction conditions. This combined approach was recently applied to E1ec, DXP synthase, and GCL.[89,60,91]

A major conclusion from these studies is that the rate of individual steps can be assessed directly from detailed time course studies, providing a virtually unprecedented opportunity to gain insight into ThDP-dependent reactions, including interrogation of individual enzyme residues regarding their catalytic roles. This issue has again become important in view of growing evidence that our understanding of the role of even His side chains is lacking, as signaled by studies on two ThDP dependent decarboxylases: (a) saturation mutagenesis studies on BFDC showed that a His residue did not have acid−base role,[92] and (b) similar studies on the E1 component of the *E. coli* 2-oxoglutarate dehydrogenase revealed that only one of the two His at the substrate's putative γ-carboxylate binding site (subjected to saturation mutagenesis) could not be replaced by hydrophobic side chains, that is, in both enzymes studied, at least one His had a role different from that assumed by biochemical intuition, such as acid−base, hydrogen bonding, or nucleophilic.[90]

Evidence for Correlation of Active Center Loop Dynamics and Reactivity in E1ec.
We wished to investigate the possibility that loop movement/dynamics may control catalysis (i.e., such movement may be rate-limiting) rather than chemical steps.

Structural Evidence Pointing to Mobile Loops. In the X-ray structure of E1ec with ThDP, there were three regions of poorly defined electron density among the 886 amino acid residues per monomer, corresponding to residues 1−55, 401−413, and 541−557.[93] A remarkable change was seen in the structure of E1ec in complex with a predecarboxylation intermediate phosphonolactylThDP (PLThDP: the adduct of MAP with ThDP), revealing the structure and important interactions of the two loops 401−413 (inner loop) and 541−557 (outer loop) not seen before.[94] This signaled a disorder-to-order transition on forming the predecarboxylation PLThDP intermediate (Scheme 2). On E1ec, the bound PLThDP is stabilized by interactions with inner loop residues E401, H407 (via direct hydrogen bond formation), and Q408 (via a water molecule).[94] The two newly ordered regions interact with each other and propagate from the active site to the enzyme surface, forming a tunnel to accept lipoamide from E2ec for the next reaction (Figure 2). This led the authors to propose that the
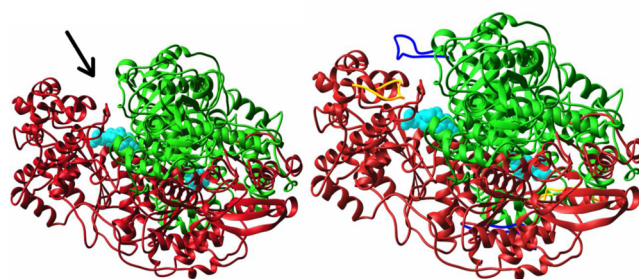

Figure 2. (Left) E1ec·ThDP with inner, outer loops fully disordered (not seen). Arrow indicates active site entrance. Subunits in red and green, ThDP (cyan) space filling. (Right) E1ec−PLThDP complex, newly ordered inner (yellow) and outer loop (blue).[52]

ordering observed in the E1ec-PLThDP complex would also be present when the true intermediate LThDP formed. In addition, the crystal structure of the H407A E1ec in complex with PLThDP provided evidence that in the absence of H407, the two loops are not ordered; hence, the H-bond from H407 to LThDP is the key trigger for the disorder-to-order transformation. The E1ec−PLThDP structure (mimicking LThDP) was among the first examples of a covalently bound predecarboxylation reaction intermediate analogue in any ThDP-dependent enzyme.[93]

Studies on the Inner Active Center Mobile Loop of E1ec.[46,95,96] The above results prompted a study on the role and dynamics of the inner loop, starting with mapping of the conserved residues on the loop. Kinetic, spectroscopic, and crystallographic studies on some inner loop variants led to the conclusion that charged residues flanking H407 are important for stabilization/ordering of the inner loop, thereby facilitating completion of the active site. The results further suggested that a disorder-to-order transition of the dynamic inner loop is essential for (a) forming LThDP, (b) sequestering active site chemistry from undesirable side carboligase side reactions, and (c) communication between the E1ec and E2ec components. The experiments carried out to reach these conclusions are presented briefly because they should be useful for studies of related enzymes.

Both a Michaelis complex (MC) and predecarboxylation intermediate analogue could be observed on addition of MAP: formation of PLThDP from MAP on E1ec (k_1^{obs} = 3.6 ± 0.2 s^{-1} and k_2^{obs} = 0.35 ± 0.06 s^{-1}) was slower than MC formation and significantly slower in E401K (k_1^{obs} = 0.37 ± 0.05 s^{-1} and k_2^{obs}

