# Elucidation of the Chemistry of Enzyme-Bound Thiamin Diphosphate Prior to Substrate Binding: Defining Internal Equilibria among Tautomeric and Ionization States†

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*Received May 3, 2007; Revised Manuscript Received July 2, 2007*

ABSTRACT: Both solution and crystallographic studies suggest that the 4′-aminopyrimidine ring of the thiamin diphosphate coenzyme participates in catalysis, likely as an intramolecular general acid−base catalyst via the unusual 1′,4′-iminopyrimidine tautomer. It is indeed uncommon for a coenzyme to be identified in its rare tautomeric form on its reaction pathways, yet this has been possible with thiamin diphosphate, in some cases even in the absence of substrate [Nemeria, N., Chakraborty, S., Baykal, A., Korotchkina, L., Patel, M. S., and Jordan, F. (2007) *Proc. Natl. Acad. Sci. U.S.A. 104*, 78−82.]. The ability to detect both the aminopyrimidine and iminopyrimidine tautomeric forms of thiamin diphosphate on enzymes has enabled us to assign the predominant tautomeric form present in individual intermediates on the pathway. Herein, we report the pH dependence of these tautomeric forms providing the first data for the internal thermodynamic equilibria on thiamin diphosphate enzymes for the various ionization and tautomeric forms of this coenzyme on four enzymes: benzaldehyde lyase, benzoylformate decarboxylase, pyruvate oxidase, and the E1 component of the human pyruvate dehydrogenase multienzyme complex. Evidence is provided for an important function of the enzyme environment in altering both the ionization and tautomeric equilibria on the coenzyme even prior to addition of substrate. The p$K_a$ for the 4′-aminopyrimidinium moiety coincides with the pH for optimum activity thereby ensuring that all ionization states and tautomeric states are accessible during the catalytic cycle. The dramatic influence of the protein on the internal equilibria also points to conditions under which the long-elusive ylide intermediate could be stabilized.

The coenzyme thiamin diphosphate (ThDP[1]) is composed of two aromatic rings, a 4-aminopyrimidine ring and a thiazolium ring (Scheme 1). The likelihood for participation of the 4-aminopyrimidine ring as its 1′,4′-iminopyrimidine tautomeric form (IP) in catalysis has gained considerable support with the appearance of X-ray crystal structures of ThDP enzymes in the early 1990s. Yet, only in 2002 was reported the first direct spectroscopic observation of the 1′,4′-iminopyrimidine tautomeric form of ThDP on enzymes (*1*). In the intervening years evidence was reported on several ThDP enzymes supporting the complex tautomeric equilibria depicted in the left-hand side of Scheme 1 (*2*−*7*). The tautomerization is probably catalyzed by the presence of a highly conserved glutamate residue within hydrogen-bonding distance of the N1′ atom of the 4′-aminopyrimidine (AP) ring (*8*), while the participation of the IP form in catalysis is suggested by the proximity of the N4′ atom to the thiazolium C2 atom, whose C2H ionization is believed to initiate the catalytic cycle (*9*).

Key to our elucidation of the tautomeric equilibrium on ThDP enzymes is the recognition of a new, hitherto unreported electronic transition pertinent to the IP tautomeric form with $\lambda_{max}$ at 300−312 nm, an assignment supported by model studies (*1*, *6*). Because of the proximity of this $\lambda_{max}$ to the UV absorptions of the protein, as well as of aromatic cofactors including ThDP, it has become clear that circular dichroism (CD) is the preferred method for observation of the enzyme-bound IP tautomeric form (*1*−*7*). At the same time, a systematic reexamination of a long-recognized negative CD band on ThDP enzymes with maximum at 320−330 nm was undertaken at Rutgers. It was shown that this band could be produced in two ways: either the AP form of

† Supported by Grants NIH GM050380 (Rutgers) and NSF EF-0425719 (Michigan).

