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Spectroscopic Evidence for Participation of the 1′,4′-Imino Tautomer of Thiamin Diphosphate in Catalysis by Yeast Pyruvate Decarboxylase

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The 1′,4′-iminopyrimidine tautomeric form of the coenzyme thiamin diphosphate (ThDP), implicated in catalysis on the basis of the conformation of enzyme-bound ThDP, has been observed by both ultraviolet absorption and circular dichroism spectroscopy. On yeast pyruvate decarboxylase, the unusual tautomer is observed in an active center variant in which catalysis in the post-decarboxylation regime of the reaction is compromised. In a model system consisting of N1-methyl-4-aminopyrimidinium or N1-methyl-N4-*n*-butylpyrimidinium salts, on treatment with either NaOH in water, or DBU in DMSO there is an intermediate formed with λ_{max} near 310 nm, and this intermediate reverts back to the starting salt on acidification. Proton NMR chemical shifts are consistent with the intermediate representing the 1-methyl-4-imino tautomer. On the enzyme, the intermediate could be observed by rapid-scan stopped flow with UV detection when reacting holoenzyme of the E477Q active center variant with pyruvate, and by circular dichroism even in the absence of pyruvate. This represents the first direct observation of the imino tautomeric form of ThDP both on the enzyme and in models, although some years ago, this laboratory had already reported some pertinent acid-base properties for its formation [Jordan, F., and Mariam, Y. H. (1978) *J. Am. Chem. Soc.* **100,** 2534–2541]. The work also represents the first instance in which a rare tautomer implicated in catalysis is identified and suggests that such tautomeric catalysis may be more common in biology than hitherto recognized.

INTRODUCTION

High-resolution X-ray structures of thiamin diphosphate(ThDP)[3]-dependent enzymes only appeared in the early 1990s. Among the most striking and universally

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[2] The first author dedicates this paper to Professor Frank H. Westheimer, a highly valued postdoctoral mentor, on the occasion of his 90th birthday. The approach used in this paper, the search for chemical rationale for enzymatic catalysis by carrying out model studies in parallel with studies of the enzyme, is one of many ‘Westheimer legacies’ handed down to successive generations of his students.

[3] Abbreviations used. ThDP, thiamin diphosphate; YPDC, pyruvate decarboxylase from the yeast *Saccharomyces cerevisiae* overexpressed in *Escherichia coli*; WT, wild-type YPDC; E477Q is a variant of YPDC with the indicated substitution; *t*-BuOK, potassium tertiary-butoxide; DBU, 1,8-diazabicyclo[5.4.0]-undec-7-ene; DMSO-d_6, hexadeuterio dimethylsulfoxide.

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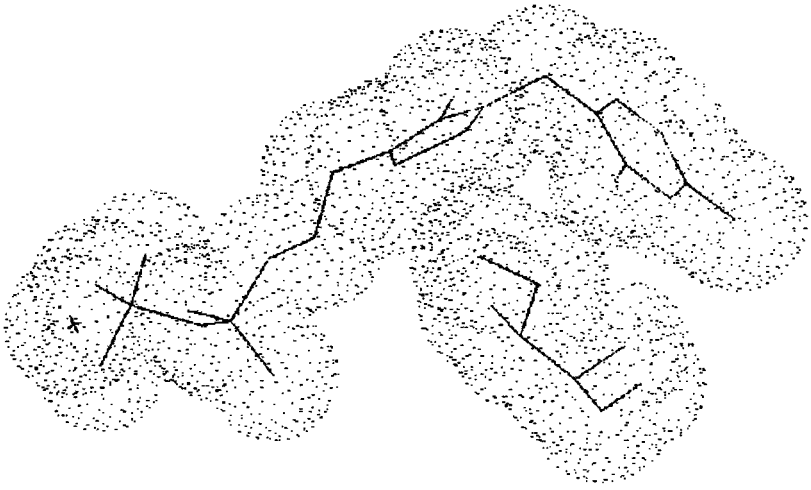
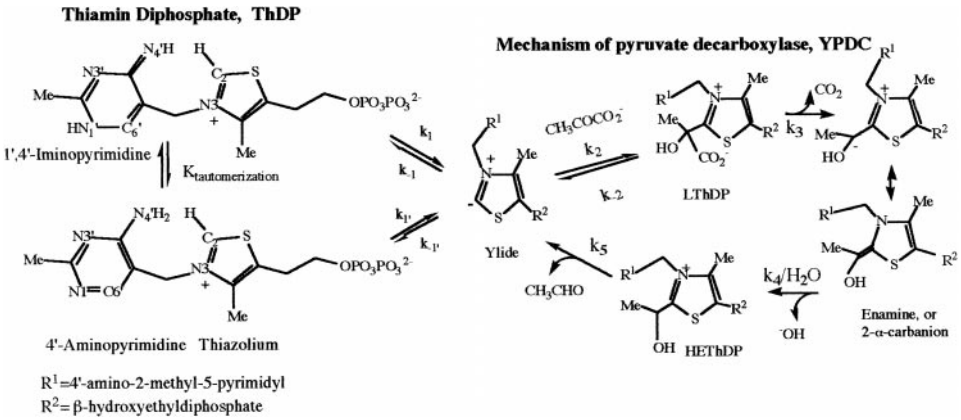


