Tetrahedral Intermediates in Thiamin Diphosphate-Dependent Decarboxylations Exist as a 1′,4′-Imino Tautomeric Form of the Coenzyme, Unlike the Michaelis Complex or the Free Coenzyme†

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ABSTRACT: Two circular dichroism signals observed on thiamin diphosphate (ThDP)-dependent enzymes, a positive band in the 300−305 nm range and a negative one in the 320−330 nm range, were investigated on yeast pyruvate decarboxylase (YPDC) and on the E1 subunit of the *Escherichia coli* pyruvate dehydrogenase complex (PDHc-E1). Addition of the tetrahedral ThDP−acetaldehyde adduct, 2-α-hydroxyethylThDP, to PDHc-E1 generates the positive band at 300 nm, consistent with the formation of the 1′,4′-iminopyrimidine tautomer, as also demonstrated for phosphonolactylthiamin diphosphate, a stable analogue of the tetrahedral ThDP−pyruvate adduct 2-α-lactylThDP (Jordan, F. et al. (2003) *J. Am. Chem. Soc. 125*, 12732−12738). Therefore, we suggest that all tetrahedral ThDP-bound covalent complexes will also prefer this tautomer, and that the 4′-aminopyrimidine of ThDP participates in multiple steps of acid−base catalysis on ThDP enzymes. Studies with YPDC and PDHc-E1, and their active center variants, in conjunction with chemical models, enabled assignment of the negative band at 330 nm to a charge-transfer transition between the 4′-aminopyrimidine tautomer (presumed electron donor) and the thiazolium ring (presumed electron acceptor) of ThDP, with no significant contributions from any amino acid side chain of the proteins. However, in both YPDC and PDHc-E1, the presence of substrate or substrate surrogate was required to enable detection, suggesting that the band at 320−330 nm be used as a reporter for the Michaelis complex, involving the amino tautomer, on both enzymes. As the positive band near 300 nm reports on the 1′,4′-imino tautomer of ThDP, methods are now available for kinetic monitoring of both tautomeric forms.

The typical thiamin diphosphate (ThDP)-dependent 2-oxo-acid decarboxylases proceed by coenzyme-mediated electrophilic catalysis, and there are a series of covalent complexes formed between the coenzyme and the substrate/intermediates/product on such pathways, as depicted in Scheme 1 for yeast pyruvate decarboxylase (YPDC) (*1*). On the YPDC pathway, usually three such intermediates are written: the substrate−ThDP adduct 2-α-lactylThDP or LThDP, the enamine or C2α carbanion, and the product−ThDP adduct 2-α-hydroxyethylThDP or HEThDP. On the pyruvate dehydrogenase complex (PDHc) from *Escherichia coli*, depending on conditions selected, there may be LThDP, the enamine, HEThDP, as well as the additional 2-acetyl-ThDP (AcThDP) as shown in Scheme 2 (*1*−*3*). Recently, we reported that the phosphonolactylThDP (PLThDP), an analogue of LThDP that cannot be decarboxylated, exists in the 1′,4′-imino tautomeric form on the PDHc-E1 subunit, suggesting that so does the LThDP itself (*4*). Henceforth, the possibility that each intermediate may exist in either its 1′,4′-imino- or 4′-aminopyrimidine tautomer should also be recognized.

Circular dichroism (CD) spectroscopy has become an important tool in the identification of chiral enzyme-bound intermediates on both YPDC and PDHc-E1. Our first observations in this regard were made with the potent inhibitor thiamin 2-thiothiazolone diphosphate bound to PDHc-E1, where the enzyme-bound form, but not the unbound inhibitor, gives a positive CD peak centered at 330 nm (*5*). Compared to the natural coenzyme, the only alteration is replacement of the C2H with C═S, with no chiral centers being introduced, allowing us to conclude that

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[1] Abbreviations: ThDP, thiamin diphosphate; YPDC, pyruvate decarboxylase from the yeast *Saccharomyces cerevisiae*; *E. coli*, *Escherichia coli*; LThDP, 2-α-lactylthiamin diphosphate; NaMAP, sodium methyl acetylphosphonate; PLThDP, phoshphonolactylThDP, the covalent adduct of NaMAP onto the C2 carbon of ThDP; HEThDP, 2-α-hydroxyethylthiamin diphosphate; PDHc, pyruvate dehydrogenase complex from *E. coli*; PDHc-E1, the first ThDP-dependent subunit of PDHc; CD, circular dichroism; 1′,4′-iminoThDP, the imino tautomeric form of ThDP; n_H, Hill coefficient; DCPIP, 2,6-dichlorophenolindophenol; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; TK, transketolase.

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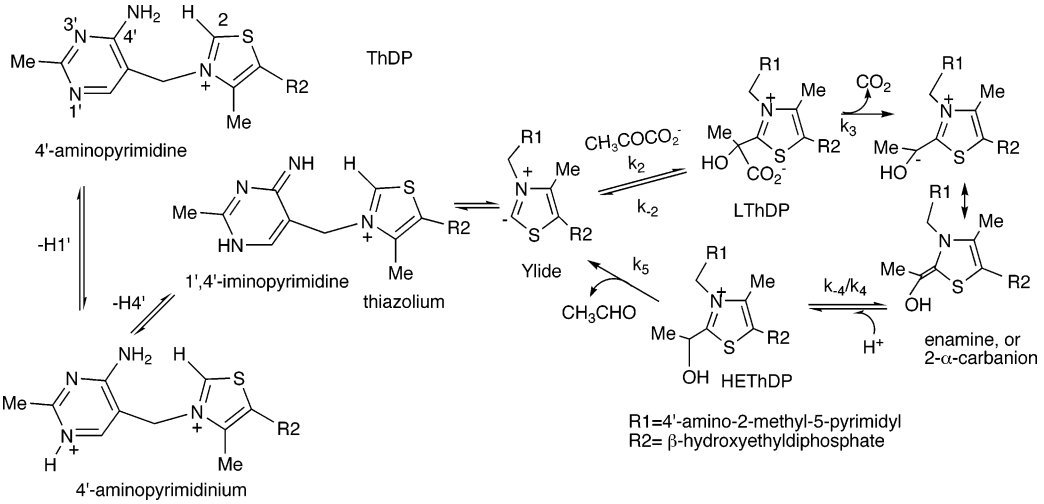
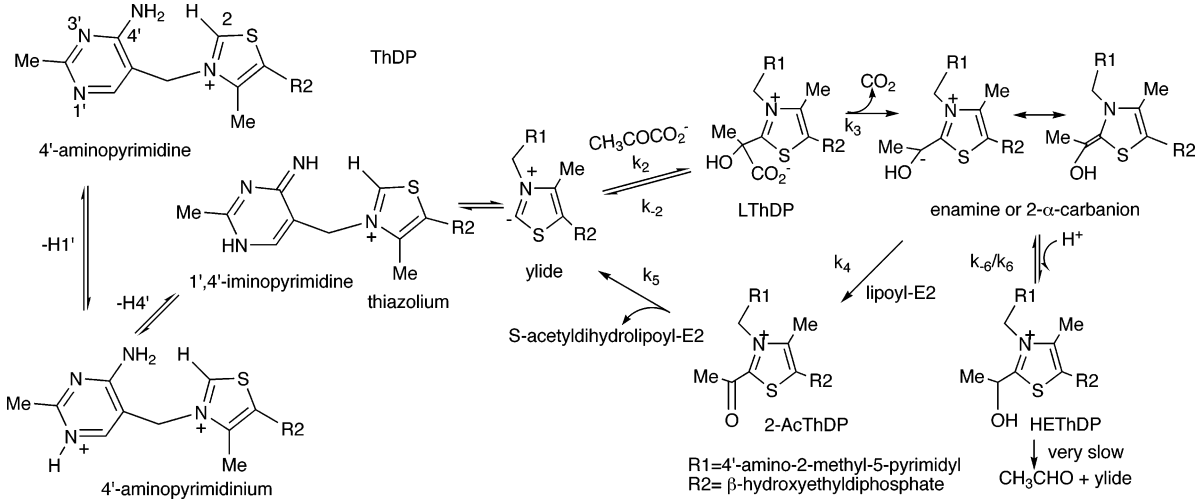
Scheme 1: Mechanism of Pyruvate Decarboxylase, YPDC