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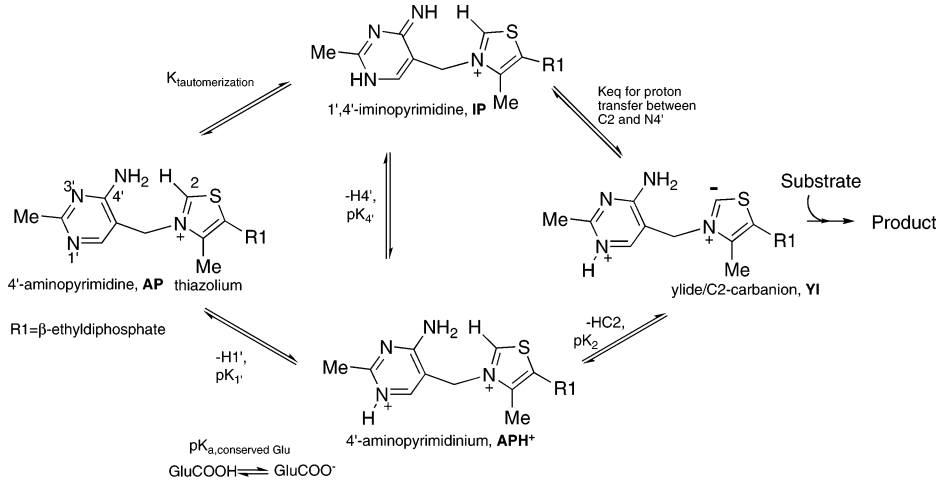
[1] Abbreviations: BAL, benzaldehyde lyase; BFDC, benzoylformate decarboxylase; POX, pyruvate oxidase; PDHc, the pyruvate dehydrogenase complex; PDHc-E1, the first component of the human pyruvate dehydrogenase complex; YPDC, pyruvate decarboxylase from the yeast *Saccharomyces cerevisiae*; ZmPDC, *Zymomonas mobilis* pyruvate decarboxylase; CD, circular dichroism; ThDP, thiamin diphosphate; AP, the 4′-aminopyrimidine form of ThDP; IP, the 1′,4′-iminopyrimidine tautomeric form of ThDP; $APH^+$, the N1′-protonated 4′-aminopyrimidinium form of ThDP; ThTTDP, thiamin 2-thiothiazolone diphosphate.

10.1021/bi700838q CCC: $37.00 
Published on Web 08/23/2007

Scheme 1: Tautomeric and Ionization States of ThDP Prior to Substrate Addition




ThDP by itself, or in a Michaelis complex of ThDP with a negatively charged substrate or substrate analogue present. Given (a) the proximity of these two electronic transitions to each other, the recognition that the band corresponding to the IP form has a positive, the one for the AP form a negative phase in the CD spectrum, and (b) the fact that only on the enzymes can these CD bands be seen since the two tautomers have no chiral centers (Scheme 1), CD spectroscopy has become our tool of choice for such studies (*1*−*7*). We have reported two important observations of applicability to ThDP enzymes so far: (a) on some enzymes (the E1 component of the human pyruvate dehydrogenase complex and on pyruvate oxidase from *Lactobacillus plantarum*), the IP and AP tautomeric forms coexist (*7*), perhaps signaling active center communication and half-of-the-sites reactivity, and (b) the IP form is the obligatory tautomer with all enzyme-bound intermediates carrying a tetrahedral C2α substituent on ThDP (on six ThDP enzymes examined so far at Rutgers) (*4*).

On the basis of both model studies and studies on six enzymes, our current view of the mechanism is as follows (Scheme 1): (a) A highly conserved glutamic acid protonates the N1′ atom of AP to form a glutamate$^{-}$•4′-aminopyrimidinium$^{+}$ ($APH^{+}$) ion pair. The $APH^{+}$ form is the species required to interconvert the IP and AP tautomers, and it could also directly lead to the ylide/C2-carbanion, Yl; (b) To ionize the weakly acidic thiazolium C2H, we suggest that the pathway proceeds as $APH^{+} \rightarrow$ IP $\rightarrow$ Yl. Because of the simple thermodynamic relationship among the four species depicted in Scheme 1, our new proposal provides an important framework to enable estimation of hitherto undetermined internal equilibrium constants among the species when enzyme bound. Crucial to such analysis is the availability of p$K_a$ values for the ([AP] + [IP])/[$APH^{+}$] equilibrium. We here report that such p$K_a$ values for benzaldehyde lyase, benzoylformate decarboxylase, pyruvate oxidase, and human pyruvate dehydrogenase complex E1 component are elevated to 5.6−7.5, compared to the value of 4.85 in water (*10*). These p$K_a$values for the $APH^{+}$ form on ThDP, and the here-reported p$K_a$ for the conserved glutamate in benzaldehyde lyase, demonstrate that proton transfer from the conserved glutamic acid to the ThDP N1′ atom is thermodynamically favored, and also suggest that the N4′-imino nitrogen also becomes a stronger base by a similar amount.