Scheme 3. Carboligase Side Reactions on YPDC and Other 2-Oxoacid Decarboxylases

R1 = 4'-amino-2-methyl-5-pyrimidyl
R2 = b-hydroxyethyldiphosphate
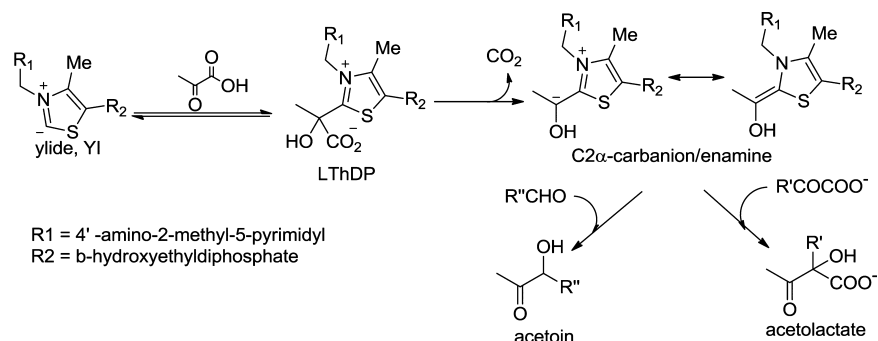
= 0.04 ± 0.01 s^{-1}). Clearly, formation of PLThDP; hence, formation of a C−C covalent bond, and not formation of MC (k_{MC}), is the rate-limiting predecarboxylation step. Since C−C bond formation, but not MC formation, is dramatically slowed down (10-fold compared with E1ec) in E401K, loop dynamics apparently greatly influence covalent addition of substrate to the enzyme-bound ThDP.

The mobile inner loop was found to control access to the active site, changing from the normal product distribution to more carboligase side product (Scheme 3), but the loop was even found to impact on the facial selectivity toward the electrophile.

To study communication between the E1 and E2 components, MALDI-TOF MS was utilized to determine the time course of reductive acetylation of the E2 component by the E1 component and pyruvate.[72,97] Although the wild-type E1ec can reductively acetylate the E2ec component in less than 30 s, with the inner loop variants, both unacetylated and acetylated forms are clearly visible, even after 30 min, that is, the reductive acetylation was incomplete even after this time period.[95] Next, the X-ray crystal structure of a second loop variant E401K was solved, with ThDP or PLThDP present. Again, the inner and outer loops were unorganized, even with PLThDP present (as with the H407A variant).[95]

To investigate the rate of loop movement, a cysteine-less E1ec was constructed in which five cysteines were substituted to alanine and the sixth, Cys259, to Asn47. This cysteine-less construct still possessed 22% activity for NADH production in the reconstituted PDHc-ec assay.

Monitoring the kinetics of E1ec and its loop variants at various solution viscosities, it was shown that the rate of a chemical step is modulated by loop dynamics. The cysteine-less E1ec construct was site-specifically labeled on the inner loop with an electron paramagnetic resonance active nitroxide label revealing ligand-induced conformational dynamics of the loop and slow "open−close" conformational equilibrium in the unliganded state. A CF$_3$C(=O)CH$_2$− (trifluoroacetonyl) ^{19}F NMR label placed at the same residue revealed that the interconversion of the open and closed forms of the inner loop takes place with a rate constant of 0.5−1.0 s^{-1}, very similar to the turnover number from the E1-specific assay (0.38 s^{-1}). These results suggest a quantitative correlation of E1ec catalysis and loop dynamics for the 200 000 Da protein. Thermochemical studies (isothermal titration calorimetry) revealed that these motions may promote covalent addition of substrate to the enzyme-bound ThDP by reducing the free energy of activation. The results are consistent with efficient coupling of catalysis and regulation with enzyme dynamics in E1ec, suggest the mechanism by which it is achieved, and reinforce the

hypothesis ascribing catalytic and regulatory roles to enzyme dynamics.[46]

Do Thiamin Enzymes Provide a Paradigm for "Enzymatic Solvent Effects"? In 1970, there appeared two publications modeling ThDP-catalyzed reactions in solvents of different dielectric solvent. Both Lienhard[98,99] and co-workers and Kemp and O'Brien[100] presented studies that suggested that a solvent of low dielectric constant accelerated thiamin model catalyzed reactions. In the same year, Ullrich and Donner presented the first enzymatic evidence suggesting, on the basis of studies with a fluorescent label,[101] that the YPDC has a hydrophobic active center. The authors' group has also probed the active center environment of ThDP enzymes using a variety of experimental approaches.

Evidence from Observation of the Enamine on YPDC. With the understanding that YPDC has an active center that accepts 2-oxo acids with large substituents replacing the methyl group and that the λ_{max} of the enamine derived from HBThDP is near 380 nm, we tested whether the enzyme would convert the second postdecarboxylation intermediate HBThDP to the enamine. When racemic HBThDP was added to the E91D YPDC (this variant can readily exchange its ThDP with its analogues), the result was a slow development of the A$_{380}$, suggesting formation of the enamine, that is, reversal of the protonation in Scheme 1.[69] Given that the pK_a for this ionization is 15−16 in the absence of enzyme,[63,64] this result strongly suggested that the YPDC could stabilize the enamine relative to an aqueous environment. To estimate the nature of the YPDC active center environment, the fluorescence emission of thiochrome diphosphate, a competitive inhibitor (competing with ThDP) of a number of ThDP enzymes, was measured on YPDC and in a series of 1-alkanols ranging from methanol to 1-hexanol.[69] The correlation of solvent dielectric with emission maximum for the 1-alkanols was virtually linear, enabling us to interpolate the value on YPDC as falling between the values for 1-hexanol and 1-pentanol, that is, a dielectric constant of 11−13. Even with the limitations of such an approach, the message is unmistakable: the active center environment of YPDC resembles that of 1-pentanol or 1-hexanol more than it resembles the aqueous milieu.