Scheme 2: Mechanism of Pyruvate Dehydrogenase Complex−E1 Subunit



the *fixed "V" coenzyme conformation* (*6*−*10*) *enforced by the enzyme environment is sufficient to induce chirality and to lead to CD signals*. At the same time, difference CD spectra with the D28A and E477Q YPDC variants clearly suggested the presence of multiple chiral intermediates on addition of substrate to this enzyme (*11*). We recently reported formation of a positive CD band centered near 305 nm, which we attributed to the 1′,4′-imino tautomer of the coenzyme supported by chemical model studies (*4*, *12*).

In this report, we summarize results on two issues: (1) evidence for the positive CD signal near 300 nm with the second tetrahedral intermediate HEThDP on PDHc-E1 and (2) evidence on several PDHc-E1 and YPDC variants for a negative CD band in the 320−330 nm range, a band that has been observed on the enzyme transketolase (TK) (*13*) and on mammalian pyruvate dehydrogenase (*14*) for many years. On both PDHc-E1 and YPDC, the band is prominent only in the presence of a substrate or substrate analogue. We could demonstrate that *the positive band at 305 nm and the negative one at 320*−*330 nm pertain to different species on the enzymes*, the latter most likely due to a Michaelis-type noncovalent complex between pyruvate and the ThDP. Our model studies suggest that the negative band near 320 nm corresponds to a charge-transfer transition, produced by the 4′-aminopyrimidine ring as an electron donor and the thiazolium ring as an electron acceptor. When added to PDHc-E1, neither PLThDP, a stable analogue of LThDP, nor HEThDP gave rise to the negative 320−300 nm CD band, but both did produce a positive band at 305 nm, consistent with the notion that for both enzyme-bound covalent intermediates, HEThDP and LThDP, the 1′,4′-imino tautomer of the coenzyme (1′,4′-iminoThDP) is the preferred tautomer.

EXPERIMENTAL PROCEDURES

Materials. The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Imidazole and 4-aminopyrimidine were purchased from Acros (Fisher Scientific). 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was from Aldrich (Milwaukee, WI). Thiamin chloride was from Sigma (St. Louis, MO). 1-Ethyl-3-methylimidazolium tosylate was from Fluka (Sigma). The Wizard 373 DNA purification system was from Promega (Madison, WI). Sodium methyl acetylphosphonate [NaMAP, $CH_3C(=O)P(O_2^-)OCH_3$] was synthesized according to Kluger and Wasserstein (*15*), while HEThDP was synthesized according to Gruys et al. (*16*). The 4-amino-*N*1-methyl-pyrimidinium tosylate was synthesized as reported earlier (*12*), as was 3,4,5-trimethylthiazolium triflate (*17*).

Bacteria and Plasmids. *E. coli* strain JRG 3456 transformed with pGS878 was used for overexpression and site-

directed mutagenesis of the *aceE* gene encoding the E1 subunit of *E. coli* PDHc. Construction of the E571A, H106A, H640A, E521A, and F602Y variants of PDHc E1 will be presented elsewhere (*18*). Construction of the H407A variant of PDHc E1 was described previously (*19*). Construction of the E91D YPDC variant was described by Li et al. (*20*), of the E51D YPDC variant by Gao (*21*), and of the E51A/E91D variant by Liu (*22*).

Overexpression and Purification of the PDHc-E1 and its Active Center Variants. Overexpression of the PDHc-E1 and its E571A, H407A, H106A, H640A, E521A, and F602Y variants was carried out following the protocol described previously for *E. coli* PDHc-E1 (*19*). Purification of PDHc-E1 and its active center variants was carried out following the protocol described previously (*23*).

Activity and Related Measurements. The activity of PDHc-E1 and its active center variants was measured by reconstituting overall PDHc activity with added E2−E3 subcomplex and monitoring the pyruvate-dependent reduction of NAD^+ at 340 nm as published previously (*5*). The PDHc-E1-specific activity was measured in the model reaction monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm (*5*).

Circular Dichroism Experiments with E. coli PDHc-E1. CD experiments were carried out on an AVIV 202 spectrometer. First, ThDP was separated from PDHc-E1 and its active center variants on a Sephadex G-25 column. Next, ThDP (1−300 μM) was added to the PDHc-E1 and its active center variants (20.1 μM dimer concentration or 40.2 μM active sites) in 10 mM potassium phosphate buffer (pH 7.0) containing $MgCl_2$ (2 mM) and pyruvate (0.20 mM). Finally, 2 mM pyruvate was added after the last portion of ThDP (200−300 μM final concentration) was added. Spectra were recorded in the 250−400 nm range immediately after the addition of ThDP and/or pyruvate. After overnight incubation at 4 °C of PDHc-E1 or its active center variants with 200−300 μM ThDP and 2 mM pyruvate, the protein was removed by Centricon YM-30, and CD spectra of the supernatant were recorded to identify the chiral products of the carboligase side reactions.

HEThDP (1−400 μM) was added to the PDHc E1 (20.1 μM dimers or 40.2 μM active sites) in 10 mM potassium phosphate buffer (pH 7.0) containing $MgCl_2$ (2 mM). CD spectra were recorded immediately in the 250−400 nm wavelength range.

The values of $S_{0.5}$ for HEThDP and NaMAP binding were determined using the Hill eq 1.

$$v_0 = (V_{max} \times [S]^n)/(S_{0.5} + [S]^n) \quad (1)$$

where S is HEThDP or NaMAP and n_H is the Hill coefficient.

Circular Dichroism Titration of YPDC and Its Variants with Sodium Methyl Acetylphosphonate. To YPDC or its E91D, E51D, and E51A/E91D variants (concentration of enzyme = 35.8 μM, concentration of active sites = 143.2 μM) in 100 mM MES buffer (pH 6.0), also containing ThDP (0.10 mM ThDP for wild-type YPDC and 1 mM ThDP for variants) and $MgCl_2$ (1 mM) in a total volume of 2.5 mL, was added 1−40 mM NaMAP. Spectra were recorded immediately after the addition of NaMAP in the 250−400 nm wavelength range.

Model Studies Attempting to Produce a Charge-Transfer Band in the 330 nm Region. A series of binary combinations of reagents were used to see which if any of these produced an absorbance at 330 nm, with the premise of creating a potential electron donor and acceptor that produced a charge-transfer band. The two rings of the ThDP were used in their different charged states, along with imidazole and dialkylimidazolium ion to model the neutral and protonated forms of histidine. Histidine residue(s) is(are) present in the active centers of all ThDP enzymes where the negative CD signal at 330 nm has been observed (TK, YPDC, and PDHc-E1). The following combinations were used (first, reagent at fixed concentration in 1 mL volume, followed by reagent at variable concentration and number of increments added; **D** is for electron donor and **A** is for electron acceptor in a putative donor−acceptor transition):

(1) To 200 mM 3,4,5-trimethylthiazolium triflate **A** were added 10 × 5 μL of 2 M 4-aminopyrimidine **D**, as a model for interaction of the thiazolium with neutral 4-aminopyrimidine.