## EXPERIMENTAL PROCEDURES

*Expression and Purification of Proteins.* The plasmid pKKBAL-His encoding the BAL was transformed into BL 21(DE3)pLysS cells (Novagen). The BAL was expressed and purified as described in ref *11*. The BFDC was expressed and purified as in ref *11*. The POX from *Lactobacillus plantarum* was a gift from Boehringer Mannheim (now Roche Diagnostics). A coexpression vector for PDHc-E1 (pET-28b-PDHA1/PHDHB) harboring coding sequences of both human E1α and human E1$\beta$ subunits was constructed as described previously (*12*). The PDHc-E1 was overexpressed in *Escherichia coli* BL21 cells and purified using Ni-NTA-agarose chromatography as described previously (*12*).

*Circular Dichroism Experiments.* CD experiments were carried out on a Chirascan CD Spectrometer from Applied Photophysics (Leatherhead, U.K.) in 1 cm path length cell in the near-UV (250−450 nm) wavelength region. The experimental conditions for each protein are presented under figure legends. The observed ellipticity was converted to the molar ellipticity [Θ] using eq 1,

$$[\Theta] = (100\Theta)/(cl) \quad (1)$$

where [Θ] is the molar ellipticity in deg•cm$^{2}$•dmol$^{-1}$, Θ is the observed ellipticity in deg, $c$ is the concentration in mol/L (M), and $l$ is the path length in cm. The observed ellipticity Θ was corrected by subtraction of baseline.

To study the pH dependence of the CD spectral bands, the pH of each protein was adjusted using a sympHony pH electrode (VWR) and CD spectra were recorded after each adjustment. The p$K_a$ was determined from the fit of the log of ellipticity versus pH according to eq 2 for a single ionizing group,

$$\log(\text{ellipticity}) = \log(\text{ellipticity}_{max}) - \log(1 + 10^{(pK1-x)}) \quad (2)$$

where $x$ is the pH.

## RESULTS AND DISCUSSION

*Benzaldehyde lyase* (BAL**)** from *Pseudomonas fluorescens* (EC 4.1.2.38) catalyzes the ThDP-dependent cleavage of (*R*)-benzoin to benzaldehyde and the reverse reaction of the

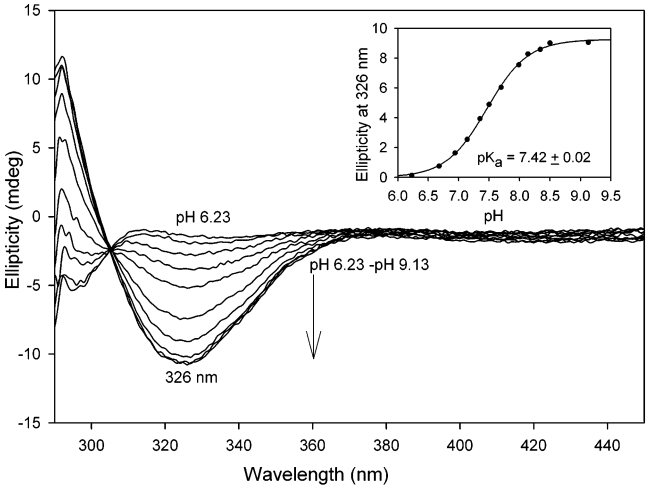



FIGURE 1: Near-UV CD spectra of BAL at different pH values. To BAL diluted to a concentration of 2 mg/mL (concentration of active centers = 33.9 $\mu$M) in 50 mM three-component buffer (pH 6.23) comprising acetic acid, MES, and Tris were added 250 $\mu$M ThDP and 1 mM $MgCl_2$. CD spectra were recorded after adjustment of the pH to the desired value (pH 6.23−pH 9.13) using a sympHony electrode. Inset: pH dependence of AP formation at 326 nm. The p$K_a$ was determined using eq 2.