Evidence from Solvent Effects on Decarboxylation Rate Constants in Model Compounds. The effect of solvent on model decarboxylation reactions carried out earlier by Lienhard[98] and Kluger[4] was reexamined by the authors' group in both C2α-lactylthiazolium salts and C2α-lactylthiamin.[59] This model isolates the decarboxylation step of Schemes 1 and 2 by monitoring enamine formation via oxidative trapping with DCPIP: first-order rate constants were achieved for decarboxylation in low dielectric media that were

Scheme 4. PLP-Dependent α-Amino Acid Decarboxylation
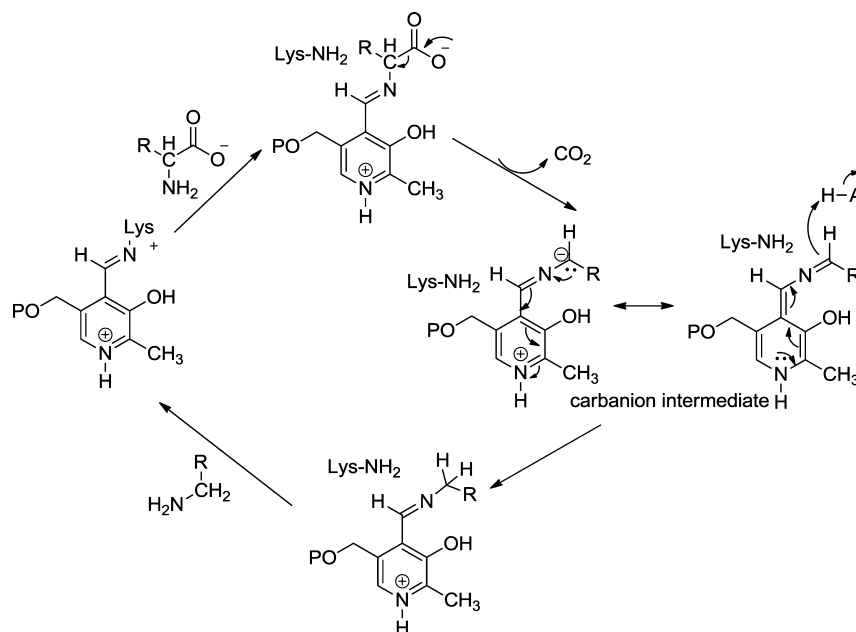
virtually the same as the turnover number per subunit for a typical decarboxylase such as YPDC. It was concluded that acceleration by the enzymatic environment per se was so large as to need little additional catalysis.

Evidence from Acceleration of the Reaction Step Leading to the Enamine on E1ec. Although HEThDP is not directly on the pathway of the E1 component of the 2-oxo acid dehydrogenase complexes, ionization of its C2αH could be used to test the reversibility of enamine protonation and to probe the effect of the active center on this reaction. To test whether the E1ec could accelerate the C2αH ionization step in Scheme 2, HEThDP was synthesized with four deuterons in the C2α-hydroxyalkyl side chain by condensing ThDP with acetaldehyde-d_4. This deuterated HEThDP was then rapidly mixed with apo-E1ec (devoid of ThDP) on a KINTEK rapid quench instrument in H_2O, and the reaction was stopped by acid quench. The hypothesis was that the rate of D-to-H exchange would reflect the rate of the C2αH ionization step. The exchange was quantified using FT-MS. Compared with model systems, the rate of this reaction was accelerated on the enzyme by ca. 10^7. Since there is no amino acid in the active site of E1ec charged with the specific function of ionizing this C2αH bond, it was concluded that the enzyme environment was largely responsible for the observed rate acceleration, again pointing to as much as 10 kcal/mol rate acceleration due to the enzyme environment.[70]

Finally, the authors recall that the IP form of ThDP is stabilized on the enzyme. This must also be a consequence of the protein environment, since there is simply no model for partitioning of the APH$^+$ form to the IP, rather than the AP tautomer in the absence of enzymes.

These few examples suggest that (a) Indeed, there is an important "environmental" contribution to the catalytic rate acceleration on ThDP enzymes, stabilizing high-energy intermediates, including the ylide and the enamine. (b) Such factors could account for as much as $10^7 - 10^9$ of the total rate acceleration. This constitutes a very significant fraction of the total rate acceleration provided by the protein over and above that afforded by ThDP itself, which on YPDC was estimated to

be $10^{12} - 10^{13}$-fold.[83,84] (c) Finally, such effects are almost certainly present on other ThDP enzymes, and researchers should be on the lookout for them.