(2) To 0.2 mM *N*1-methyl-4-aminopyrimidinium triflate were added 20 × 5 μL of 10 mM DBU for generation of the 1′,4′-iminopyrimidine tautomer.

(3) To 0.2 mM *N*1-methyl-4-aminopyrimidinium triflate was added 5 μL of 7.2 M NaOH to produce the 1′,4′-iminopyrimidine tautomer **D**, to which were added 5 × 5 μL of 4 mM 1-ethyl-3-methylimidazolium tosylate **A**.

(4) To 0.0375 M thiamin chloride **D** were added 10 × 5 μL of 0.084 M 1-ethyl-3-methylimidazolium tosylate **A**.

(5) To 100 mM *N*1-methyl-4-aminopyrimidinium triflate **A** were added 10 × 5 μL of 2 M imidazole **D**.

(6) To 0.075 M imidazole **D** were added 10 × 5 μL of 1.5 M *N*1-methyl-4-aminopyrimidinium triflate **A**.

(7) To 100 mM 4-aminopyrimidine **D** were added 10 × 5 μL of 2 M 1-ethyl-3-methylimidazolium tosylate **A**.

Spectra were recorded on a Cary 300 Bio UV−Visible Spectrophotometer, using cells with a 1 cm path length. All solutions were made up in water except the one for the DBU titration.

RESULTS

Circular Dichroism Spectra of PDHc-E1 with HEThDP. Our recent evidence on PLThDP (a stable LThDP analogue) when bound to PDHc-E1 suggested that the 4′-aminopyrimidine ring is in the 1′, 4′-imino tautomeric form, as evidenced by the appearance of a positive CD band near 305 nm (*4*) and assigned with the help of model studies (*12*). It is important to note that no changes in the CD spectrum were noted in the 300−400 nm region when saturating concentrations of ThDP itself were added to the enzyme (*4*). We next wished to determine which tautomeric form of HEThDP is present on PDHc-E1. Addition of increasing concentrations of HEThDP (1−400 μM) to PDHc-E1 did produce changes in the spectral region of 295−320 nm (Figure 1A). Difference spectra obtained by subtraction of the spectrum of PDHc-E1 in the absence of HEThDP displayed the maximum at 300 nm, which can be assigned to the 1′,4′-imino tautomeric form of HEThDP (Figure 1B). The slow rate at which HEThDP is converted to products enabled us to observe this intermediate. Incubation of the PDHc-E1 subunit with HEThDP in the absence of any

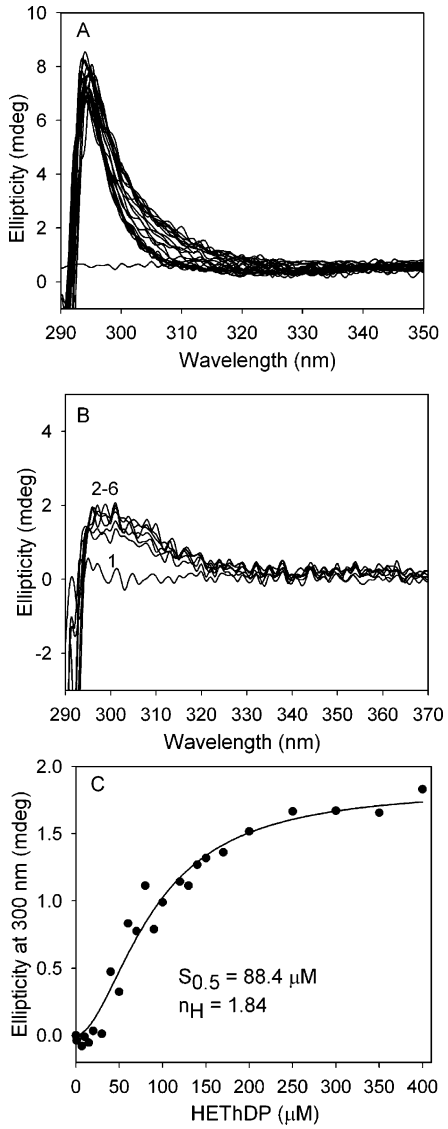


FIGURE 1: Near-UV CD spectra of PDHc-E1 titrated with HEThDP. (A) PDHc-E1 (concentration of active centers = 40.2 μM) in 10 mM KH_2PO_4 buffer (pH 7.0) was titrated with HEThDP at 1−400 μM concentrations in the presence of 2.0 mM $MgCl_2$. (B) Difference spectra obtained on addition of 1 μM (curve 1) and 100−300 μM HEThDP (curves 2−6). (C) Dependence of ellipticity at 300 nm on the concentration of HEThDP.

oxidizing agents (either E2−E3 subcomplex to reconstitute the entire complex or an external oxidizing agent such as DCPIP) produces free acetaldehyde and ThDP bound to PDHc-E1. The coupled alcohol dehydrogenase/NADH assay showed that acetaldehyde was produced from HEThDP at a rate of 0.0019 units/mg of protein (k_{cat} = 0.0064 s^{-1}), indicating slow transformation of HEThDP in the active sites of PDHc-E1. A plot of the ellipticity at 300 nm versus HEThDP concentration displayed saturation binding, with $S_{0.5,HEThDP}$ = 88.4 ± 8.70 μM and n_H = 1.84 ± 0.12, suggesting positive cooperativity of active sites (Figure 1C). Given the modest amplitude of the CD signal, the experiments were carried out in triplicates and the observations were proven to be reproducible. In independent determinations, values of $S_{0.5,HEThDP}$ of 49.25 ± 6.60 and 56.51 ± 6.8 μM were obtained. Using the protocol reported by us earlier (*5*), a $K_{d,HEThDP}$ of 35.20 ± 8.81 μM was obtained by fluorescence quenching experiments (data not shown). On the basis of these results and those reported for PLThDP binding (*4*), it is evident that both tetrahedral intermediates LThDP and HEThDP exist in their imino tautomeric form when bound to the enzyme and could be identified by the positive CD signal at 305 and 300 nm, respectively.

Circular Dichroism Titration of E. coli PDHc-E1 with ThDP. Having determined the tautomeric preferences of the tetrahedral ThDP-bound intermediates, we next attempted to identify chiral intermediates when starting with pyruvate and ThDP and varying the order of addition of the two. To the PDHc-E1, with 0.20 mM pyruvate present, were added increments of ThDP resulting in final concentrations of 1−300 μM. The rationale for the presence of low concentrations of pyruvate is that the substrate is believed to strengthen ThDP binding (*24*). A spectrum of the PDHc-E1 (ThDP was separated by gel-filtration chromatography) before and after addition of 0.2 mM pyruvate is presented in Figure 2A, left-hand panel. No changes were observed in the 310−400 nm range, consistent with our earlier report (*4*). Next, increasing concentrations of ThDP were added and led to modest changes in the 295−320 nm spectral region (data not shown), probably due to HEThDP formation in the active sites (see preceding paragraph and data on the F602Y variant presented below). Subsequent addition of 2 mM pyruvate resulted in the appearance of a broad negative CD band with a maximum at 327 nm (curve 3 in Figure 2B, left-hand panel), similar in sign, shape, and position to that observed for TK on addition of ThDP (*13*). On the basis of a number of observations, we concluded that the negative CD band at 327 nm became readily apparent only on addition of 2 mM pyruvate to the PDHc-E1−ThDP complex. It appears likely that formation of HEThDP in one of two PDHc-E1 active sites is required before the negative band at 327 nm can be generated, and the presence of the virtually dead-end HEThDP product slows down the turnover in the vacant active site. With time, the negative CD band at 327 nm was gradually converted to a negative band at 310 nm (curve 4 in Figure 2B, left-hand panel). This conversion was completed within 35 min, and the spectrum was unchanged after overnight incubation at 4 °C. After removal of the protein from the supernatant, the spectrum was recorded and displayed two bands, one negative at 310 nm and one positive at 280 nm (probably due to the formation of chiral acetolactate and acetoin, respectively) (Figure 2C, left-hand panel), similar to those observed earlier with YPDC (*11*) and more recently with PDHc-E1 (*25*) in our studies of "carboligase" side reactions. Formation of chiral acetolactate and chiral acetoin requires that the enzyme-bound enamine intermediate react with a second molecule of pyruvate or acetaldehyde (the latter is a side product of PDHc), respectively. That free acetaldehyde is indeed being produced by the PDHc-E1 under the experimental conditions was confirmed using a coupled yeast alcohol dehydrogenase/NADH assay for acetaldehyde (rate = 0.082 ± 0.006 units/mg of E1, which suggests a k_{cat} = 0.27 s^{-1}).