formation of (*R*)-benzoin from two molecules of benzaldehyde. We first discuss BAL, which has an active center that is virtually devoid of acid−base residues, with the exception of the highly conserved E50, and H29 (*11*, *13*). This makes interpretation of the spectroscopic and kinetic data the least ambiguous of any of the known ThDP enzymes.

Our principal results on BAL are the following.

(1) The negative band at 326 nm in the CD spectrum of the BAL·ThDP complex (Figure 1) revealed the presence of the AP form, but the CD band corresponding to the IP form was not detected. However, the IP form was observed with BAL complexed to a stable ThDP-bound reaction intermediate (unpublished). The following experiments support assignment of the negative CD band at 326 nm to the AP form of ThDP bound to BAL: (i) the CD band is no longer visible after dialysis against 50 mM Tris-HCl (pH 8.0) but reappears on addition of ThDP (data not shown); (ii) the negative CD band is replaced by a positive one at 322 nm on addition of the thiamin 2-thiothiazolone diphosphate (ThTTDP), an analogue of the enamine intermediate with C2−H bond substituted by a C2═S bond (Figure 2), indicating the formation of the BAL·ThTTDP complex; (iii) on addition of ThTTDP to apo-BAL (free of ThDP) a positive band at 322 nm developed and reached a maximum (data not shown); no negative CD band at 326 nm was observed.

(2) The amplitude of the CD band for the AP form is pH dependent. It is not detected at pH 6.23 (where the predominant form is $APH^+$, for which we have no spectroscopic assignment), but increases with increasing pH and reaches a limiting value at pH 8.5 (Figure 1, inset), consistent with full conversion to the AP form (*3*). The titration data for the AP form fits to a single proton titrating with p$K_a$ of 7.42 ± 0.02 for the ([AP] + [IP])/[$APH^+$] equilibrium. It is reasonable to assume that, at pH 8.5, all four ThDPs of the BAL homotetramer (each subunit binds one ThDP and $Mg^{2+}$) are fully in the AP form. Our results permit determination of the molar ellipticity of the AP tautomeric form for the

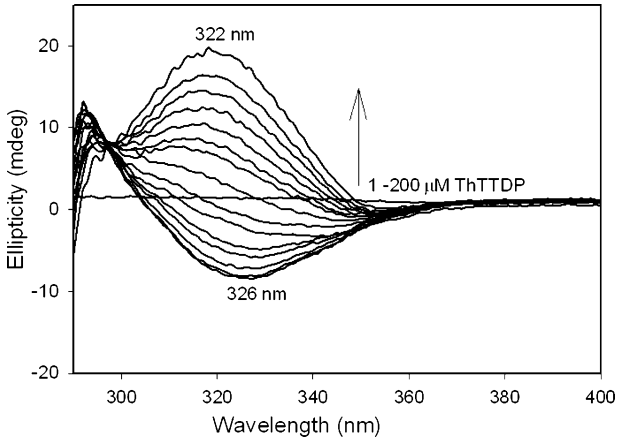



FIGURE 2: CD spectra of BAL on replacement of ThDP by ThTTDP. To BAL (2 mg/mL, concentration of active centers = 33.9 $\mu$M) in 50 mM Tris-HCl (pH 8.0) containing $MgSO_4$ (1 mM) were added increments of ThTTDP (1−200 $\mu$M).