■ INTRODUCTION TO PYRIDOXAL-5-PHOSPHATE-DEPENDENT α-AMINO ACID α-DECARBOXYLASES

Pyridoxal-5′-phosphate (vitamin B6)-dependent enzymes play an important role in amino acid metabolism of both humans and microorganisms. These enzymes catalyze a variety of reactions of α-amino acids, including transaminations, racemizations, α-decarboxylations, β-decarboxylations, etc. Several enzymes are targets for inhibitor design to treat a variety of diseases.[102,103] Alignment of the amino acid sequences of PLP-dependent decarboxylases identified four distinct groups,[104] among which three belong to type I fold[105] (among five distinct folds[106−108]) and that react at the α- and γ-carbon atoms of the substrate.[106] The PLP-dependent α-decarboxylation mechanism is shown in Scheme 4, in which PLP stabilizes the carbanions by negative charge delocalization. A common theme among PLP-dependent enzymes is protection of the aldehyde of PLP in an "internal aldimine" (covalent enzyme−PLP complex) in which PLP is bound to a highly conserved active site Lys residue, forming an imine (Schiff base). The stable enzyme−PLP complex then reacts with the α-amino group of the substrate via transimination, forming the so-called "external aldimine". This aldimine provides the necessary driving force for decarboxylation by placing the imine electron-withdrawing group β to the departing CO_2 and leading to a resonance-stabilized carbanion. Once the carbanion is protonated at Cα, the decarboxylated product is released by a reverse of the transimination reaction with the conserved lysine, hence, reforming the internal aldimine. Unlike in the more complicated transamination mechanism, for α-decarboxylation, there is no cycling required between the pyridoxal and pyridoxamine forms of the cofactor; the aldehyde form is sufficient to carry out the entire reaction sequence.
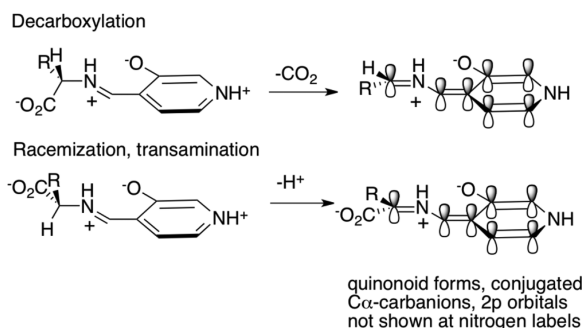
There is an enormous amount of literature on the mechanisms of PLP-catalyzed reactions; here, only some more recent results dealing with the α-decarboxylation will be discussed. The general mechanism of PLP enzymes was proposed by David Metzler, and the aldehyde carbon atom, the pyridine nitrogen, and the 3-OH group were proposed to be important to catalysis.[109]

Catalysis of the Conversion of the Internal Aldimine to the External Aldimine and Its Reversal after Decarboxylation. This preliminary reaction, universal to all PLP enzymes, is itself a typical nucleophilic attack leading to a tetrahedral diamine, which is partitioned to one imine and an amine in each direction. Such reactions in solution are weak-acid-catalyzed to enhance the leaving group ability of the departing amine.

The conserved residue Cys360 in ornithine decarboxylase (ODC) was suggested to be important for the decarboxylation step: the C360A substitution decreased the enzyme's k_{cat} by 50-fold.[110−112] Recently, Eduardo et al. suggested that the conserved active site Cys360 residue has a role in the interconversion of the internal/external aldimines.[113]

Selection of the Appropriate Conformation at Cα and Catalysis of CO_2 Loss. In an excellent review, Eliot and Kirsch[114] introduce the reader to key issues in PLP-catalyzed reactions. One of the unique features of the entire class of PLP enzymes is the selection of the type of reaction, that is, decarboxylation at the Cα position, racemization or trans-amination (both reaction types starting with Cα-H ionization), or reactions at Cβ or Cγ. A brilliant hypothesis by Dunathan[115] in 1966 presented a simple solution to the conundrum by selection of the desired conformer with respect to the Cα−N single bond, as outlined in Scheme 5. For decarboxylation, the

Scheme 5. Dunathan Hypothesis for Conformational Selection in PLP Enzymes

Decarboxylation

Racemization, transamination

quinonoid forms, conjugated Cα-carbanions, 2p orbitals not shown at nitrogen labels

$C_{carboxyl}$−Cα bond is lined up such that the incipient 2p orbital will be conjugated with the already conjugated external aldimine (the resulting intermediate is called the quinonoid structure and has λ_{max} near 500 nm, clearly informing of extended conjugation). By contrast, for racemization and transamination, the Cα−H must be ionized; hence, that conformer must be selected where this Cα−H bond is aligned such that its scission will provide the lone pair of electrons to participate in the π system. By this stereochemical selection, the energy of the transition state for bond scission is lowered because it is approaching the ideal 2p-π orbital overlap. This stereochemical imperative is ensured by having tailored subsites around each of the three substituents at Cα, and it appears to be seldom violated.

A fascinating example of this hypothesis is presented by the enzyme dialkylglycine decarboxylase, an enzyme which first decarboxylates the substrate then carries out a transamination reaction. This ping-pong mechanism uses different subsites in the consecutive parts of the chemistry: first, the CO_2 fits into its subsite and decarboxylation ensues; next, the Cα−H fits into its subsite and transamination follows, providing excellent support for the hypothesis.[116]

It was reported that in ornithine decarboxylase, the carboxyl group of the bound substrate is buried in a region comprising hydrophobic and electron-rich residues.[117] The residue Phe-397 was found to form a close contact with the carboxylate. Its substitution not only reduced the rate of decarboxylation but also affected product distribution, since there was a transimination side reaction producing pyridoxamine. The authors suggested that the environment enhances the decarboxylation rate to accommodate the charge-dispersed carbanion intermediate in favor of the negatively charged predecarboxylation carboxylate.[117] This theme appears to be somewhat akin to findings on ThDP enzymes where the environment also appears to have an important impact on decarboxylation. In the case of ThDP, the decarboxylation concentrates charges in going from the zwitterionic LThDP to the dipolar enamine.

In contrast, dialkylglycine decarboxylase has evolved an Arg group proximal to the bound carboxylate,[118] and although the enzyme is an unusual PLP-dependent decarboxylase, this finding points out how difficult it is to generalize regarding themes for acceleration of decarboxylation.