Control experiments were carried out omitting 0.2 mM pyruvate from the initial samples. Addition of saturating concentrations of ThDP (200 μM) to PDHc-E1 (Figure 2A, right-hand panel) with subsequent addition of 2 mM pyruvate (Figure 2B, right-hand panel) failed to produce the negative band at 327 nm. Apparently, under these conditions, pyruvate is rapidly converted to products. After the protein was

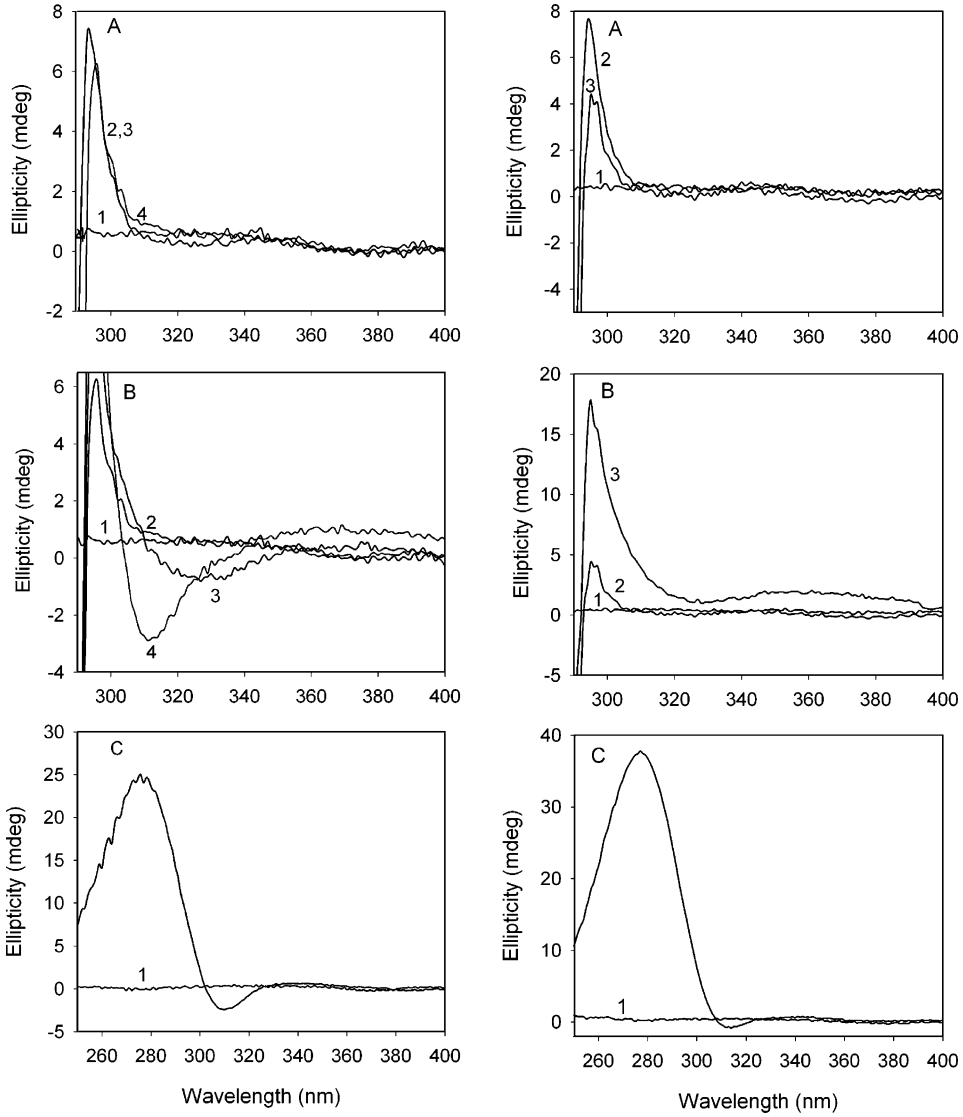


FIGURE 2: Near-UV CD spectra of PDHc-E1 at a 40.4 μM active center concentration in the presence of pyruvate and ThDP. Left-Hand Panel: (A) Spectra in the absence (curve 2) and in the presence of 0.20 mM pyruvate (curve 3) or in the presence of 0.20 mM pyruvate, 2.0 mM $MgCl_2$, and 300 μM ThDP (curve 4). (B) Spectrum in the presence of 0.20 mM pyruvate, 2.0 mM $MgCl_2$, and 300 μM ThDP (curve 2); spectrum of the same solution after addition of 2 mM pyruvate (curve 3); spectrum of the same solution after 35 min of incubation with 2 mM pyruvate (curve 4). (C) Spectrum resulting from removal of PDHc-E1 from the medium with a Centricon YM-30 unit displaying chiral acetoin (larger positive band at 280 nm) and chiral acetolactate (smaller negative band at 310 nm). Right-Hand Panel: (A) Spectra in the absence (curve 2) and in the presence of 200 μM ThDP and 2 mM $MgCl_2$ (curve 3). (B) Spectra with 200 μM ThDP and 2 mM $MgCl_2$ added (curve 2) and then with subsequent 2 mM pyruvate added (curve 3). (C) CD spectrum of chiral acetoin and chiral acetolactate after PDHc-E1 was removed from the solution with a Centricon YM-30 unit.

removed, the presence mostly of chiral acetoin, along with a significantly smaller amount of chiral acetolactate, was in evidence in the supernatant (Figure 2C, right-hand panel).

On the basis of the data presented above, we suggest that the negative band at 327 nm is related to the formation of PDHc-E1−ThDP−pyruvate Michaelis complex in one-half of the PDHc-E1 active sites, while the second site is occupied by HEThDP.

Circular Dichroism Evidence for the Interaction of ThDP with the E571A Variant of PDHc-E1. The residue E571 in PDHc-E1 is located within hydrogen bonding distance of the N1′ atom of ThDP's 4′-aminopyrimidine ring and forms a highly conserved interaction in all ThDP-dependent enzymes (Figure 3). The near-UV CD spectra of the E571A variant were different from those observed with the parental PDHc-E1 (Figure 4A). Addition of 0.20 mM pyruvate even in the absence of exogenous ThDP induced the negative band at 327 nm (Figure 4A), indicating the presence of tightly bound ThDP in the active sites of E571A PDHc-E1. Addition of ThDP (1−350 μM) to this solution decreased the magnitude of the negative band at 327 nm, and the band disappeared once the concentration of ThDP reached 350 μM (Figure 4B). Subsequent addition of 2 mM pyruvate induced the negative band at 327 nm again (ellipticity = −1.97 mdeg) (Figure 4C and Table 1), indicating the formation of PDHc-E1−ThDP−pyruvate Michaelis complex, more probably in both active sites. With this variant, formation of the 1′,4′-iminoThDP tautomer was not in evidence on addition of PLThDP since there appeared no positive band at 305 nm (*4*). The results with this variant therefore provide strong evidence against the negative band at 327 nm being pertinent to the 1′,4′-iminoThDP and affirm that the positive band at 300−310 nm and the negative one at 320−330 nm pertain to different species.