FIG. 1. Van der Waals picture of the V conformation of ThDP with I415.

observed early findings on these structures on yeast pyruvate decarboxylase (YPDC; Refs. *1,2*) transketolase (*3*) and pyruvate oxidase (*4*) was the V coenzyme conformation (see Fig. 1), only seldom observed in ThDP derivatives in the absence of enzymes. While the variety of conformations that the thiazolium and 4′-aminopyrimidine rings can assume with respect to the bridging methylene group has long been the object of both experimental (*5*) and theoretical studies (*6,7*), the latter certainly did not place the V conformation at a minimum energy. The X-ray studies revealed three highly conserved hydrogen bonds to the 4′-aminopyrimidine nitrogen atoms, and a hydrophobic amino acid that appears to support the V conformation. The only systematic study to date to examine this issue used YPDC variants with progressively smaller side chains substituting the I415 in the wild-type enzyme (*8*). Both experimental and computational approaches suggested that I415, while a conformational pivot, is not the only source of the stability of the V conformation on the enzyme. In terms of catalytic impact, the specific activity of the variants decreased with decreasing size of the 415 residue. Perhaps most importantly, one consequence of the V conformation is to bring the thiazolium C2 atom within 3.0–3.4 Å of the exocyclic nitrogen atom (see Scheme 1 for nomenclature) of the 4′-aminopyrimidine ring (*2*). Arguments were made that for the shorter distance, it is most unlikely that there could be more than two hydrogen atoms accommodated between the C2 and N4′ atoms (*2*). This is counter to the expectation from the conventional structures and ionization states, where in a F or S conformation (*5*), and indeed in free thiamin, there should be three hydrogen atoms accommodated, two at N4′ and one at C2. This finding raised the possibility, also supported by the presence of the highly conserved, typically Glu, side chain at hydrogen bonding distance from the N1′ atom, that, at least in some reaction steps catalyzed by ThDP (see the example of YPDC in Scheme 1), the 4′-aminopyrimidine exists in its 1′4′-iminopyrimidine tautomeric form. The notion of the participation of the 4′-aminopyrimidine in enzymatic catalysis was dismissed by



SCHEME 1.

most prior to the publication of the X-ray structures of the enzymes, with notable exceptions of the group of Schellenberger and Hübner at Halle, Germany (*9*), and our group at Rutgers (*10–12*).

