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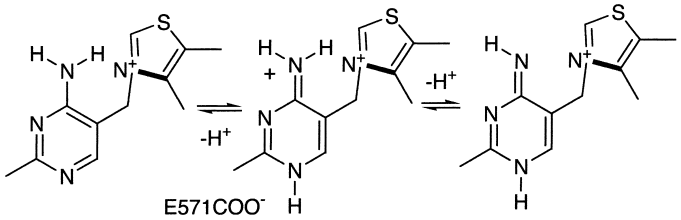
Catalysis of coenzyme tautomerization in thiamin diphosphate-dependent enzymes



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Dual Catalytic Apparatus of the Thiamin Diphosphate Coenzyme: Acid−Base via the 1′,4′-Iminopyrimidine Tautomer along with Its Electrophilic Role

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Abstract: It was recently reported (Jordan, F.; Zhang, Z.; Sergienko, E. A. *Bioorg. Chem.* **2002**, *30*, 188−198) that addition to the E477Q active-center variant of yeast pyruvate decarboxylase of (a) pyruvate on a rapid-scan UV stopped-flow, or (b) acetaldehyde or benzoylformate on a circular dichroism (CD) instrument, generates a new band with λ_{max} near 300−310 nm. A chemical model demonstrated that the wavelength is appropriate to the 1′,4′-iminopyrimidine tautomer of the 4′-aminopyrimidine ring in thiamin diphosphate. Herein, we report the formation of a new positive CD band centered at 305 nm when the *Escherichia coli* pyruvate dehydrogenase complex first E1 subunit and its variants are exposed to phosphonolactylthiamin diphosphate, a stable analogue of the covalent adduct formed between the substrate pyruvate and the C2 atom of thiamin diphosphate. The behavior of this CD band, whether it suggests saturation of the enzyme by phosphonolactylthiamin diphosphate, or its very existence (the band is not seen with the E571A E1 variant, where E571 is hydrogen bonded to the N1′ atom of the 4′-aminopyrimidine ring), as well as its position are consistent with its assignment to the 1′,4′-imino thiamin diphosphate tautomer on the enzyme, chiral by virtue of its fixed V conformation. The mechanism of binding of phosphonolactylthiamin diphosphate closely resembles that of thiamin diphosphate itself.

Since the demonstration by R. Breslow[1] that the C2 thiazolium position is key to the reactivity of the thiamin diphosphate coenzyme (ThDP; see Scheme 1 for structures and mechanism relevant to this paper), two groups raised the possibility that the 4′-aminopyrimidine ring of the coenzyme may also have catalytic function.[2,3] As we showed many years ago, in 4′-aminopyrimidine, the exocyclic nitrogen is not suitable for acid−base catalysis because the N1′ ring nitrogen atom is most basic. Yet, once N1′-protonated, the 4′-amino group becomes a weak acid, with a pK_a near 12, the ionization converting the 4′-aminopyrimidinium to the 1′,4′-iminopyrimidine tautomer.[2] One of the revelations in the structures of the three ThDP enzymes first published[4−7] was the highly conserved "V" enzyme-bound conformation of ThDP, bringing the C2 and N4′ atoms to within 3.4−3.5 Å of each other.[7] Also, a highly conserved glutamate residue at a short hydrogen-bonding distance from the N1′ atom on all ThDP enzymes suggests that this residue may indeed catalyze the amino−imino tautomerization. All ThDP-enzyme structures published to date have confirmed these features, which are certainly consistent with the notion of intramolecular proton transfer(s) involving the imino tautomer of the 4′-aminopyrimidine ring during the reaction sequence, starting with abstraction of the proton from the C2 thiazolium atom (Scheme 1). Yet, demonstrating the existence of the 1′,4′-imino tautomeric form of ThDP on the enzymes themselves is quite challenging.

Our recent work on the E477Q active-center variant of yeast pyruvate decarboxylase (YPDC) revealed a hitherto unreported UV and a positive circular dichroism (CD) band[8] between 302 and 310 nm. There are only two possible candidates for this absorption on the reaction pathway of the YPDC, the enamine and the 1′,4′-imino tautomer of the ThDP. The λ_{max} for the enamine derived from pyruvate is 295 nm, and from benzoyl-