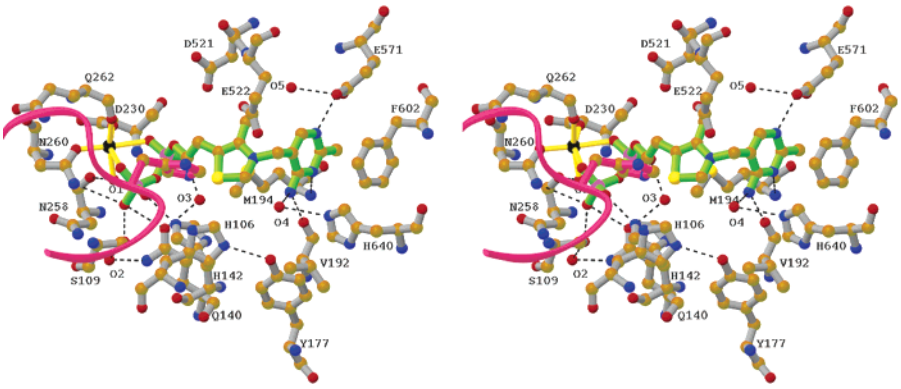


FIGURE 3: Stereo drawing of the *E. coli* PDHc-E1 active center environment with the residue H407 modeled into the active center as in ref 19.

CD Titration of H407A PDHc-E1 by ThDP. Just as with the E571A substitution, addition of PLThDP to the H407A PDHc-E1 variant also failed to produce the CD signal at 305 nm, the signature of the 1′,4′-iminoThDP (*4*, *12*). As with the E571A variant, addition of 0.20 mM pyruvate (but no additional ThDP) to the H407A variant induced the CD band at 323 nm (ellipticity = −0.620 mdeg; data not shown). The intensity of the band was gradually reduced with increasing concentrations of ThDP, presumably due to slow turnover of pyruvate. Subsequent addition of 2 mM pyruvate led to reformation of the negative band with a maximum at 324 nm (ellipticity = −1.27 mdeg; see Table 1), and the band was still visible even after overnight incubation. In this case, neither chiral acetoin nor chiral acetolactate were detected in the supernatant after removal of the protein. According to our earlier report, the H407A substitution in PDHc-E1 very much reduced the rate of the overall PDHc reaction (0.15%) but not of the DCPIP reaction (12%), indicating formation and decarboxylation of LThDP in the active sites of H407A PDHc-E1 (*19*). The H407A substitution rendered the PDHc-E1 enzyme incapable of forming chiral acetoin or chiral acetolactate in carboligase reactions, perhaps by interfering with the addition of the second substrate to the enamine.

CD Spectra of Other Active Center Variants of PDHc-E1. The residue H106 is one of four histidine residues located in the active center of PDHc-E1. Addition of 2 mM pyruvate to ThDP.PDHc-E1 with the H106A substitution results in formation of the PDHc-E1−ThDP−pyruvate Michaelis complex according to the negative CD band formed at 327 nm, similar to that observed with the parental enzyme. After overnight incubation at 4 °C, no CD signal remained in the 300−400 nm range; hence, acetolactate was not produced. Subsequent addition of 2 mM pyruvate produced the negative CD band with a maximum at 326 nm. On removal of the protein, formation of chiral acetoin was detected, but formation of chiral acetolactate was not detected (data not shown).

The behavior of the H640A and D521A PDHc-E1 variants resembled that of the E571A variant, since the negative CD band at 326 nm appeared on addition of 0.20 mM pyruvate, even in the absence of added ThDP (Table 1).

The F602Y PDHc-E1 variant displayed changes in the 295−320 nm range on addition of 0.2 mM pyruvate followed by 0.2 mM ThDP (Figure 5), providing strong evidence for formation of the imino tautomer of HEThDP (see section on HEThDP). This substitution was created specifically to mimic the interaction of the highly conserved tyrosine 448 and phenylalanine 445 in TK; both of these residues are stacked against the 4′-aminopyrimidine ring in TK, a ThDP-dependent enzyme in which the negative CD band at 320 nm is very strong. The residue F602 indeed is stacked against the 4′-aminopyrimidine ring in PDHc-E1 (*8*), and we hypothesized that its conversion to tyrosine should lead to a stronger CD signal, if the signal in TK is due to the interaction of Y448 with ThDP (*26*). The results show that interaction of ThDP with neither F602 nor Y602 is required for formation of the negative CD band at 330 nm, ruling out this interaction as the source of the phenomenon (Table 1).

With the E522A and E636A PDHc-E1 variants, we could not observe the CD band at 327 nm. This is likely due to the fact that these variants produced the acetolactate carboligase product immediately on mixing. The negative CD maximum of chiral acetolactate produced here is in the 300−310 nm range, and it tends to mask the smaller band at 327 nm, if indeed the latter even exists (detailed discussions of the carboligase side reactions of these variants and their stereochemical implications will be presented elsewhere).

Circular Dichroism Spectra of Yeast Pyruvate Decarboxylase on Addition of Sodium Methyl Acetylphosphonate. Next, we wished to determine whether the CD signals induced on PDHc-E1 could also be seen on YPDC. As it is more difficult to generate the apo form of YPDC than of PDHc-E1, we used an alternative method to generate the phosphono analogue of LThDP, PLThDP. Kluger and Pike showed that the PDHc from *E. coli* could synthesize PLThDP from sodium methyl acetylphosphonate (NaMAP) and ThDP (*27*); therefore, we used NaMAP in the experiments carried out with YPDC. The wild-type YPDC (from *Saccharomyces cerevisiae*) and its two singly substituted variants E91D and E51D and one doubly substituted variant E51A/E91D were used. The presence of the 1′,4′-imino tautomer of PLThDP (synthesized by the enzyme from ThDP and NaMAP) would be given credence by the positive CD band centered between 300 and 310 nm, as reported recently for *E. coli* PDHc-E1 (*4*), while the negative CD band near 325 nm would provide evidence for the Michaelis complex between NaMAP (as a pyruvate surrogate) and ThDP on the enzyme. Prior to our assignment of these bands, we had observed them on the slow E477Q variant of YPDC derived from benzoylformate and in the presence of the substrate activator surrogate pyruvamide, even in the absence of pyruvate (*12*).