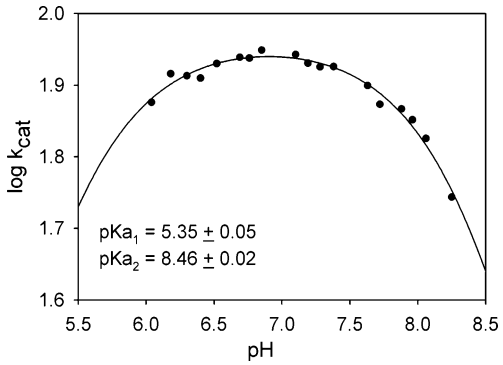



FIGURE 3: pH dependence of $k_{cat}$ for BAL. The reaction was carried out in 50 mM three-component buffer comprising acetic acid, MES, and Tris in the pH range of 6.0−8.25. The reaction medium contained 1 mg/mL of bovine serum albumin, 1 mM $MgCl_2$, 0.50 mM ThDP, 0.25 unit of horse liver alcohol dehydrogenase, 0.60 mM benzoin (0.01 M stock in 99.9% DMSO), and 0.35 mM NADH. The reaction was initiated by the addition of 0.5 $\mu$g of BAL and was recorded for 5 min at 340 nm and 30 °C. The specific activity of BAL was approximately 30−40 units/mg protein. The values of $k_{cat}$ were fitted to a bell-shaped curve defined by two ionizing groups according to $\log k_{cat} = \log(k_{cat,max}) - \log(1 + 10^{(pK1-x)} + 10^{(x-pK2)})$, where $x$ is the value of pH.

first time: $[\Theta]_{AP} = -26{,}594$ deg·cm$^2$·dmol$^{-1}$ per active center (*4*). The pH-dependence of $k_{cat}$ showed maximal activity at pH 6.5−7.5 and derived p$K_{a1}$ = 5.35 and p$K_{a2}$ = 8.50, corresponding to the E·S complex (Figure 3). Due to the paucity of acid/base residues on BAL, we could assign the p$K_{a1}$ = 5.35 to the highly conserved E50, located within hydrogen-bonding distance of the N1′ atom of ThDP. The value is similar to that determined for the corresponding residue of *Zymomonas mobilis* pyruvate decarboxylase (ZmPDC) using a thiazolium C2H→D exchange experiment (*14*). The p$K_{a2}$ = 8.50 is less reliable, since at pH > 8.0 the BAL activity decreases due to lower stability. We note that a plot of $k_{cat}/K_m$ vs pH should parallel the $k_{cat}$ vs pH plot since the $K_m$ values for (*R*)-benzoin were found to be invariant within experimental error at pH 8.0 (0.095 mM) and pH 6.0 (0.084 mM).

We conclude that the acid limb in the $k_{cat}$−pH plot (or $k_{cat}/K_m$−pH plot) controls the BAL activity and it pertains to E50. However, the p$K_a$ for the ([AP] + [IP])/[$APH^+$] equilibrium is not far from the pH optimum for enzyme

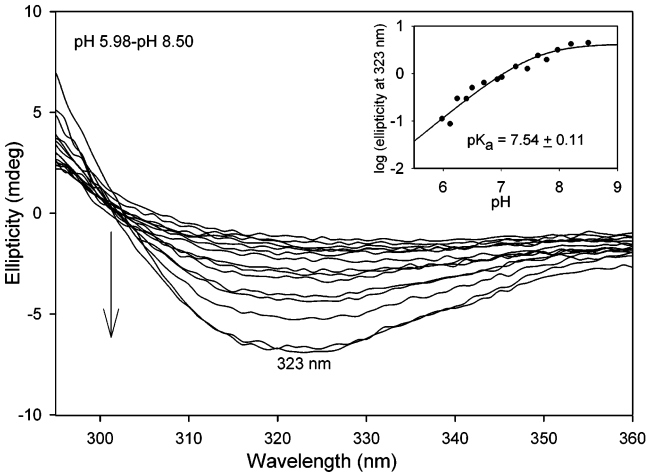



FIGURE 4: Near-UV CD spectra of BFDC at different pH values. To BFDC diluted to a concentration of 2 mg/mL (concentration of active centers = 35.5 $\mu$M) in 50 mM three-component buffer (pH 6.0) were added 250 $\mu$M ThDP and 1 mM $MgCl_2$, and CD spectra were recorded after adjustment of pH in the range of 5.98−8.5. Inset: pH dependence of the AP formation at 323 nm. The p$K_a$ was determined from the fit of the experimental data to eq 2.