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(1) Breslow, R. *J. Am. Chem. Soc.* **1958**, *80*, 3719−3726.
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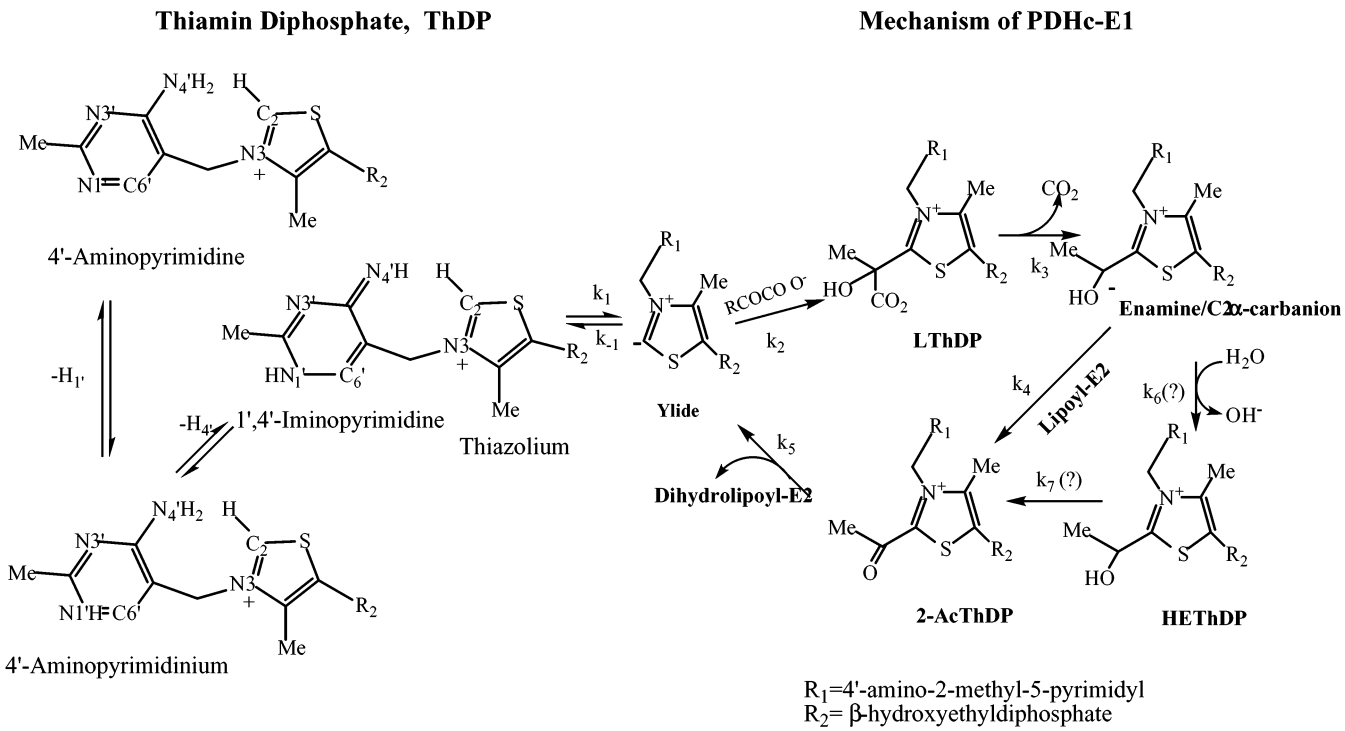
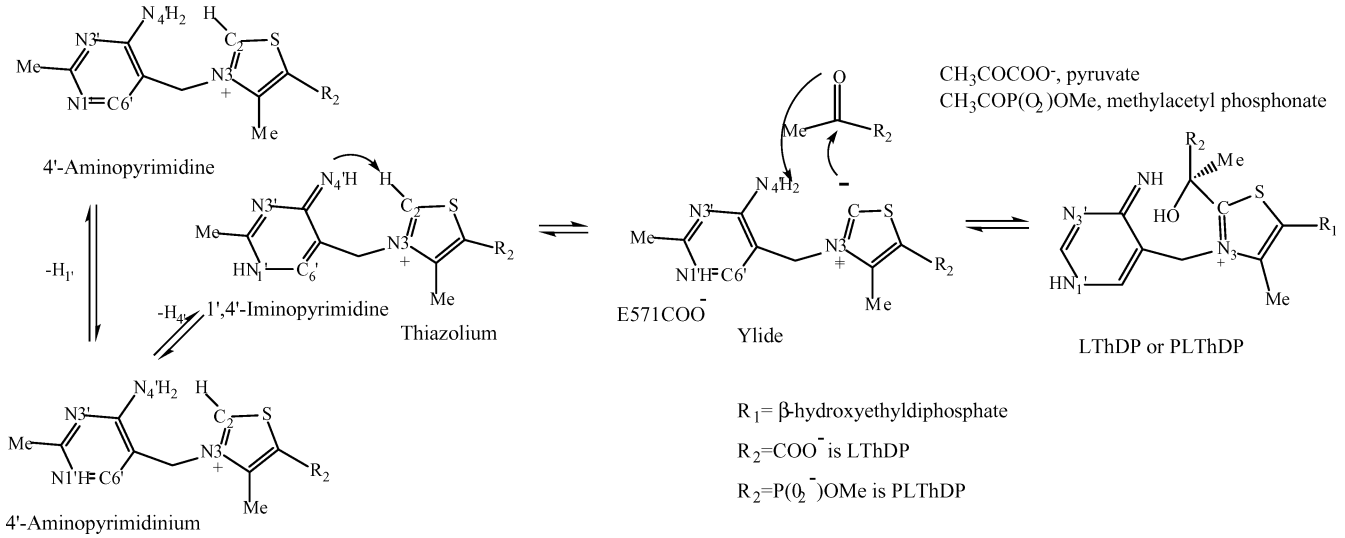
(8) Jordan, F.; Zhang, Z.; Sergienko, E. A. *Bioorg. Chem.* **2002**, *30*, 188−198.