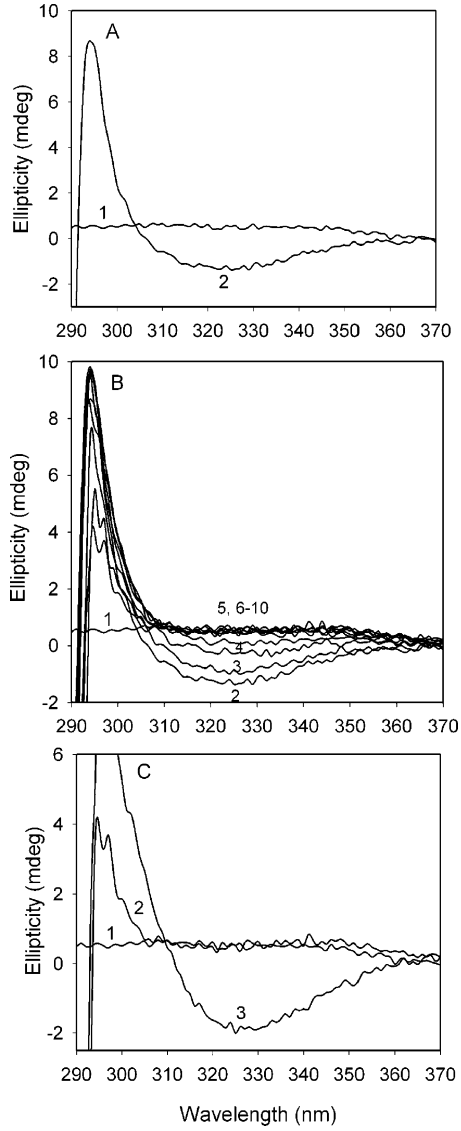


FIGURE 4: Near-UV CD spectra of E571A PDHc-E1 titrated with pyruvate and ThDP. (A) Spectrum of E571A PDHc-E1 (concentration of active centers = 40.4 μM) in the presence of 0.20 mM pyruvate (curve 2). (B) Spectra after addition of 0.20 mM pyruvate (curve 2) and of increasing concentrations of ThDP in the presence of 2 mM $MgCl_2$: 10 μM (curve 3); 20 μM (curve 4); 30 μM (curve 5); 50 μM (curve 6); 100 μM (curve 7); 200 μM (8); 300 μM (curve 9); 350 μM (curve 10). (C) Spectra in the presence of 0.20 mM pyruvate and 350 μM ThDP before (curve 2, also curve 10 in panel B) and after addition of 2 mM pyruvate (curve 3).

Table 1: Circular Dichroism Maximum for E1−ThDP−Pyruvate Michaelis Complex Formation in PDHc E1 and Its Active Center Variants[a]

variant	overall PDHc activity (%)	CD maximum (nm)	ellipticity (mdeg)
parental E1	100	327	−0.81
F602Y	57	327	−1.00
E571A[b]	1[d]	327	−1.97
H640A[b]	12	327	−0.40
H407A[b]	0.15	324	−1.27
H106A	10	330	−0.71
D521A[b]	5	326	−1.50
E522A[c]	20	not determined	not determined
E636A[c]	3[d]	not determined	not determined

[a] All variants were used at the same protein concentration of 20 μM and concentration of active sites of 40 μM. [b] Variants of PDHc-E1 with which the negative CD band at 327 nm was observed on addition of 0.20 mM pyruvate in the absence of added ThDP. [c] CD band at 327 nm could not be seen due to overlap with chiral acetolactate being formed immediately on mixing. [d] Activity of E571A and E636A variants was measured in the presence of 0.20 mM ThDP and may be enchanced 6-fold and 8-fold, respectively, with a ThDP concentration of 5 mM, as the v_o − [ThDP] plots did not display saturation.

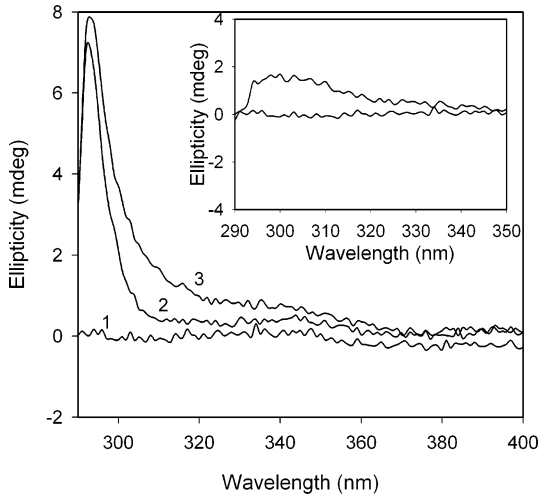


FIGURE 5: Near-UV CD spectra of F602Y PDHc-E1 titrated with pyruvate and ThDP. Spectra of F602Y PDHc-E1 (concentration of active centers = 36 μM) in the absence (curve 2) and in the presence of 0.20 mM pyruvate, 200 μM ThDP, and 2 mM $MgCl_2$ (curve 3). Inset: difference spectrum of F602YE1 obtained on addition of 200 μM ThDP (curve 3 minus curve 2).

Addition of 5−30 mM NaMAP to the E91D variant of YPDC produced major changes in the CD spectrum in the 300−400 nm range (Figure 6A). This variant had been shown by Li et al. to have exceptional properties in being able to exchange its ThDP (*20*). We used NaMAP since PLThDP, even at a concentration range of 2−65 μM, displayed no saturation according to fluorescence titrations of the E91D YPDC variant. Difference CD spectra obtained at varying NaMAP concentrations displayed two bands: the negative one at 330 nm and the positive one at 300 nm (see Figure 6B for results on the E91D variant). The negative band at 330 nm suggests formation of YPDC−ThDP−NaMAP Michaelis complex in one-half of the active sites, as seen above for addition of pyruvate to the PDHc-E1. The positive band at 300 nm clearly signals the presence of the 1′,4′-imino tautomer of PLThDP in the second half of the active sites, a result of the ability of YPDC to synthesize PLThDP from NaMAP and ThDP. A plot of ellipticity at 300 nm versus NaMAP concentration displayed saturation with $S_{0.5,NAMAP}$ = 5.56 ± 0.61 mM, n_H = 1.48 ± 0.23 (Figure 6C), while the plot of ellipticity at 330 nm versus NaMAP concentration did not display saturation with the concentrations used (Figure 6C). Similar CD spectra were obtained with wild-type YPDC on addition of NaMAP (data not shown).

Unlike with the wild-type YPDC and the E91D YPDC variant in the previous paragraph, the E51D YPDC variant required addition of 40 mM NaMAP to produce the two CD bands (Figure 7). The need for a higher concentration of NaMAP to produce the CD signatures with the E51D YPDC variant is not surprising in view of the fact that the E → D substitution moves the carboxyl group by perhaps 1 Å from the N1′ atom of the coenzyme.