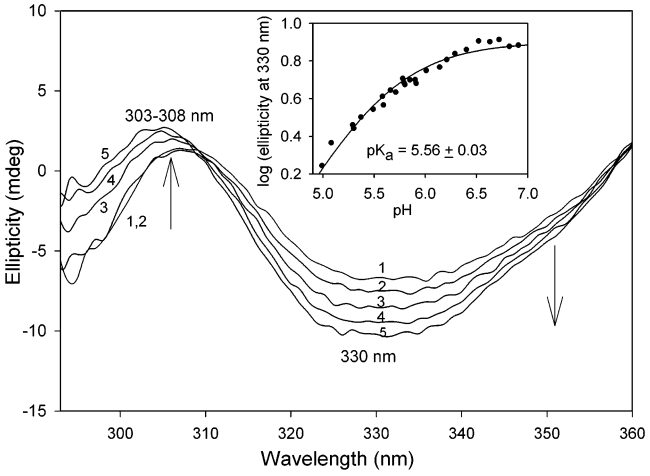



FIGURE 5: Near-UV CD spectra of POX at different pH values. The POX from stock in 100 mM $KH_2PO_4$ buffer (pH 6.0) containing 20% glycerol was diluted to a concentration of 2.24 mg/mL in 50 mM $KH_2PO_4$ buffer (pH 6.0) (concentration of active centers = 34.1 $\mu$M). Line number (pH): 1 (5.46); 2 (5.56); 3 (5.77); 4 (5.88); 5 (5.96). Inset: pH dependence of AP formation at 330 nm. The experimental data were fitted to eq 2.

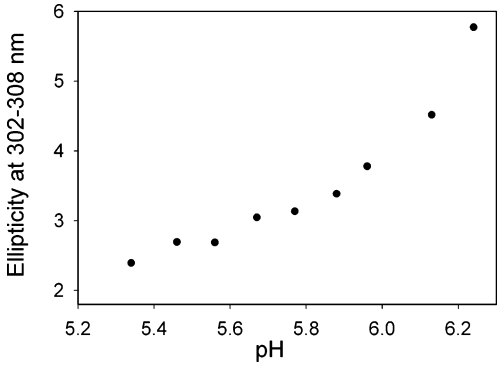



FIGURE 6: pH dependence of the CD band at 303−308 nm pertaining to the IP form of ThDP on POX. The CD spectra of POX (concentration = 3.90 mg/mL, concentration of active centers = 59.5 $\mu$M) were recorded in 50 mM $KH_2PO_4$ in the pH range of 5.34−6.24.

activity; consequently all three forms are readily accessible during the catalytic cycle. The deductions from the BAL experiments most likely apply to other ThDP enzymes. For example, it is likely that the acidic limb found in the $k_{cat}$−pH plot and $k_{cat}/K_m$−pH plots with yeast pyruvate decarboxylase (YPDC) is due to E51 (*15*).

*Benzoylformate decarboxylase* (BFDC) from *Pseudomonas putida* (EC 4.1.1.7) catalyzes the nonoxidative decarboxylation of benzoylformate to benzaldehyde and $CO_2$. The CD spectra of the BFDC·ThDP complex revealed that the $APH^+$ form is dominant to pH 7.0, while at higher pH the AP form appears and reaches a limiting value of pH 8.5 (Figure 4). The p$K_a$ of 7.54 ± 0.11 for the ([AP] + [IP])/[$APH^+$] equilibrium is similar to that observed on BAL and correlates with the pH of maximum activity at pH 6.0−8.5 (*16*). The studies revealed that at pH 8.5, for the same concentration of active centers, the amplitude of the CD band for the AP form of BFDC (−4.642 mdeg) was only half that of BAL (−9.016 mdeg). It is plausible that two of the four binding sites for ThDPs are disordered on BFDC as reflected by the inability to observe the charge-transfer band reflecting the AP form. This is consistent with the suggested mechanism of alternating active sites in a functional dimer of BFDC (*17*, *18*).