10.1021/ja0346126 CCC: $25.00

Scheme 1



Scheme 2. Proposed Proton Transfers in LThDP and PLThDP Formation



formate it is 380 nm.[9−11] However, the position of the positive CD band for YPDC is the same when produced with pyruvate or benzoylformate, ruling out the enamine as the source of this new spectral feature. To model the new absorbance, base was added to two N1′-methylpyrimidinium salts in aqueous or organic media, leading to a new absorbance with λ_{max} at 302 and 307 nm; these absorbances reverted to their initial values on neutralization. Both UV and NMR properties of the compounds generated were consistent with their assignment to the 1,4-imino tautomer,[8] thus providing an appropriate model for the observations on enzymes.

The E1 component of the pyruvate dehydrogenase multienzyme complex (PDHc-E1) from *Escherichia coli* carries out a decarboxylation reaction typical of ThDP-dependent enzymes, and we recently published its high-resolution structure.[12] We here report circular dichroism results with PDHc-E1 in the presence of phosphonolactylthiamin diphosphate (PLThDP),[13] a stable analogue of LThDP, the covalent adduct between substrate pyruvate and ThDP (Scheme 2). Addition of PLThDP to PDHc-E1 provides the clearest spectroscopic evidence to date for the existence of the 1′,4′-iminoThDP. Because PLThDP cannot suffer C−P bond cleavage, the observations rule out the enamine as being responsible for the observations. With the help

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(10) Barletta, G. L.; Huskey, W. P.; Jordan, F. *J. Am. Chem. Soc.* **1997**, *119*, 2356−2362.
(11) Zou, Y. Ph.D. Dissertation, Rutgers University Graduate Faculty at Newark, 1999.
(12) Arjunan, P.; Nemeria, N.; Brunskill, A.; Chandrasekhar, K.; Sax, M.; Yan, Y.; Jordan, F.; Guest J. R.; Furey, W. *Biochemistry* **2002**, *41*, 5213−5221.
(13) PLThDP is a covalent adduct of methylacetyl phosphonate to C2 of ThDP; its synthesis was described by Kluger and Pike.[14]

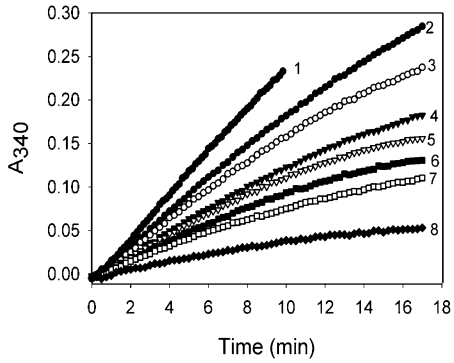

Figure 1. Progress curves of NADH production in the overall PDHc reaction in the absence (line 1) and in the presence of 10 (line 2), 15 (line 3), 20 (line 4), 30 (line 5), 40 (line 6), 60 (line 7), and 80 (line 8) μM PLThDP.

of active-center variants of the PDHc-E1, protein residues required for stabilizing the 1′,4′-iminoThDP form could be assigned.

Results

Interaction of PLThDP with PDHc-E1 and Its Variants. It was first shown that PLThDP is an inhibitor of PDHc-E1 and that 2 mol of PLThDP/mol of PDHc-E1 dimer is required for the inhibition. However, complete inhibition of PDHc-E1was not reached under the experimental conditions. The PDHc-E1 activity in the presence of 50 μM PLThDP was about 28% and did not change significantly after 16 h of incubation at 4 °C (23% activity remaining, probably due to tightly bound ThDP; see below). It was also shown that inhibition of PDHc-E1 by PLThDP was reversible, because enzyme that had been first inhibited to 28% activity then recovered 25% additional activity upon 16 h of dialysis against 20 mM potassium phosphate buffer (pH 7.5).

We carried out experiments to test whether PDHc-E1 catalyzed the conversion of PLThDP to ThDP (see Experimental Procedures). When PDHc-E1 was incubated with PLThDP, approximately 22% of ThDP was detected at the initiation of the incubation, indicating the release of tightly bound ThDP from the enzyme. This ThDP did not originate from PLThDP. After 1 h of incubation of PDHc-E1 with PLThDP, 19% of ThDP was detected, after 2 h 21% was detected, and after overnight incubation 24.8% of ThDP was detected. Taking into account (a) that the synthetic PLThDP had no detectable ThDP contamination according to ^{1}H NMR, (b) that with the amount of enzyme used, a quantitative material balance was obtained for PLThDP and ThDP, and (c) the reversibility discussed in the previous paragraph, we conclude that under the conditions of the experiment no significant conversion of PLThDP to ThDP had taken place.