The Tautomeric Equilibrium between the Imine Nitrogen and the 3-OH of PLP. There is information indicating that the 3-OH proton is sometimes transferred to the imine nitrogen of the external aldimine, increasing its electron-withdrawing potential.[65] In a recent report on carboxynorspermidine decarboxylase, an Arg was identified water-separated from the 3-OH, suggesting that this Arg residue stabilizes the ionized form of 3-OH, which would be very useful to stabilize, in turn, the positive charge on an iminium ion derived from the α-amino group.[119] Computational and kinetic isotope effect studies on L-DOPA decarboxylase also suggest that such a proton transfer contributes to catalysis.[120,121] Dopa decarboxylase (DDC) catalyzes the irreversible decarboxylation of aromatic L-amino acid substrates. The crystal structure of DDC in complex with its inhibitor carbidopa (PDB entry: 1JS3)[122] shows that the N1 in the pyridine ring of PLP interacts with the side chain of acidic amino acid Asp271 (Figure 3).

A very extensive study of the protonation/tautomerization states of PLP both in solution and on aspartate amino-transferase was carried out by Limbach, Toney, and co-workers[65] using a combination of solution and solid-state NMR methods, mostly relying on ^{15}N labeling at the Cα−N and at the pyridinium N1 position, and supported by very extensive model studies. The methods developed enabled this group to suggest chemical shift−bond length correlations. Their results are fully consistent with an Asp residue hydrogen-bonded to the pyridinium nitrogen atom and the zwitterionic charge distribution in the external aldimine, that is, a 3-oxy anion opposite the iminium (protonated Schiff base) ion at a distance of perhaps 2.6 Å or less, as diagrammed in Scheme 5. The pK_a of the pyridinium ion is raised compared with that in water, an increase attributed to the Asp residue within the hydrogen bonding distance. These NMR studies, along with the studies reported on ThDP mechanisms, constitute some of the most
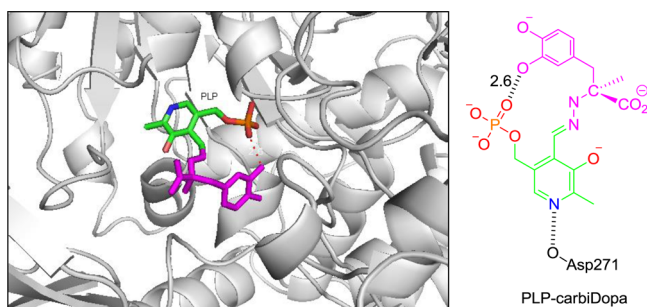
Figure 3. (Left) View of dopa decarboxylase in complex with external PLP-carbiDopa (PDB: 1JS3)[122] shown as stick, PLP in green, and carbiDopa in magenta. The image was created using the program PyMOL. (Right) Schematic diagram showing interaction of N1 of PLP with Asp271. The value 2.6 Å represents the distance between connected atoms.

strong basicity of the imine nitrogen, a valuable piece of data for mechanistic understanding.

INTRODUCTION TO OROTIDINE 5′-MONOPHOSPHATE DECARBOXYLASE

The cofactor independent enzyme orotidine 5′-monophosphate decarboxylase (OMPDC) catalyzes the decarboxylation of orotidine 5′-monophosphate to uridine 5′-monophosphate during pyrimidine nucleotide biosynthesis (Scheme 6).[127,128]

Scheme 6. OMPDC-Catalyzed Reaction Mechanism

precise definition of the state of ionization and tautomerization of coenzymes on such large enzymes.

Must the Pyridine Ring Be Protonated in All PLP Reactions? For many years, it was assumed that the aromatic ring of PLP exists as a pyridinium ion, a protonation that would increase the electron withdrawing ability of the cofactor and could be useful for drawing resonance structures for the intermediates, especially the so-called quinonoid form. On the basis of the above-discussed ThDP catalysis, this would be manifested by a carboxylic acid amino acid (Asp or Glu) within hydrogen bonding distance of the pyridinium ion. With the multiple structures solved for various classes of PLP enzymes, it became obvious that not all PLP enzymes use this catalytic mechanism, but, in fact, the α-decarboxylases do appear to use it. A very ingenious way to test this mechanism was developed by M. Toney and his group, via the de novo synthesis of 1-deaza-PLP then reconstitution of the PLP enzymes with this coenzyme analogue. Indeed, some PLP enzymes could function at reduced rates with this 1-deazaPLP, demonstrating that protonation at N1 is not a universal requirement for PLP enzymes.[123]

At the same time, in several α-decarboxylases, there is clear evidence of a Glu side chain carboxylic acid within hydrogen-bonding distance of the pyridine nitrogen atom.[119,124]

Evaluation of the Kinetic Fates of PLP-Bound Intermediates. Determination of the kinetics of individual steps in PLP enzymes is facilitated by observations in the VIS spectroscopic range. Detailed analysis by multiwavelength VIS spectroscopy at both steady state and pre-steady-state time scales has taken advantage of the recognition of the different wavelength maxima of different intermediates on the pathway (see ref 123 for a good summary of the spectral properties of key PLP-bound intermediates). Using a variety of sophisticated mechanistic probes to study the enzyme dialkylglycyl decarboxylase, the authors concluded that decarboxylation and C4′ (aldehyde carbon) protonation were concerted.[125]
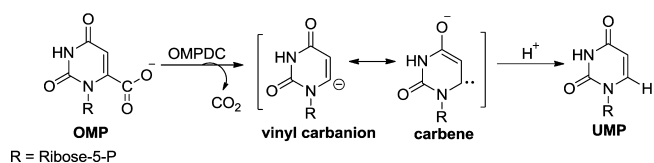
State of Ionization of the Phosphate Group of PLP. The environment of the phosphate of bound PLP has been examined by ^{31}P NMR spectroscopy.[126] Of immediate interest is the state of ionization of the phosphate monoester. Application to dialkylglycine decarboxylase indicated that the pK_a for a second ionization is ca. 6.0−7.0, depending on the positive counterion. Equally important, the method in favorable cases provides information regarding the state of ionization of the iminium ion form of the external aldimine, confirming