Recognizing the need for acid-base catalysis in ThDP-dependent enzymatic reactions, we suggested that one way to utilize the 4′-aminopyrimidine ring is to impose a positive charge at N1′, and that this charge would convert the exocyclic amino group to a weak acid. In a series of model studies, we: (a) Used N1′-methylated thiaminium and N1-methylated 4-aminopyrimidinium salts to study the effect of the N1′-protonated state on thiamin reactions, noting modest rate enhancement on the C2H/D exchange, and, more importantly, a pK_a suppression at the exocyclic amine to 12−13 according to potentiometric titrations (*10*); (b) Demonstrated that the structurally related imino tautomer of N1-methyladenosine (readily and commercially available, and used in place of the ThDP imino tautomer) could catalyze some ThDP-dependent model reactions (*11*); (c) Also demonstrated that the amino hydrogen atoms on N1′-methylthiaminium salt and thiamin itself (under conditions of N1′-protonation) are coplanar with the aromatic ring since the two have different chemical shifts. These protons are subject to differential rates of buffer catalysis, and, rotation around the C4′–N4′ bond is highly restricted with a barrier of about 14 kcal/mol (*12*).

The thiazolium C2 atom has been recognized as the catalytically crucial center of ThDP since Breslow's seminal studies (*13*). However, generating experimental evidence for the participation not only of the 4′-aminopyrimidine ring, but for the imino tautomer as well, remains an important challenge. Recently, we obtained evidence pertinent to this issue while studying the reactions of YPDC active center variants by rapid-scan stopped flow UV spectroscopy, in which we observed a hitherto unreported intermediate, different from any that we had seen before. We then proceeded to model the putative iminopyrimidine intermediate and here report the current status of these studies.

TABLE 1

Proton Magnetic Resonance Chemical Shifts of Compounds I (N1-Methyl-4-aminopyrimidinium triflate) and II (N1-Methyl-N4-*n*-butyl-pyrimidinium triflate)

Compound/conditions	Chemical Shifts (ppm)				
	C2-H	C5-H	C6-H	$N1\text{-}CH_3$	N4-H
I ($DMSO\text{-}d_6$)	8.72 (s)	6.72 (d)	8.18 (d)	3.77 (s)	8.87 (d)
I + DBU ($DMSO\text{-}d_6$)	7.83 (s)	5.97 (d)	7.22 (d)	3.38 (s)	
II ($DMSO\text{-}d_6$)	8.77 (s)	6.76 (d)	8.09 (d)	3.77 (s)	9.40 (s)
II + DBU ($DMSO\text{-}d_6$)	7.78 (s)	5.79 (d)	6.98 (d)	3.31 (s)	
I (D_2O)	8.42 (s)	6.71 (d)	7.92 (d)	3.75 (s)	
I + NaOH (D_2O)	7.93 (s)	6.22 (d)	7.30 (d)	3.49 (s)	

MATERIALS AND METHODS

Synthesis of Models for Imino Tautomer Formation (14)

*N1-Methyl-4-aminopyrimidinium triflate (**I**).* 4-Aminopyrimidine (0.48 g, 5 mmol) was dissolved in 10 ml of chloroform. Methyltriflate (5 mmol) was added and then the solution was refluxed for 2h, followed by evaporation of the solvent at a rotary evaporator. The product was recrystallized from methanol to yield N1-methyl-4-aminopyrimidinium triflate (0.42 g, 73%).

4-Chloropyrimidine. To 4-hydroxypyrimidine (1.0 g) was added phosphorous oxychloride (4.0 ml) at 100°C until dissolution occurred. The solution was cooled after 20 min, then filtered yielding a yellow precipitate. The yellow precipitate was sublimed at 110°C yielding white needle crystals (0.4 g, 25%). The product melted with decomposition over the range of 160–170°C.

N4-n-Butylpyrimidine. 4-Chloropyrimidine (0.4 g) was added slowly to *n*-butylamine (2 ml) at 0°C and then the mixture was dried under vacuum. The product was recrystallized from methanol to yield a white precipitate (0.4 g, 80%). ^{1}H-NMR ($DMSO\text{-}d_6$), 8.40 (1H, s), 8.02 (1H, br), 7.36 (1H, s), 6.42 (1H, d), 3.25 (2H, br), 1.48 (2H, m), 1.32 (2H, m), 0.95 (3H, t).