A progress curve analysis was carried out to determine the kinetics of binding of PLThDP to PDHc-E1 by monitoring NADH production at 340 nm in the overall PDHc reaction (Figure 1). The analysis revealed that the initial velocity of NADH production decreased with increasing concentration of PLThDP, suggesting rapid equilibrium conditions for the equilibria drawn below:

$$\text{PDHc-E1} + \text{ThDP} = \text{PDHc-E1}\cdot\text{ThDP} \quad (1)$$

$$\text{PDHc-E1} + \text{PLThDP} = \text{PDHc-E1}\cdot\text{PLThDP} \quad (2)$$

Table 1. Dissociation Constants and Circular Dichroism Maximum for PLThDP Binding to PDHc-E1 and Its Active-Center Variants

variant	overall PDHc activity, %	K_d, PLThDP fluorescence (μM)	CD maximum (nm)	K_d, PLThDP, CD (μM)
E1	100	3.91	305−306	1.37
F602Y	57	5.33	308	0.76
H407A	0.14	9.96	not detected	
H640A	12	4.06	304−305	not determined[b]
H106A	10	not determined	302	4.93
Y599A	3	3.83	306	not determined[b]
E571A	1	2.13	not detected	
E571D	1.8	19.48	305[a]	not determined[b]

[a] Maximum at 305 nm observed at concentrations of PLThDP > 35 μM. [b] The plot of ellipticity versus PLThDP concentration did not display saturation; hence, $K_{d\text{-PLThDP}}$ could not be determined. For these variants, the maximum amplitude achieved for the accessible PLThDP concentrations was considerably less than that with PDHc-E1.

The pseudo-first-order rate constant (k_{app}) for inhibition calculated from the progress curves exhibited hyperbolic dependence on the PLThDP concentration. Data were analyzed according to the Kitz−Wilson treatment,[15] suggesting that PLThDP first forms a Michaelis-type complex ($K_i = 6.69 \pm 2.39$ μM), which is converted to the inhibited enzyme form with a rate constant of $k_{inhibition} = 0.068 \pm 0.0057$ min^{-1}. The value of K_i determined from progress curves was significantly larger than that reported for thiamin 2-thiazolone diphosphate ($K_i = 0.003 \pm 0.001$ μM) and thiamin 2-thiothiazolone diphosphate ($K_i = 0.064 \pm 0.022$ μM) using the same method.[16] The K_d values for binding PLThDP to PDHc-E1 and its variants were also determined from the quenching of intrinsic protein fluorescence (Table 1).

Circular Dichroism Studies of the Interaction of PLThDP and PDHc-E1 and Its Variants. The CD spectra of PDHc-E1 with PLThDP are shown in Figure 2. We had earlier reported that addition of ThDP to PDHc-E1 produces no CD bands in the 300−350 nm region.[16] We also showed that the enzyme-bound "V" conformation of the ThDP analogue with a C2═S in place of C2−H is sufficient to produce a strong positive CD signal at 330 nm.[16] In contrast to the observations with ThDP, addition of PLThDP to PDHc-E1 produced significant changes in the 300−350 nm region, which on subtraction of the spectrum of the PDHc-E1 revealed a peak centered at 305 nm. A plot of the ellipticity at 305 nm versus PLThDP concentration suggested saturation with a $K_{d\cdot PLThDP} = 1.37 \pm 0.29$ μM (inset to Figure 2), a value consistent with those determined by quenching of intrinsic fluorescence ($K_{d\cdot PLThDP} = 3.91 \pm 0.14$ μM and $K_{d\cdot ThDP} = 1.84 \pm 0.27$ μM) and from the kinetic progress curve analysis reported above ($K_i = 6.69 \pm 2.39$ μM). The steady-state initial velocity data suggested that PLThDP is a "mixed"-type reversible inhibitor, with a $K_i = 5$ μM. We consider the agreement among these values to be satisfactory in view of (a) the very different nature of the spectroscopic and kinetic experiments, and (b) the different concentrations of enzyme and PLThDP used in the experiments. For example, in the CD experiments, the concentrations of PLThDP and PDHc-E1 (18 μM active sites) were comparable, the fluorescence titrations used PDHc-E1 with 0.40 μM active sites with a considerable excess of PLThDP, while the progress curve analysis used

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(15) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245−3249.
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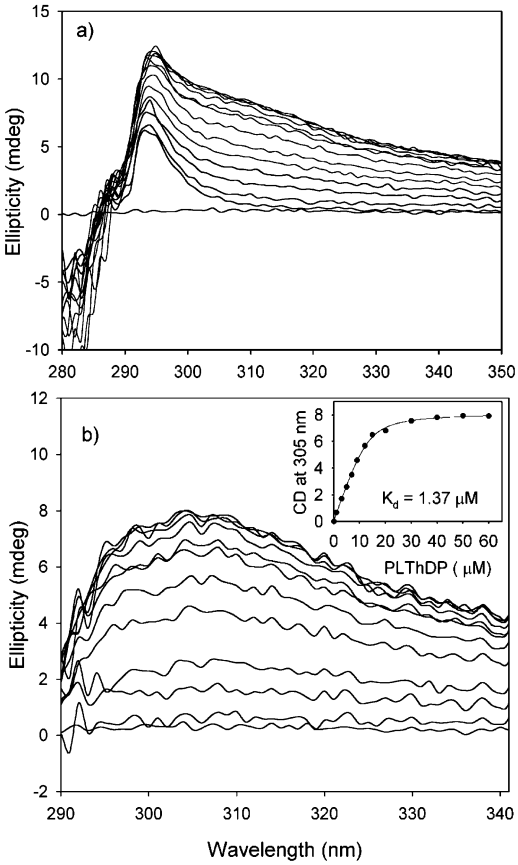

Figure 2. (a) Titration of PDHc-E1 with PLThDP (1−50 μM). (b) Difference spectra resulting from subtraction of the spectrum of PDHc-E1. Inset: variation of the CD signal at 305 nm with PLThDP concentration.