The finding that this enzyme catalyzes the enzymatic reaction by a factor of 10^{17} over the nonenzymic rate makes this enzyme perhaps the most proficient one known to date[129,130] and also carries out a reaction in the apparent absence of any obvious electron sink in the system, akin to those created in ThDP and PLP enzymes. Wolfenden's discovery spawned enormous interest, producing many structures,[131,132] many novel experimental tests, and even computational tests. For this brief review, the authors had to be selective and apologize to all research groups whose work is not referred to. There are a number of reviews on the subject, including refs 133 and 134. The authors will present what they perceive to be the key current theories to explain the catalytic power of OMPDC. Experiments indicating that this is a single substrate enzyme with no known cofactor or metal ion requirement make such studies more tractable.

Early Chemical Model Studies. Early chemical model studies[135] led to a mechanism, according to which an initial protonation at the pyrimidine O2 or O4 atoms creates a potential electron sink via an ylide involving the N1 atom, and this drives the subsequent decarboxylation reaction. Solvent kinetic isotope effects suggest that there is an isotope-sensitive, rate-determining step, perhaps consistent with the protonation idea.[136] Arguments against protonation include (a) the absence of any suitably positioned amino acid that could donate the proton to O2 or O4, albeit hydrogen bonding is a possibility via amides according to the X-ray structures of OMPDC in the presence of substrate, and (b) ^{15}N kinetic isotope effects appear to rule out ylide formation in the rate-determining step.[137]

Mechanistic Hypotheses Based on Active Center Amino Acid Side Chains. The structure of OMPDC from *Saccharomyces cerevisiae* (ScOMPDC) in a complex with 6-hydroxyuridine-5′-phosphate (BMP) suggests that Lys 93 (Lys 72 in *Methanothermobacter thermautotrophicus*, MtOMPDC,[138] as in Figure 4) is positioned to stabilize the negative charge at C-6 and also to protonate the intermediate, which then forms the product UMP.[139] Saturation mutagenesis experiments were carried out on 24 residues of the *E. coli* OMPDC[140] in and around the active site. All positions could be substituted to some extent when the library mutants were expressed from a multicopy plasmid. For the conserved quartet of charged residues Lys44, Asp71, Lys73, and Asp76 (Lys42, Asp70, Lys72, and Asp75 inMtOMPDC), a cysteine substitution was found to provide function at positions 71 and 76. The authors suggested that a lower pK_a for both cysteine variants supports a
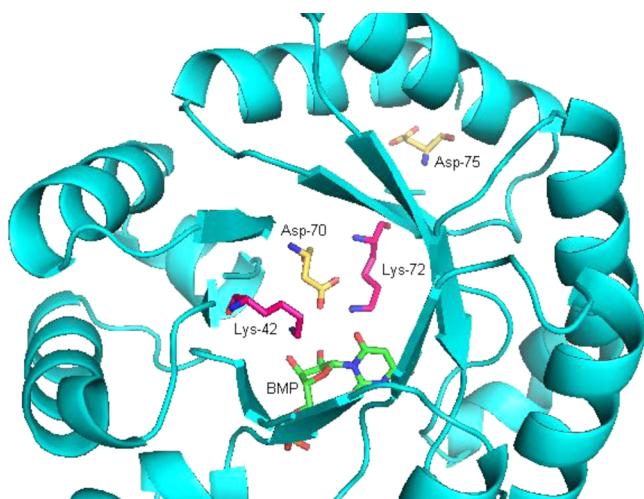
Figure 4. Partial view of *M. thermautotrophicus* OMPDC in complex with BMP (PDB: 1LOR)[138] showing four charged side chains Lys-42, Asp-70, Lys-72, and Asp-75.

mechanism whereby the thiolate group of cysteine substitutes for the negatively charged aspartate side chain. The partial function variants such as D71C and D76C exhibit reduced catalytic efficiency relative to wild type OMPDC but still reacted 10^{15} times as fast as the uncatalyzed rate, indicating the catalytic proficiency of the enzyme is tolerant to amino acid substitutions.

A different proposal invokes "electrostatic stress" by nearby negative charges,[141,142] in which the carboxylate group of the substrate to be removed is forced into the immediate proximity of the Asp-91 (yeast) by the bound phosphate of the substrate. Assuming both carboxylates are, indeed, negatively charged, this electrostatic repulsion would raise the energy of the ground state, thereby lowering the energy of activation. Results of computational analyses predict that electrostatic stress furnishes the majority of the catalytic driving force for OMP decarboxylation. Several independent experimental and computational studies, along with careful thermodynamic considerations, have questioned the significance of electrostatic stress as a catalytic device for OMPDC.[143,144]

On the basis of studies of H-to-D exchange rates at the C6 product position, as well as in the 5-F substituted product, the authors concluded that both carbanion stabilization and substrate destabilization by the proximal Asp contribute to the overall catalytic efficiency.[145] On the basis of experiments with a 6-phosphonic acid derivative of uridine-5′-phosphate, as an analogue of the substrate that cannot be decarboxylated, the authors claimed to also have support for the substrate destabilization idea,[146] as did a recent X-ray study at 1.0 Å resolution with product bound.[147]

When the hydrophobic environment around the substrate was investigated with site-directed mutagenesis, some evidence was obtained for the importance of a hydrogen bond between a backbone NH of the enzyme and the O4 atom,[148] along with as much as 4 orders of magnitude reduction in k_{cat}. The authors suggested that their results supported both the electrostatic repulsion idea via the conserved Asp and the presence of a transient C6 carbanion/carbene intermediate. Iiams et al. reported that the substitution S127P has a great impact on k_{cat}/K_M,[148] supporting the explanation by Lee and Houk[149] that the hydrogen bond between O4 of the substrate and NH backbone is important in reducing the energy barrier.