*N4-n-Butyl-N1-methylpyrimidinium triflate (**II**).* N4-*n*-Butylpyrimidine (0.3 g) was dissolved in 10 ml of chloroform and methyltriflate (2 ml) was added to the solution. After 2 h of refluxing, the solvent was evaporated at a rotary evaporator. The products were separated by chromatography on silica gel to yield a brown oil **II** (0.12 g, 30%). ^{1}H-NMR ($DMSO\text{-}d_6$), 9.40 (1H, s), 8.77 (1H, s), 8.09 (1H, d), 6.76 (1H, d), 3.77 (3H, s), 3.44 (2H, m), 1.53 (2H, m), 1.32 (2H, m), 0.89 (3H, t).

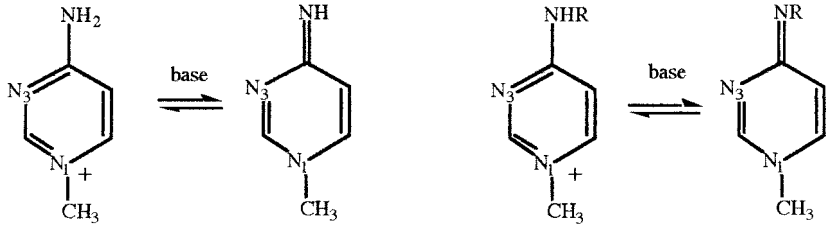
Preparation of YPDC variants and assays (15). Stopped-flow and circular dichroism instrumentation and methods were reported by Sergienko and Jordan (*16*).

RESULTS AND DISCUSSION

Modeling the Absorbance for the 1′,4′-Imino Tautomer of ThDP

The initial experiments with compounds **I** and **II** were carried out in DMSO using 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) as the base. As seen in Table 1, the

chemical shifts of all of the protons move to more shielded values on addition of DBU, with the amino proton resonance(s) disappearing, as expected. There is clear evidence for restricted rotation around the C4-N4 bond as reported many years ago (*10*,*12*), since in compound **I** there are two NH resonances, while in compound **II** there are two sets of resonances for each type of hydrogen around the pyrimidinium ring. On neutralization, the original chemical shifts reappear. It is important to note, that the pK_a of the exocyclic amino group in **I** is not much greater than 12 [as reported earlier (*10*)], since the spectrum could be generated in 10 mM hydroxide.



I **II**

When the experiment on **I** was repeated in DMSO-d_6 but using potassium tertiary-butoxide (*t*-BuOK) as base, in addition to the absorbance at 310 nm, there was also observed one with a λ_{max} at 352 nm. On neutralization of the base, the absorbance at 352 nm persisted, indicating that the strong base also produced a product, which is not a result of a simple acid-base reaction, hence these conditions were abandoned.

The ^{1}H NMR experiments clearly indicated that conditions were identified for producing the imino tautomer of compounds **I** and **II**. We next turned to generating the imino compounds in the UV cuvette, since, for the present, only this signature could be detected on YPDC. In the first experiment, compound **I** (0.1 mM) was dissolved in 0.01 M NaOH, producing the spectrum in Fig. 2A, providing a λ_{max} of 307 nm with an $\varepsilon_{307} = 3500$. In a different experiment, to compound **II** dissolved in DMSO was added increasing concentrations of DBU (Fig. 2B), providing a λ_{max} of 302 nm with an $\varepsilon_{302} = 3000$. These results are essential for the interpretation of the data on YPDC discussed below.