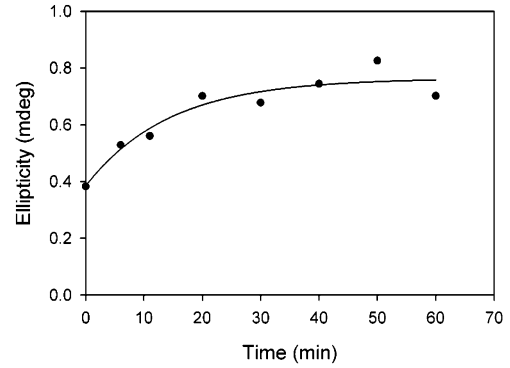

Figure 3. Time dependence of the development of the CD ellipticity at 305 nm. PLThDP at 1 μM was added to PDHc-E1 (19 μM active sites measured by protein content), yielding a $k_{app} = 0.070 \pm 0.026$ min^{-1} and a CD elllipticity maximum of 0.76 mdeg.

enzyme in the low nanomolar and PLThDP in the 1−50 μM range.

As illustrated with the example in Figure 3 ([PLThDP] = 1 μM), the development of the full CD amplitude at 305 nm was time dependent; the time dependence was not readily apparent at concentrations of PLThDP > 5 μM. The time-course in Figure 3 shows that approximately one-half of the full amplitude is achieved prior to recording the first spectrum, while a first-order rate constant of 0.070 ± 0.026 min^{-1} describes the development of the second half of the amplitude. These results strongly suggest fast saturation by PLThDP of one of the two active sites/dimer of PDHc-E1, followed by slower addition to the second active site.

The CD feature here identified can be associated with the 1′,4′-iminoPLThDP in the V conformation at the active site of E1, chiral by virtue of the fixed conformation.[17] Importantly, the CD measurements provide a more direct measurement of $K_{d\cdot PLThDP}$ than the other methods because the CD signal is attributed directly to the species being formed at the active center.

Next, we interrogated the PDHc-E1 as to which amino acid is required for stabilizing the 1′,4′-iminoThDP. With the F602Y, H640A, H106A, Y599A, and E571D variants, the band near 305 nm is evident. With the H640A, Y599A, and E571D variants, the CD signal near 305 nm did not reach its maximal amplitude achieved with the PDHc-E1, or display saturation behavior with increasing PLThDP concentration up to 50 μM (the usable range of this reagent). Yet, these variants still bound PLThDP according to the fluorescence titrations (see Table 1 for $K_{d\cdot PLThDP}$ values determined by fluorescence).

With the H407A and E571A variants, no CD signal could be detected in the presence of even 50 μM PLThDP, while the E571D variant required a concentration of PLThDP > 35 μM. For the typical variants, the CD signal could be readily detected even at low micromolar concentrations of PLThDP.

Discussion

Background Regarding the Rare 1′,4′-IminoThDP Tautomer. For nearly 5 decades, chemists and biochemists have accepted the seminal contribution by R. Breslow concerning the role for ThDP as an electrophilic catalyst (Scheme 1), creating the electron sink β to the departing carboxyl group in the LThDP intermediate. More generally, the electrophilic center could stabilize the C2α-carbanion, the key intermediate, and transition states resembling the C2α-carbanion on all ThDP-dependent pathways. The first essential step in this Breslow mechanism is the generation of the C2-carbanion of the thiazolium ring of ThDP; the latter is a weak acid whose pK_a was estimated to fall between 17 and 19 by Washabaugh and Jencks.[18] Recently, two important reports appeared concerning this ionization process: (a) Arduengo and co-workers reported generation and spectroscopic characterization of the C2-carbanion/ylide for the first time, with a striking ^{13}C chemical shift of 253 ppm for the C2 atom.[19] (b) Kern et al. carried out ^{13}C NMR studies on [2-^{13}C]ThDP bound to yeast pyruvate decarboxylase, and, on the basis of the observations that the chemical shift of the resonance for the bound ThDP was the same as that of the free ThDP (157 ppm), suggested that at pH 6, the optimum for this enzyme, the cofactor was in its undissociated state.[20]

At the same time, an important issue all along has been how the enzyme catalyzes the ionization of this very weak acid at the key C2 position. For the most part, researchers dismissed the possibility of the participation of the 4′-aminopyrimidine in catalysis. In pioneering studies using de novo synthesized ThDP analogues, in which the 4′-aminopyrimidine nitrogen atoms were replaced one at a time, Schellenberger, Hübner, and collaborators had shown that the nitrogen atoms at the 4′ and

(17) While PLThDP is synthesized as a racemic mixture, the CD signature of either enantiomer should have a wavelength well below 300 nm.
(18) Washabaugh, M. W.; Jencks, W. P. *Biochemistry* **1988**, *27*, 5044−5053.
(19) Arduengo, A. J.; Goerlich, J. R.; Marshall, W. J. *Liebigs Ann./Recueil* **1997**, 365−374.
(20) Kern, D.; Kern, G.; Neef, H.; Tittmann, K.; Killenberg-Jabs, M.; Wikner, C.; Schneider, G.; Hübner, G. *Science* **1997**, *275*, 67−70.