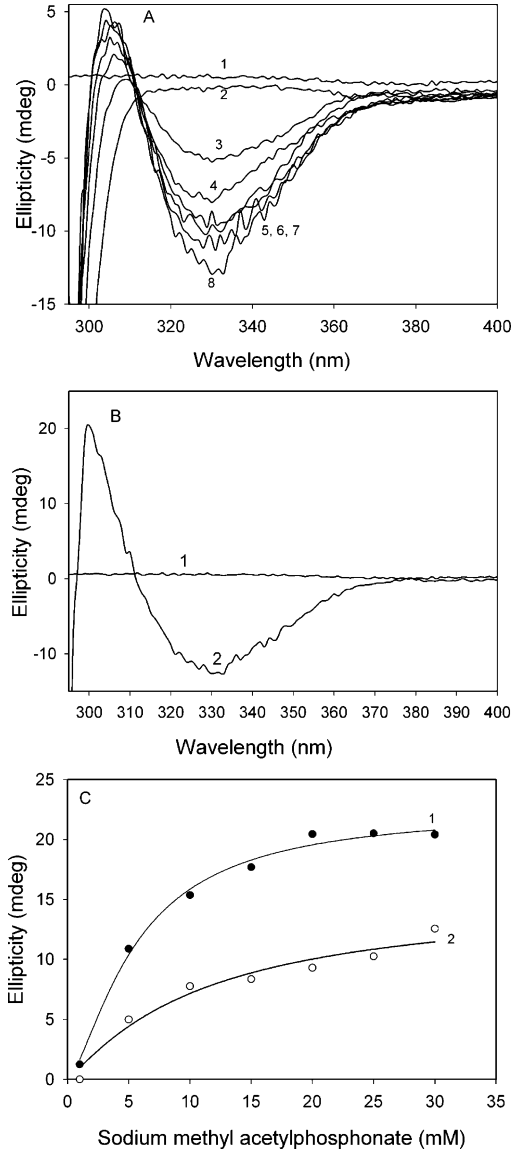


FIGURE 6: Near-UV CD spectra of the E91D YPDC variant in the presence of sodium methyl acetylphosphonate. (A) Spectra of E91D YPDC (concentration of active centers = 143.2 μM) in 100 mM MES buffer (pH 6.0), containing $MgCl_2$ (1 mM) and ThDP (1 mM) in the absence of (curve 2) and with increasing concentrations of NaMAP: 5 mM (curve 3); 10 mM (curve 4); 15 mM (curve 5); 20 mM (curve 6); 25 mM (curve 7); 30 mM (curve 8). (B) Difference spectrum obtained on addition of 30 mM NaMAP. (C) Dependence of ellipticity at 300 nm (curve 1) and at 330 nm (curve 2) on the concentration of NaMAP.

With the E51A/E91D doubly substituted YPDC variant, no signal at 300 nm and only a weak one at 330 nm was apparent even with 30−40 mM NaMAP, indicating that this variant did not form the 1′,4′-imino tautomer of PLThDP, or indeed PLThDP itself (see Figure 8 for the difference spectra at 35 mM NaMAP).

Search for a Relevant Binary Complex to Produce a Charge-Transfer Band in the 330 nm Wavelength Range. The premise for these model studies is that the active centers of the three enzymes that have displayed the negative CD band at 320−330 nm (TK, YPDC and PDHc-E1) share only two features: thiamin diphosphate and histidine residues. We therefore created binary complexes comprising a putative electron donor **D** and electron acceptor **A** with all permutations of three components, the 4-aminopyrimidine (**D**) as a

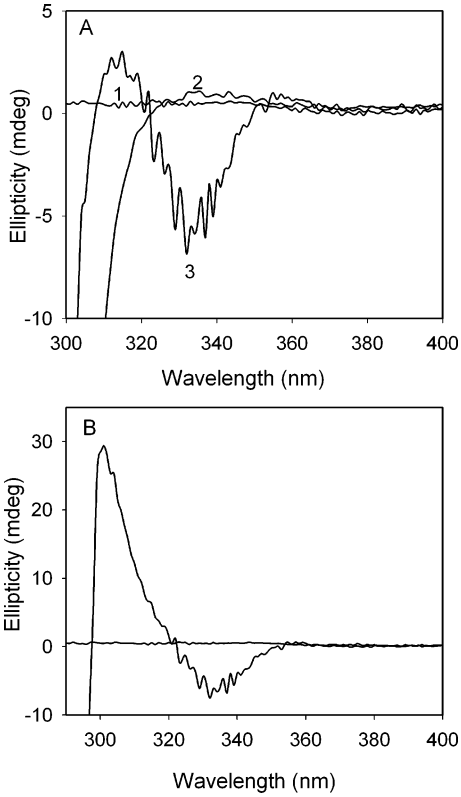


FIGURE 7: Near-UV CD spectra of the E51D YPDC variant titrated with sodium methyl acetylphosphonate and ThDP. (A) Spectra of E51D YPDC (concentration of active centers = 143.2 μM) in 100 mM MES buffer (pH 6.0) after addition of 1 mM ThDP in the presence of 1 mM $MgCl_2$ (curve 2) and with further addition of 40 mM NaMAP (curve 3). (B) Difference spectrum obtained on addition of 40 mM NaMAP (curve 3 minus curve 2).

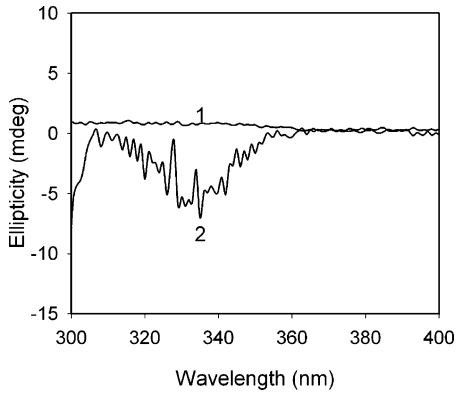


FIGURE 8: Near-UV CD difference spectrum of the E51A/E91D YPDC variant with ThDP and sodium methyl acetylphosphonate. Concentration of active centers is 143.2 μM with 35 mM NaMAP added in 100 mM MES buffer (pH 6.0), 1 mM $MgCl_2$, and 5 mM ThDP.

neutral species, an *N*1-methyl-4-aminopyrimidinium ion as a surrogate for the N1-protonated species (**A**), the 1′,4′-iminopyrimidine tautomer (**D**), 3,4,5-trimethylthiazolium ion (**A**), 1-methylimidazole (**D**), and 1-ethyl-3-methylimidazolium ion (**A**) as a surrogate for protonated histidine. The clearest evidence for such a donor−acceptor interaction, as reflected by a new charge-transfer band, is provided by mixing of 4-aminopyrimidine (**D**) as a neutral species and 3,4,5-trimethylthiazolium ion (**A**) as a positively charged one as illustrated in Figure 9. The wavelength maximum is near 340 nm. While the concentrations needed are very large in

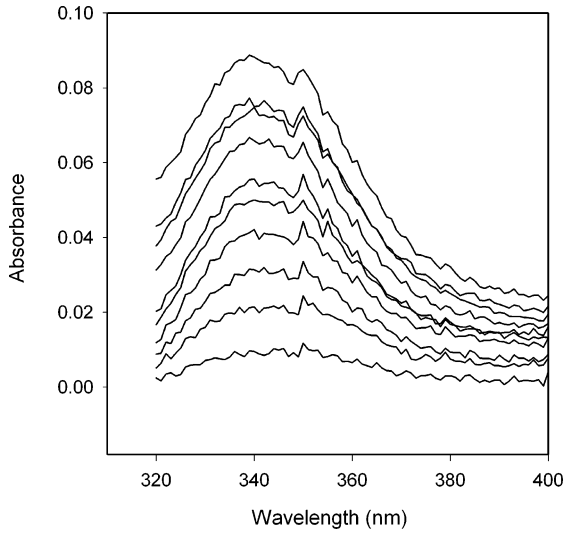


FIGURE 9: Absorption spectra resulting from addition of 4-aminopyrimidine to 3,4,5-trimethylthiazolium triflate. To 1 mL of 200 mM 3,4,5-trimethylthiazolium triflate were added 10×5 μL of 2 M 4-aminopyrimidine, resulting in final concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mM. The baseline for the constant component was flat.

these intermolecular models, almost certainly the intramolecular interactions and the V conformation of ThDP enforced by the enzyme could very much enhance the intensity of the charge-transfer band on the enzymes.

DISCUSSION

Evidence has now been presented suggesting that both LThDP (information obtained via its unreactive analogues PLThDP and NaMAP) and HEThDP exist as their 1′,4′-imino tautomers when bound to the enzyme. The results tempt us to further speculate that all tetrahedral ThDP adducts on all pathways involving the ThDP coenzyme will likewise exist as their 1′,4′-imino tautomers.