*Pyruvate oxidase* (POX) from *Lactobacillus plantarum* (EC 1.2.3.3) contains one tightly bound FAD, ThDP and $Mg^{2+}$ per subunit and catalyzes the oxidative decarboxylation of pyruvate and produces $CO_2$, hydrogen peroxide and acetylphosphate. The CD spectra of POX ($\alpha_4$ homotetramer) revealed CD bands corresponding to both the IP and AP forms (Figure 5; ref *7*). The bands for both AP and IP forms increased in amplitude with increasing pH and reached a limiting value at pH 6.50 (Figure 5 inset for AP and Figure 6 for the IP form), leading to a p$K_a$ = 5.56 ± 0.03 for the ([AP] + [IP])/[$APH^+$] equilibrium (POX is active in the pH range of 5.6−6.2; ref *19*). In the pH range 5.3−6.3 the ratio of [AP]/[IP] is pH independent and suggests that the p$K_a$ of 5.56 applies to both the [IP]/[$APH^+$] and [AP]/[$APH^+$] equilibria. Using the molar ellipticity $[\Theta]_{AP}$ for BAL, and assuming that [$APH^+$] = [AP] + [IP] at pH = p$K_a$, of the 58.6 $\mu$M concentration of active centers of POX, the occupancy is calculated as [$APH^+$] = 29.3 $\mu$M, [AP] = 22.6 $\mu$M, and [IP] = 6.70 $\mu$M. We estimated a molar ellipticity $[\Theta]_{IP}$ = 34,800 deg·cm$^2$·dmol$^{-1}$ per active center for the IP form of ThDP.

*Human Pyruvate Dehydrogenase E1 Component (PDHc-E1).* (EC 1.2.4.1; $\alpha_2\beta_2$ structure with two catalytic sites, each providing ThDP and $Mg^{2+}$ and catalyzes two reactions, the decarboxylation of pyruvate to the enamine intermediate releasing $CO_2$ and the subsequent reductive acetylation of the lipoyl groups of the second component of the complex) (*20*). A recent study on the rate of the thiazolium C2H→D exchange suggested half-of-the-sites reactivity for ThDP activation on PDHc-E1 (*21*). Recently, we observed simultaneous presence of both the IP and AP forms on PDHc-E1 (*7*) and concluded that the two active centers are occupied by different tautomeric forms of ThDP. Apparently, the concentration of AP form increases with increasing pH, reaching a limiting value at pH 7.5, describing part of a titration curve (Figure 7), and leads to p$K_a$ = 7.07 ± 0.07 for the ([AP] + [IP])/[$APH^+$] equilibrium. Using the molar

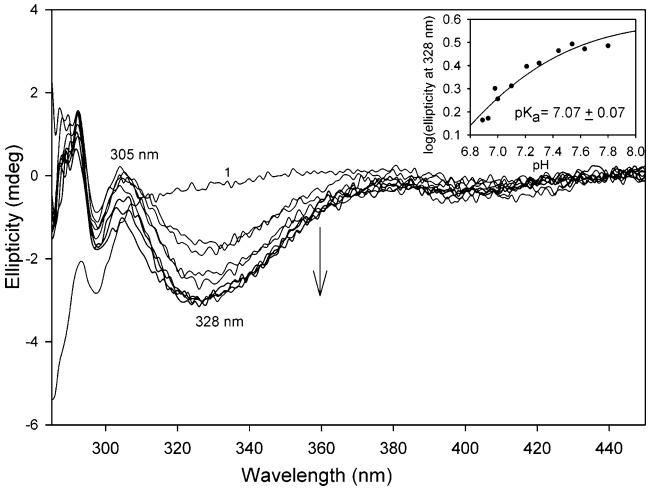



FIGURE 7: Near-UV CD spectra of PDHc-E1 at different pH values. To PDHc-E1 diluted to a concentration of 1.45 mg/mL (concentration of active centers = 19 $\mu$M) in 20 mM $KH_2PO_4$ (pH 7.0) were added ThDP (0.10 mM) and $MgCl_2$ (1.0 mM). CD spectra were recorded after adjustment of the pH to the desired value (pH 6.89−pH 7.80). Line 1 shows the CD spectrum in the absence of ThDP. Inset: pH dependence of AP formation at 328 nm. The pH range used was limited by protein stability. The experimental data were fitted to eq 2.