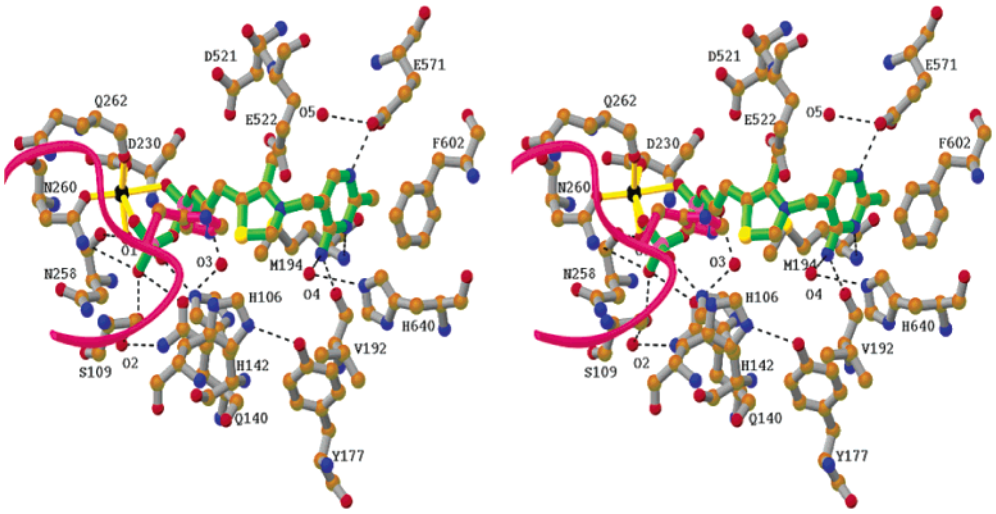

Figure 4. Stereodrawing of the *E. coli* PDHc-E1 active-center environment with the residue H407 modeled into the active center as in ref 22.

1′ position are required for activity, while that at the 3′ position is less important.[21] The implication of their studies was clear: the 4′-aminopyrimidine almost certainly has a role in ThDP catalysis. Starting in the mid-1970s, the group at Rutgers also has worked on this hypothesis.[2] We suggested that the 4′-amino group could be activated for acid−base catalysis once the ring was protonated at the N1′ atom. We had reported and recently reconfirmed that N1′-methylThDP and analogues can readily be converted to their 1′,4′-iminoThDP tautomer with a pK_a of 12−13.[2a] Our X-ray structures[6,7,12] and the conserved features below gave credence to the hypothesis advanced by these two groups for more than 25 years. (a) There is a glutamate residue across from the N1′ atom in all ThDP enzyme structures. (b) There is an enforced "V" coenzyme conformation in all structures, which brings the N4′ and C2 atoms to within 3.5 Å of each other, a plausible distance for either direct or mediated proton transfer between the two atoms. In addition, extensive mutagenesis studies on several enzymes, including YPDC and PDHc-E1 by us, have affirmed that substitution of no acid−base group at the active center abolishes fully the activity, again suggesting that the coenzyme itself participates in proton transfer(s) along the pathways.

Kern et al. reported that the rate of C2H/D exchange, as a measure of the rate of ylide formation, was greatly impaired when the residue corresponding to residue E571 of PDHcE1 was substituted on YPDC, pyruvate decarboxylase from *Zymomonas mobilis*, and transketolase.[20] Given the position of E571 (Figure 4), the results certainly suggest the involvement of tautomerization in the mechanism.

Recently, the Rutgers group reported the observation of a heretofore unreported spectroscopic signal centered near 305 nm, both by rapid-scan UV−vis stopped-flow and by CD measurements on the E477Q YPDC variant.[8] At the same time, a model system was reported for the first time to demonstrate that the 1′,4′-imino tautomer indeed would have a λ_{max} at this wavelength.

Deductions Regarding the Stabilization of the 1′,4′-IminoThDP Tautomer on PDHc-E1 from Circular Dichroism Evidence. The evidence presented for the origin of the CD band centered near 305 nm as derived from PLThDP bound to PDHc-E1, and the data in Table 1 and Figure 4, lead us to the following conclusions.

The residue F602 is stacked onto the 4′-aminopyrimidine ring of ThDP, and the F602Y variant resembles the unsubstituted PDHc-E1 in every respect; hence, this residue appears to have no impact on the observation of the rare tautomer.

The residue H106 is one of four histidine residues seen in the active center of the PDHc-E1·ThDP complex. The results with the H106A variant show that it can still generate the 1′,4′-iminoPLThDP from PLThDP, albeit the K_d is increased by 4-fold, approximately the same as the reduction in specific activity as compared to PDHc-E1. Hence, some interaction of the residue H106 with PLThDP is suggested.