As was mentioned in Results, the reason for our ability to observe the positive CD signal at 300 nm on addition of HEThDP to PDHc-E1 is the slow rate at which HEThDP is converted to acetaldehyde and ThDP bound to PDHc-E1. The observation of the 1′,4′-imino ThDP tautomer with both tetrahedral complexes, LThDP (via its phosphonate analogue) and HEThDP, also suggests participation of the 4′-aminopyrimidine moiety of ThDP in acid−base reactions throughout the reaction sequences. Similar CD spectra with a positive maximum at 300 nm were also observed when apo-TK or holo-TK were titrated with C2-[α,β-dihydroxyethyl]-thiamin diphosphate, an intermediate on the TK reaction pathway (*28*). In view of our cumulative results, those results strongly suggest that the CD signal at 300 nm implies a preference of the 1′,4′-imino tautomer for C2-[α, β-dihydroxyethyl]-thiamin diphosphate as well.

Our observations on YPDC with NaMAP suggest that the negative CD band with a maximum at 320−330 nm represents a charge-transfer complex due to the interaction of two aromatic rings of ThDP with each other, and enhanced by the presence of substrate, i.e., a complex with all of the elements of the Michaelis complex present.

The experiments carried out on PDHc-E1 specifically rule out several amino acid side chains as being responsible for the charge-transfer interaction leading to the negative CD band at 320−330 nm. We point out that the active center of YPDC has no aromatic residues near ThDP but has carboxylate groups (denoting Asp or Glu) and histidines; PDHc-E1 has carboxylates, histidines, a phenylalanine, and a tyrosine nearby, while TK has several types of amino acids with the exception of carboxylates (a highly conserved glutamate 418 opposite the N1′ atom is considered separately since it is insulated from the substrate-binding locus). We believe that the results in Table 1 rule out phenylalanine, tyrosine, histidines, and carboxylates, leaving ThDP itself as the source of the phenomenon. The model studies in Figure 9 suggest that the interaction between the neutral conventional tautomer of the 4′-aminopyrimidine with the thiazolium ring is sufficient to produce a charge-transfer band with λ_{max} in the wavelength range appropriate to the CD observations. Many years ago, we reported such a charge-transfer band formed between 4′-aminopyrimidines (including thiamin) and indoles as models for tryptophan, mistakenly believing (in the absence of three-dimensional structures) that there was a tryptophan in the YPDC active site on the basis of fluorescence experiments (*29*). In fact, there is no tryptophan near ThDP on YPDC, ruling out this interaction as well. We also note relevant rapid-scan stopped-flow experiments carried out with the catalytically impaired E477Q YPDC active center variant displaying two overlapping absorptions, centered near 300−310 and 340−350 nm, both with the same phase in the UV−vis absorption mode (*11*, *12*). Their time-course of formation and depletion also indicated that the signals pertained to different species, now assignable to the 1′,4′-iminoThDP, and to the "canonical" thiamin with neutral 4′-aminopyrimidine and thiazolium ion interaction, respectively.

Observation of the negative CD band at 320−330 nm on YPDC with NaMAP as a starting material is very useful to our interpretation since this substrate analogue can participate on the enzyme in only two forms: (1) Free as a negatively charged pyruvate analogue and (2) as the PLThDP covalent adduct of NaMAP with ThDP, a LThDP analogue that cannot undergo C−P bond cleavage. These experiments allow us to rule out any alternative conjugated intermediate such as the enamine as the source of the signals in the 320−330 nm range (which could complicate the interpretation of the signals). The fact that both the positive band at 300−310 nm and the negative one at 320−330 nm refer to enzyme-bound species enables simple interpretation. On addition of NaMAP to YPDC, both the positive band at 300 nm and the negative one at 330 nm appeared, supporting the simultaneous presence on the enzyme of both PLThDP and the Michaelis complex between ThDP and NaMAP, respectively. Since YPDC is a homotetramer and has been shown to display kinetic behavior consistent with an "alternating active site in a functional dimer" (*30*), these findings provide excellent structural support for such half-of-the-sites behavior, support based on electronic spectroscopy and buttressed by chemical models. The other issue clarified by the studies with YPDC and NaMAP is the finding that with the E51A/E91D substitutions, only the negative CD band at 330 nm is visible, reflecting the fact that this substitution could not stabilize PLThDP; hence, the 1′,4′-imino tautomer is not in evidence at 300−310 nm. This is strong evidence for participation of the conserved E51 residue (E571 on PDHc-E1 from *E. coli*) in stabilization of the 1′,4′-imino tautomer

of ThDP. Importantly, the results also confirm that the two CD bands discussed here pertain to different species on these enzymes.

As seen in Table 1, the observations regarding the negative CD band between 320 and 330 nm on PDHc-E1 fall into three distinct classes: (1) when there is no negative CD band evident until a larger 2 mM concentration of pyruvate is added (see parental PDHc-E1 and the F602Y and H106A variants); (2) when there is a strong signal observed immediately on addition of 0.2 mM pyruvate (see the E571A, H640A, H407A and D521A variants); and (3) when the signal is not resolved, probably due to the immediate production of chiral acetolactate, whose CD signal is also negative and represents accumulated material released from the enzyme (see the E522A and E636A variants). As shown in Figure 2, the products of the reaction under scenarios 1 and 2 are the same, so it is the order of addition and the amount of pyruvate present that determine whether the signal persists. We believe that the reason the signal can be seen in the class 1 experiments is that the small initial concentration of pyruvate is rapidly converted to HEThDP, which in the absence of an oxidizing partner has a longer half-life on the enzyme but apparently allows only slow conversion of pyruvate to products (Figure 2, left-hand panel). Addition of ThDP prior to addition of any pyruvate apparently activates the enzyme (*24*) so that conversion to product(s) proceeds with no apparent evidence for the Michaelis complex, which under these conditions must be short-lived. Finally, it is likely that the variants in class 2 have failed to lose their ThDP, so that addition of only 0.2 mM pyruvate already could produce the negative CD signal, implying the presence of the Michaelis complex.

Evidence for dissymmetry in active sites is readily apparent in the YPDC studies, while the evidence is subtler in the case of the PDHc-E1 results. For example, in Figure 2A, left-hand panel, there is an increase at 300 nm on addition of ThDP, but the magnitude of the signal is weak, perhaps due to occupation of only half of the sites, compared to the experiments with HEThDP (see Figure 1). The evidence is much stronger with the F602Y mutant, suggesting synthesis of HEThDP and the concomitant appearance of the 300 nm band, which in turn signals presence of the 1′,4′-imino tautomer. Also, the magnitude of the signals in Table 1 suggests that in class 2, at least with some variants such as E571A PDHc-E1, there is significantly higher occupancy of the Michaelis complex, perhaps due to full occupancy of both active sites by the complex under the experimental conditions; these variants are all considerably less active than the wild-type enzyme.

CONCLUSION

From our data presented here and those reported on PLThDP binding to PDHc-E1 (*4*), it is evident that both intermediates, LThDP and HEThDP, exist as their 1′,4′-iminopyrimidine tautomers according to the positive CD signal in the 300−305 nm range. The negative CD band at 320−330 nm could be assigned to the PDHc-E1−ThDP−pyruvate or YPDC−ThDP−pyruvate (or the substrate surrogates NaMAP or pyruvamide) Michaelis complex. Under favorable conditions, the rate of formation of the Michaelis complex could be followed both on PDHc-E1 and on YPDC by the appearance of this negative CD band. The method could also provide information regarding the presence of tightly bound ThDP, since under these conditions the negative CD band is visible even prior to the addition of ThDP. According to the results on PDHc-E1, no residue on the enzyme is required for formation of the negative CD band in the 320−330 nm region; however, on both YPDC and PDHc-E1, the signal can only be observed and quantified in the presence of substrate or substrate surrogate. Finally, it is worth emphasizing that the two intermediates seen in CD spectra are chiral by virtue of the enforced V coenzyme conformation.

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