Evidence for the Presence of the 1′4′-Imino-ThDP Tautomer on YPDC

The first hint for the formation of the imino-ThDP was observed in a rapid-scan stopped-flow UV spectrum resulting from mixing of the E477Q active-center YPDC variant with pyruvate in the presence of pyruvamide. Pyruvamide is often used as a substrate surrogate at the regulatory site (residue C221 according to a lot of accumulating evidence from this laboratory; see Ref. *17* and references therein), capable of activating the enzyme (*18*). Such a spectrum is shown in Fig. 3, showing the persistence of the signal over many seconds. Since there appear to be two new absorbances observed (near 310 and near 350 nm), we were quite puzzled by the observation. A consideration of the species in Scheme 1 indicates that the only obvious chromophore on the YPDC reaction pathway is the enamine, which we have characterized over the past two decades both in chemical models (*19*) and on YPDC (*20*,*21*), and more recently on benzoylformate decarboxylase (*22*,*23*). According to model studies, the

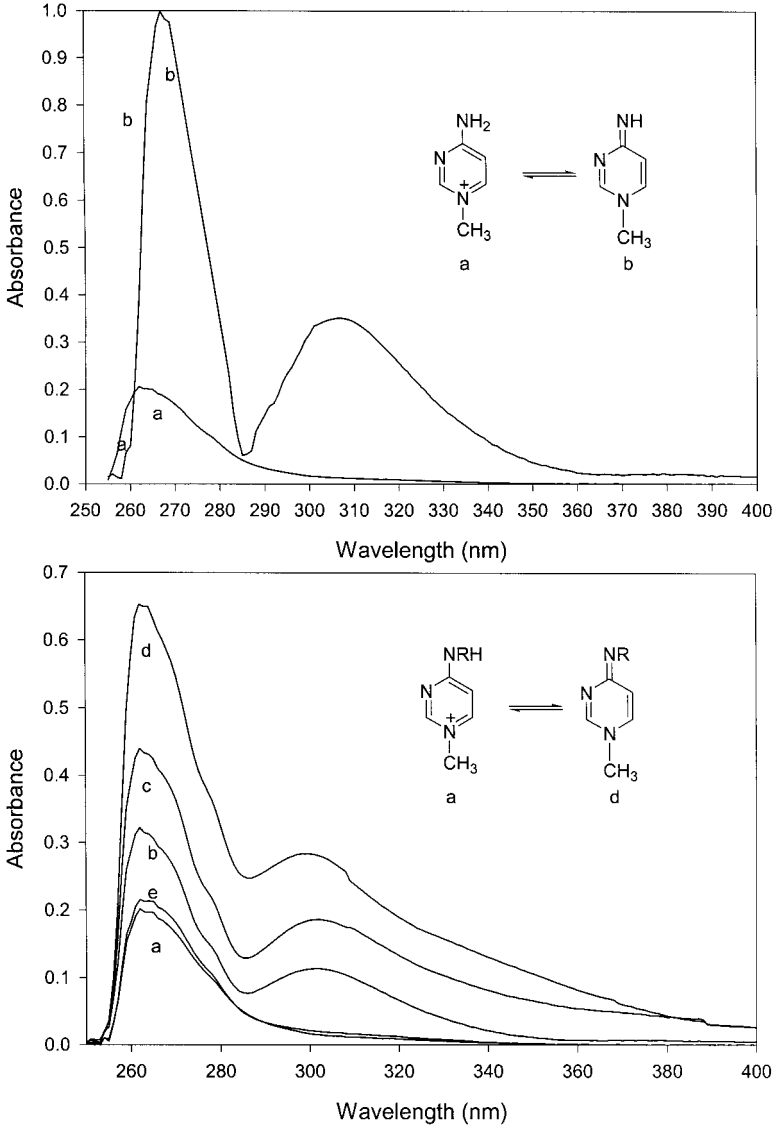


FIG. 2. (Top) Ultraviolet spectrum of compound **I** and the derived imino tautomer. (a) 0.1 mM N1-methyl-4-aminopyrimidinium triflate (**I**). (b) curve *a* with 10 mM NaOH. λ_{max} = 307 nm, ε = 3500. In the imino tautomer, the position of the NH is ambiguous, could be on the same or opposite side of N3. (Bottom) Ultraviolet spectrum of compound **II** and the derived imino tautomer. (a) 0.1 mM N4-*n*-butyl-N1-methyl-4-aminopyrimidinium triflate (**II**) dissolved in DMSO. (b) Curve *a* then 0.1 mM DBU. (c) Curve *a* with 0.5 mM DBU. (d) Curve *a* with 10 mM DBU. (e) Curve *d* with excess acid. λ_{max} = 302 nm, ε_{302} = 3000. *R* = *n*-butyl, and in the imino tautomer the position of *R* is ambiguous, could be on the same or opposite side of N3.