The residue Y599 is in the channel leading to the active center. The residue H640 is near enough to the substrate and ThDP-bound intermediates to participate in hydrogen bonding; in fact, one of its imidazole nitrogen atoms is hydrogen bonded to a water molecule, which is in turn hydrogen bonded to the amino group of ThDP. The CD signal characteristic of the 1′,4′-iminoPLThDP tautomer is still visible with both Y599A and H640A substitutions, but the amplitude of the signal is smaller than that observed with PDHc-E1, and the plots of ellipticity versus PLThDP concentration do not display saturation. We conclude therefore that residues Y599 and H640 both interact with PLThDP.

While no electron density could be detected for H407 in the PDHc-E1 crystal structure, the position for this side chain was modeled into the active center of PDHc-E1 by comparison with the structure of transketolase.[22] We have shown that the residue H407 has an important role in the reductive acetylation of E2 by E1 and pyruvate and, to a lesser extent, in the first decarboxylation phase of the reaction.[22] This was one of only two residues tested whose substitution essentially abolished the CD signature near 305 nm. Apparently, the residue H407 also interacts with the PLThDP, in turn being partly responsible for the stabilization of the 1′,4′-iminoThDP tautomer.

The residue E571 is the highly conserved glutamate within strong hydrogen-bonding distance of the N1′ atom of ThDP.

(21) Schellenberger, A.; Hübner, G.; Neef, H. *Methods Enzymol.* **1997**, *279*, 131−146.

(22) Nemeria, N.; Arjunan, P.; Brunskill, A.; Sheibane, F.; Wei, W.; Yan, Y.; Zhang, S.; Jordan, F.; Furey, W. *Biochemistry* **2002**, *41*, 15459−15467.

Very significantly, the signal was not seen with the E571A substitution (specific activity 1%). With the E571D variant (specific activity 1.8%), the negative charge is still present as with glutamate, but the charge is displaced by approximately 1 Å away from the N1′ atom as compared to glutamate. Accordingly, the CD signal at 305 nm is indeed visible, but with diminished amplitude, and the plot of ellipticity versus PLThDP concentration does not display saturation. Parenthetically, the H407A and E571D PDHc-E1 variants did not exhibit saturation with ThDP in the fluorescence titrations either. Hence, E571 must have a role in stabilizing the 1′,4′-iminoThDP tautomer and indirectly in the formation of the LThDP intermediate. A possible role for the highly conserved glutamate as a catalyst for the amino−imino tautomerization is shown in Schemes 2.

Thus, the residues E571 and H407 have the most dramatic effects on the behavior of the 1′,4′-iminoThDP (in this case, 1′,4′-iminoPLThDP), but residues H407, E571, Y599, and H640 all appear to interact with PLThDP, and by inference with LThDP, perhaps they interact with the transition states leading to LThDP.

An overview of the experimental data suggests that PLThDP binds to PDHc-E1 dimer according to a mechanism analogous to that reported by us for binding ThDP.[25] In this scheme, there is rapid formation of a Michaelis complex with one PLThDP/

$$\text{PDHc-E1} + \text{PLThDP} = \text{PDHc-E1}\cdot\text{PLThDP} \quad (3)$$

$$\text{PDHc-E1}\cdot\text{PLThDP} = \text{PDHc-E1}\cdot\text{PLThDP}^* \quad (4)$$

$$\text{PDHc-E1}\cdot\text{PLThDP}^* + \text{PLThDP} = \text{PDHc-E1}\cdot(\text{PLThDP})_2 \quad (5)$$

dimer, which then is rapidly converted to the half-saturated dimer, PDHc-E1·PLThDP*. Our experiments did not reveal any information about the rate of this first binding step. Next, the second binding site in the PDHc-E1 dimer is filled by PLThDP at a measurable and slow rate, leading to the formation of PDHc-E1·$(PLThDP)_2$, and reflected both by the kinetic progress curves and by the time dependence of the development of the CD ellipticity (Figure 3). Overall, our results strongly suggest that the affinity of PDHc-E1 for PLThDP is rather similar to its affinity for ThDP.

Conclusions

We consider that these results on PDHc-E1 with PLThDP, and those recently reported with the YPDC E477Q variant,[8] now establish the presence of the 1′,4′-iminoThDP on ThDP enzymes. So far as PLThDP is a suitable analogue for LThDP at the active center,[23] we conclude that in the first ThDP-substrate covalent tetrahedral adduct, there is a preponderance of the 1′,4′-iminoThDP tautomer, perhaps a result of steric crowding, and this tautomer is almost certainly poised to participate in catalysis (see Scheme 2 for details of suggested protonic states and proton movements leading to LThDP or PLThDP).

The imino tautomer is probably stabilized by the highly conserved hydrogen bonds to the N1′ and N4′ atoms (the latter always from the N3′ side of the ring), and perhaps by the hydrophobic environment as well. Such a hydrophobic environment certainly appears to have a dramatic effect on pK_a's in YPDC.[24]

Finally, we suggest that the Breslow mechanism for ThDP catalysis be augmented with inclusion of a role(s) for the 4′-aminopyrimidine and its 1′,4′-imino tautomer, as shown on the left-hand side of the schemes. These findings make thiamin diphosphate an astonishingly versatile coenzyme, endowed with both an electrophilic and an acid−base catalytic apparatus in a relatively small molecule, the former even in the absence of the protein component, the latter induced by the protein.