A Concerted Protonation-CO$_2$ Loss at C6 To Avoid an Unstable Carbanion. The notion that there is a distinct negative charge at C6 on CO$_2$ loss was challenged by a different model proposed by Begley and Ealick on the basis of their structure of OMPDC. This model proposes concerted protonation at C6 and C6−C-carboxyl scission, abrogating the need for stabilization of a distinct carbanion and preceding the transition state.[128,139,150,151]

Role of Conserved Lysine in Stabilizing the Carbanion. There is also a suggestion based on a highly conserved Lys, presumably positively charged, that part of the catalytic power of OMPDC resides in simple electrostatic attraction. Here, formation of the putative C6 carbanion creates favorable dipole interactions with a cationic, active site lysine residue.[152] Depending on the distance of these charges (2.7−3.0 Å) and the local dielectric constant, such interactions could, indeed, change pK_a's significantly (the authors refer the reader to a 3−5 pK_a unit elevation of the active center His of serine proteases on juxtaposing tetrahedral oxyanionic transition state analogues on the Ser residue).[153−156] A prediction made by proponents of the electrostatic attraction theory is that the lysine residue thought to pair with the carbanion will have a perturbed pK_a value from its value in solution, so as to optimize its potential as an H-bond donor. It is also argued that the solvation behavior of the OMPDC active site differs considerably from that of bulk water.[157] Indeed, it has been estimated that the pK_a for C6−H is suppressed by at least 10 units on the enzyme,[158] as compared with the value estimated by Wolfenden's group for the aqueous value,[159−161] leading to as much as 14 kcal/mol transition state stabilization.

The H-to-D exchange at the product C6 position was studied over a range of pH values, and the results were interpreted as being consistent with proton transfer from the product (UMP) C6 atom to a neutral conserved lysine nearby, thus producing an enzyme-bound vinyl-carbanion (or carbane in resonance with it) and concluding that the enzyme stabilizes the carbanionic intermediate by 10^{10}-fold, consistent with the earlier conclusion above.

Test of the Conversion of Intrinsic Binding Energy to Transition State Stabilization. In a series of papers, Amyes and Richard and co-workers tested the idea by the late WP Jencks[162] that the intrinsic binding energy could be converted into transition state stabilization. This notion appears to have been first tested on OMPDC by Sievers and Wolfenden, who reported that a substrate analogue lacking the phosphate group underwent decarboxylation at a much-impaired rate.[164] In the first communication by Amyes and Richard, 1-($α$-D-erythrofuranosyl)orotic acid (EO) was complemented with exogenous phosphite dianion, and it was found that binding of the phosphite away from the reactive center caused a significant lowering of the transition state energy for decarboxylation, consistent with the Jencks theory.[165] Further research by the group has explored both altered substrates (such as the use of an electron-withdrawing 5-F substituent on the pyrimidine ring) in conjunction with added phosphite dianion, and concluded that phosphodianion binding interactions are utilized to stabilize a rare closed enzyme form that exhibits a high catalytic activity for decarboxylation.[166−169] This continuing work not only sheds light on the OMPDC mechanism but also provides a very important protocol for others wishing to study the effects of various parts of sugar phosphates in catalysis.

The continuing fascination with this enzyme (as evidenced by the yearly publication output on it) is justified because it provides additional issues on decarboxylation mechanisms, which extensive studies of the ThDP and PLP mechanisms have not yet provided.

Comparison of the Catalytic Strategies Employed by the Three Enzymes. There is an important parallel in the catalytic strategies employed by ThDP and PLP, while some parallels also appear to exist with all three reactions.

1. Simply forming a covalent linkage with the substrate thus juxtaposing a β-electron sink, the electrophilicity assisting CO_2 loss is enhanced by many orders of magnitude. According to computational studies, formation of the external aldimine with substrate per se provides as much as 16 kcal/mol lowering of the barrier to decarboxylation of L-DOPA, whereas the protein lowers it further by an additional 8 kcal/mol, producing perhaps 5−6 orders of magnitude additional catalysis by the protein.[170] At the same time, it had been estimated that YPDC, the prototypical ThDP enzyme, lowers the rate of enzymatic decarboxylation of pyruvate by 10^{12}−10^{13}-fold compared with ThDP in solution.[171] Even with ThDP present, benzoin condensations are the prototypical model reactions for ThDP, rather than decarboxylations, and the classical benzoin condensation is also catalyzed by cyanide anion. For decarboxylation of 2-oxo acids, we appear to have no reliable experimental models with which to assess the catalytic effects of ThDP itself on the reaction. This factor is obviously absent in the case of OMPDC.