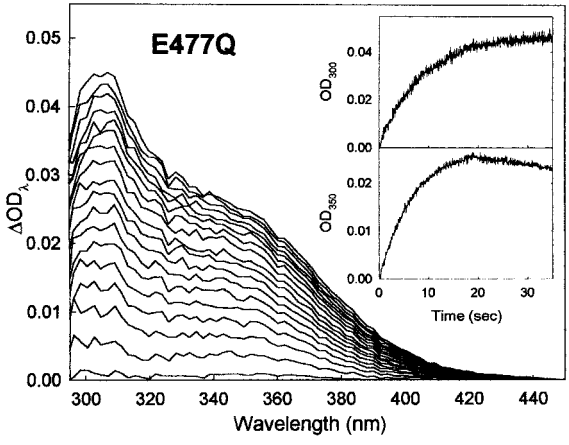


FIG. 3. Difference spectra of a reaction mixture containing E477Q variant and pyruvate. E477Q variant YPDC (85 μM) preincubated with 50 mM pyruvamide in 100 mM MES, pH 6.0, containing 5 mM $MgCl_2$ and 0.1 mM ThDP, was mixed with equal volume of 10 mM pyruvate in the same buffer. Spectral changes at 25°C were recorded using the photo diode-array accessory with the Applied Photophysics SX.18MV stopped-flow spectrometer. Time-dependent changes of absorbance at 300 and 350 nm are shown in the inset.

enamine derived from pyruvate should have a λ_{max} near 295 (*19*). We believe that the observations must pertain to either the enamine or to the imino-ThDP. While we now have models for both of these intermediates, we still do not understand the source of the absorbance near 350 nm. We do recognize, that the 350 nm region, with the high intensity lamp needed for the diode-array experiments, is complicated by photobleaching effects.

We next turned to circular dichroism studies, already exploited in our recent studies of the carboligase side reactions of the active center variants (*16*). Careful difference CD spectra of the enzyme were generated, both in the presence and in the absence of pyruvate, as shown in Fig. 4, indicating that several chiral intermediates accumulated on the enzyme variants. Especially important for this discussion is the figure on the right-hand side showing double-difference spectra and providing evidence for a chiral intermediate accumulating on the E477Q, but not on the D28A variant, with a λ_{max} near 310. This is a particularly useful and strong piece of evidence since neither pyruvate nor acetaldehyde is chiral and almost certainly we are observing a YPDC-bound intermediate. The result suggests that the imino-ThDP tautomer is chiral when enzyme-bound, a chirality due to the fixed V conformation imposed by the protein, but absent in model compounds in which there is rapid conformational interconversion. To further differentiate whether the observations pertained to the enamine or the imino-ThDP, we resorted to benzoylformate, a slow alternate substrate. The premise of the experiment is that if the 310 nm absorption/ellipticity pertained to the imino-ThDP, its λ_{max} should remain unchanged with this substrate, whereas the enamine derived from benzoylformate should have a λ_{max} near 380 nm (*24*). The circular dichroism spectrum resulting from addition of benzoylformate and pyruvamide to the