Experimental Procedures

Preparation of the PDHc-E1 and Its Variants. The PDHc-E1 and its variants were purified as described previously.[26,27] Mutagenesis reactions were carried out using the QuikChange site-directed mutagenesis kit from Stratagene and are presented elsewhere.[27] The activity of PDHc-E1 was assayed after reconstitution with E2-E3 subcomplex as before.[26]

Synthesis of PhosphonolactylThDP (PLThDP). Methyl 2-hydroxy-2-(2-thiamin diphosphate)ethylphosphonate chloride (PLThDP) was prepared according to Kluger and Pike[14] with slight modifications. ThDP (1 g, 2.2 mmol) and sodium methyl acetylphosphonate (3 g, 18.8 mmol) were dissolved in 30 mL of water. The pH was adjusted to 8.2 with NaOH (10%), and the solution was stirred under nitrogen at room temperature for 15 h. Next, the pH was lowered to 6.0 by the addition of 6 N HCl. The resulting solution was applied to a Prosphere C18 300 reverse phase HPLC column (10 μ, 22 × 250 mm, Alltech) with 50 mM potassium phosphate (pH 7.0), and PLThDP was eluted with the same solvent at a flow rate of 20 mL/min. The ^{1}H NMR and ^{31}P NMR chemical shifts matched those published by Kluger and Pike.[14] The ^{1}H NMR spectrum of PLThDP gave no evidence of any unreacted starting material after purification. At pD 7.0, no spontaneous decomposition of PLThDP to ThDP could be detected by ^{1}H NMR over a period of 7 days.

Tests for Enzyme-Catalyzed Decomposition of PLThDP to ThDP. (a) Inhibition of PDHc-E1 by PLThDP. PDHc-E1 (9.5 μM) in 10 mM potassium phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$ was titrated with 5.0−50 μM concentrations of PLThDP. After 10 min of incubation with PLThDP, aliquots of PDHc-E1 were withdrawn to measure the overall PDHc activity.

(b) Analytical Test for PDHc-E1-Catalyzed Conversion of PLThDP to ThDP. The PDHc-E1 (9.5 μM) was incubated with $MgCl_2$ (2.0 mM) and PLThDP (50 μM) in 0.60 mL of 10 mM potassium phosphate (pH 7.0). At different times of incubation, 0.10 mL aliquots were withdrawn, and 0.030 mL of a mixture of 0.40 M HCl and 22% trichloroacetic acid was added to precipitate the protein. The denaturated protein was removed by centrifugation, and the pH of the solution was readjusted to 7.0. The clarified supernatant was applied to a Zorbax SB-C18 HPLC column (5 μ, 4.6 × 150 mm, Agilent). The ThDP and PLThDP were eluted with 0−5% methanol in 50 mM potassium phosphate (pH 7.0) in 4.5 min at a rate of 1 mL/min. Under these conditions, satisfactory separation of ThDP and PLThDP was achieved.

Progress Curve Analysis of the Inhibition of PDHc-E1 by PLThDP. PDHc-E1 (0.126 μM) was preincubated with E2-E3 subcomplex (molar ratio of PDHc-E1/E2-E3 subcomplex was 1:5). An aliquot from this mixture containing 0.25 μg of PDHc-E1 and a 5-fold excess of E2-E3 subcomplex was added to 1 mL of assay medium containing all components required for assay of PDHc activity and 30 μM ThDP, in the absence or presence of 0.5−100 μM of PLThDP. The reaction was initiated by addition of coenzyme A, and the formation

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of NADH was recorded at 340 nm for 17 min at 30 °C. The progress curves were analyzed according to eq 6:[28]

$$P = (v_o/k)*(1 - e^{-kt}) \quad (6)$$

where v_o is the initial velocity and k is the pseudo-first-order rate constant for the inactivation.

Circular Dichroism (CD). CD experiments were carried out on an AVIV 202 spectrometer. First, ThDP was separated from PDHc-E1 on a G-25 column. Next, PLThDP (1−50 μM) was added to the PDHc-E1 (9.5 μM dimers or 19 μM active sites) in 10 mM potassium phosphate (pH 7.0) containing $MgCl_2$ (2.0 mM). Because of the time dependence of the CD ellipticity at low PLThDP concentrations, the CD spectra were recorded after 30 min of preincubation of enzyme with PLThDP. The value of K_d from the CD titration was determined using the quadratic eq 7, because the concentrations of enzyme and PLThDP were in the same range:

$$CD_i = [(E_t + I_t + K_d) - \{(E_t + I_t + K_d)^2 - 4E_t*I_t\}^{1/2}]/(2E_t/CD_{max}) \quad (7)$$

where E_t and I_t are the total concentrations of PDHc-E1 and PLThDP and CD_{max} is the maximum ellipticity under saturating conditions.

Fluorescence Spectroscopy. The fluorescence experiments were carried out on a Cary Eclipse spectrofluorometer as reported previously.[16]

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