2. The possibility of contribution to catalysis by substrate distortion has been raised especially in the case of OMPDC, in which a carboxylate group is juxtaposed very near the departing CO_2 of the substrate. This juxtaposition would impart severe electrostatic distortion, were both the substrate carboxylate and the protein Asp carboxylate negatively charged, not a simple question to resolve experimentally. It is to be noted that although there are a number of ThDP-dependent decarboxylases with Asp or Glu at their active sites, there are also some with no potentially ionizable or protonated side chains of any kind, such as glyoxylate carboligase, certainly ruling out this mechanism as a universal property of ThDP enzymes. At the same time, as quoted in the section on ThDP, there is evidence of distortion in the predecarboxylation covalent intermediate (specifically, of the thiazolium ring), perhaps also accelerating the CO_2 loss. Perhaps the fact that ThDP is found in the "V" conformation when enzyme-bound, already strained compared with what is observed for free ThDP and even LThDP, could also be construed as substrate distortion. This by itself then enables intramolecular acid−base catalysis.

3. All three enzymes appear to share an electrostatic stabilization by a positive charge near the departing CO_2 group. In some reported structures of PLP-dependent α-amino acid α-decarboxylases and in OMPDC, this is provided by a Lys group, and the authors suggest a similar role for the APH$^+$ form of the coenzyme in the predecarboxylation intermediate. The observation of the APH$^+$ form on all three enzymes examined so far by solid state NMR methods, along with

the elevated pK_a's observed for this form on all enzymes where such data are available (seven cases as of this writing), suggest such a possibility with ThDP, as well. Such a positive charge would provide significant electrostatic stabilization for any negative charge nearby, including the transition state preceding the carbanion product of CO_2 loss.

4. Apparently, both ThDP enzymes and OMPDC have evolved to significantly suppress pK_a's of weak carbon acids, as discussed in the review. Measurement of pK_a's on the enzymes themselves provides a very powerful tool to evaluate the effect of the enzyme environment on reactivity. Some of the acidification observed and deduced indirectly is, indeed, very impressive, perhaps as many as 9−10 orders of magnitude. Such pK_a suppressions are almost certainly due to the hydrophobic environment of the protein and provide the opportunity for quantitative assessment of catalytic contributions.

5. As mentioned in the section entitled "Do Thiamin Enzymes Provide a Paradigm for "Enzymatic Solvent Effects"?", there is significant evidence both from models and on enzymes that ThDP-dependent decarboxylases would accelerate the decarboxylation step, in which the zwitterionic LThDP and analogues would experience reduced charge separation on decarboxylation to the enamine by creating an apolar (lower effective dielectric constant) environment. Richard and Amyes had pointed out the advantages of such zwitterionic decarboxylation transition states,[172] including for PLP-dependent decarboxylases and OMPDC, in addition to ThDP-dependent enzymes. Although ThDP- and PLP-dependent decarboxylases offer clear examples, charge concentration experienced for rate reduction appears to be more modest in the case of OMPDC, according to current understanding.

6. Regarding the issue of the form of CO_2 released from the active site (whether as carbon dioxide or bicarbonate), Kluger and associates recently proposed a mechanism according to which the predecarboxylation intermediate of the enzyme benzoylformate decarboxylase (analogous to LThDP with a phenyl ring replacing the methyl group in YPDC) undergoes hydration of the carboxylate group, followed by expulsion of bicarbonate concerted with C−C scission.[173] The hypothesis is based on decarboxylation model studies for BFDC. Although an intriguing idea, the mechanism cannot be universal for all ThDP decarboxylases, given that some enzymes, such as GCL, have no serine residues at their active centers, and the 4′-imino nitrogen appears to be the only conserved nucleophile across all ThDP-dependent decarboxylases. At the same time, there were early studies by Vennesland and co-workers, who in the 1960s identified CO_2 to be released by GCL.[174] For further test of the Kluger hypothesis, more research addressing whether CO_2 or HCO_3 leaves the active center on the ThDP decarboxylases themselves, and indeed on other decarboxylases, is required.

In conclusion, notwithstanding the large amount of excellent research on all three types of enzyme-catalyzed decarboxylation mechanisms, much remains to be learned about each, and each class offers its own uniqueness and challenges: ThDP, its dual catalytic mode; PLP, its need for conformational selection; and

OMPDC, its deceptively (for a simple looking reaction) difficult mechanism.

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Notes

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GLOSSARY OF ABBREVIATIONS USED

ThDP, thiamin diphosphate

YPDC, yeast pyruvate decarboxylase from *Saccharomyces cerevisiae*

PDHc, pyruvate dehydrogenase complex

TK, transketolase

POX, pyruvate oxidase from *Lactobacillus plantarum*

BFDC, benzoylformate decarboxylase

BAL, benzaldehyde lyase

E1ec, the first component of the *E. coli* PDHc

E1h, the first component of the human PDHc

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-aminopyridinium 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, methyl acetylphosphonate

MBP, benzoylphosphonic acid monomethyl ester

PMThDP, C2α-phosphonomandelylThDP, the adduct of MBP and ThDP

PLThDP, C2α-phosphonolactylThDP, the adduct of MAP and ThDP

2-AcThDP, 2-acetylThDP

3-PKB, (*E*)-4-(pyrid-3-yl)-2-oxo-3-butenoic acid

GCL, glyoxylate carboligase

DXP synthase, 1-deoxy-D-xylulose-5-phosphate synthase

PLP, pyridoxal-5-phosphate

DDC, dopa decarboxylase

OMPDC, orotidine 5′-monophosphate decarboxylase

UMP, uridine 5′-monophosphate

EO, 1-(α-D-erythrofuranosyl)orotic acid)

BMP, 6-hydroxyuridine-5′-phosphate

PDB, protein data bank

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