Table 1: Correlation between p$K_a$ of Enzyme-bound $APH^+$ Form of ThDP and pH Optimum of Enzyme Activity

| enzyme | pH of optimum activity | p$K_a$ for the ([AP] + [IP])/($APH^+$) equilibrium |
|---|---|---|
| BAL | 6.5−7.5[a] | 7.42 ± 0.02 |
| BFDC | 6.0−8.5[b] | 7.54 ± 0.11 |
| POX | 5.6−6.2[c] | 5.56 ± 0.03 |
| PDHc-E1 | 7.0−7.5[d] | 7.07 ± 0.07 |

[a] This paper. [b] Reference 16. [c] Reference 19. [d] Korotchkina, L. G., and Patel, M. S., unpublished observations.

ellipticity $[\Theta]_{AP}$ for BAL, at the pH = p$K_a$ of 7.0 with 24.8 $\mu$M active centers of PDHc-E1, we estimate 12.4 $\mu$M of $APH^+$, 7.95 $\mu$M of AP, and 4.45 $\mu$M of IP.

## SUMMARY AND CONCLUSIONS

The results allow us to draw the following conclusions regarding the mechanism of all ThDP enzymes. (1) The p$K_a$s for the ([AP] + [IP])/[$APH^+$] equilibrium will parallel the pH of optimum activity for the enzyme (Table 1); and (2) the optimum activity requires the presence of both neutral and positively charged forms, AP, IP, and $APH^+$. The POX and PDHc-E1 are unusual in stabilizing the IP form in an amount observable by CD. It is plausible that, on the other enzymes, the concentration of IP is simply too small to detect by CD. Also, while the [AP]/[IP] equilibrium constant is pH independent in solution, on enzymes several factors, including stabilization by the enzyme environment and spectroscopic properties, influence whether or not we observe both species.

Once the different forms of ThDP in Scheme 1 reach equilibrium, the newly established p$K_a$s here reported, and a value for $K_{tautomerization}$ ([AP]/[IP]) near unity, enable us to complete the thermodynamic cycles in the scheme for POX and PDHc-E1. (1) Considering the left triangle, knowing the values of p$K_{1'}$ and $K_{tautomerization}$, we conclude that p$K_{4'}$ should be similar to p$K_{1'}$. This is a striking conclusion since in water that p$K_{4'}$ is estimated near 12 in model reactions (*6*, *22*). (2) With this new estimate of p$K_{4'}$ the right triangle can also be considered. The p$K_2$ on PDHc-E1 can be estimated from the ratio of the C2H deprotonation rate constant (50 $s^{-1}$; ref *21*) to a diffusion controlled reprotonation rate constant of the ylide/C2 carbanion ($10^{10}$ $s^{-1}$ $M^{-1}$) as an upper limit. The ratio, ca. $5 \times 10^{-9}$ M, provides a p$K_2$ between 8 and 9, suggesting that the thiazolium ion is a stronger acid by perhaps 8−9 orders of magnitude than the values of 17−19 estimated from model reactions (*23*). This p$K_a$ suppression at C2H is similar in magnitude to those observed previously for both YPDC (*24*) and on the first component of PDHc from *E. coli* (*25*). The estimates of p$K_{4'}$ and p$K_2$ also bracket the proton-transfer equilibrium constant ratio [IP]/[Yl] in the right triangle to the range of 1−10 for PDHc-E1 and suggest conditions under which the Yl form could be detected on ThDP enzymes.

## ACKNOWLEDGMENT

For Dr. Kenyon this material was based on work supported by the NSF while he was working there. Any findings or conclusions expressed here are those of the author and do not necessarily reflect the views of the NSF. The corresponding author also acknowledges helpful discussions with Prof. Phil Huskey regarding Scheme 1.

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BI700838Q