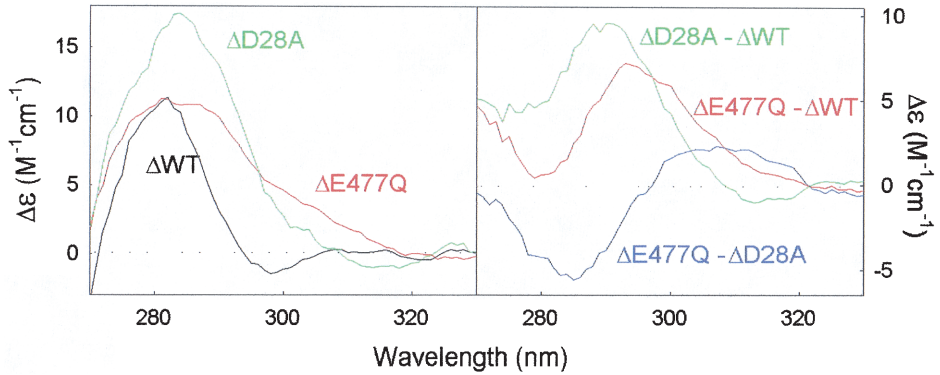


FIG. 4. CD spectral changes occurring in WT and variant enzymes upon addition of pyruvate. WT, E477Q, or D28A at concentrations of 7, 5.7, and 4.9 μM, respectively, were dissolved in standard buffer, pH 6.0, containing 5 mM $MgCl_2$ and 0.1 mM ThDP. After scanning the initial CD spectra, to the enzyme solution was added 10 mM (WT and E477Q) or 1.89 mM (D28A) pyruvate. To ensure minimal interference from carboligase products, the temperature was maintained at 4°C and several spectra were collected. Difference spectra (left) and double difference spectra (right) demonstrate accumulation of distinct intermediates.

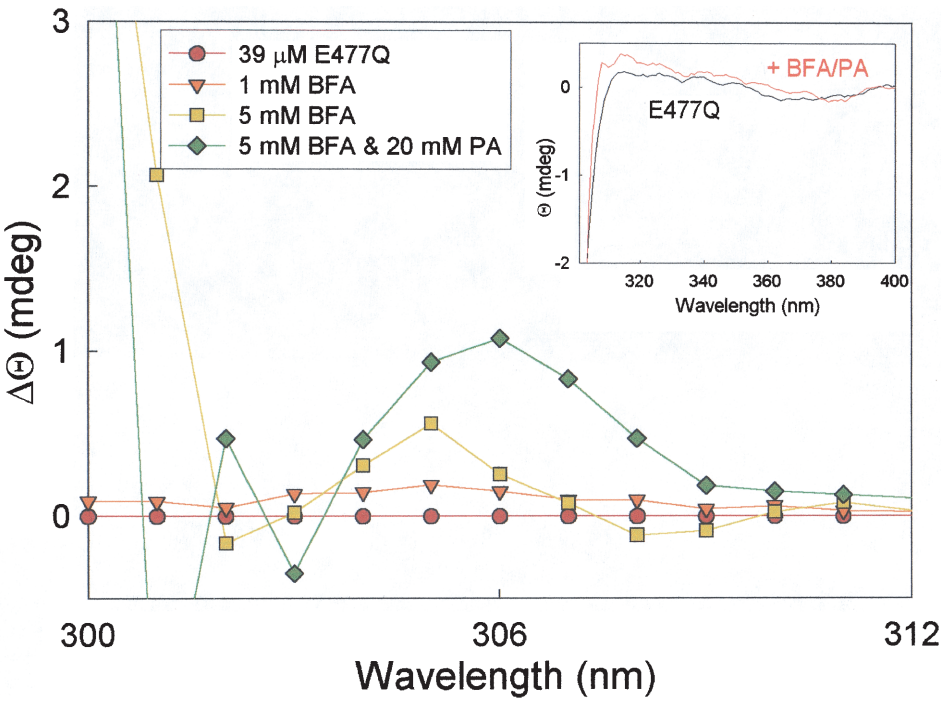


FIG. 5. Circular dichroism spectra of the E477Q YPDC variant with benzoylformic acid in the absence and presence of pyruvamide. Enzyme was dissolved in 100 mM MES, 5 mM $MgCl_2$, 0.1 mM ThDP, pH 6.0, to give 1.79 mg/ml (29 μM active sites). Benzoylformate was added in portions to reach 1 and 5 mM and then pyruvamide was added to 20 mM. The inset shows the raw initial and final spectra, while the larger spectra represent difference spectra.

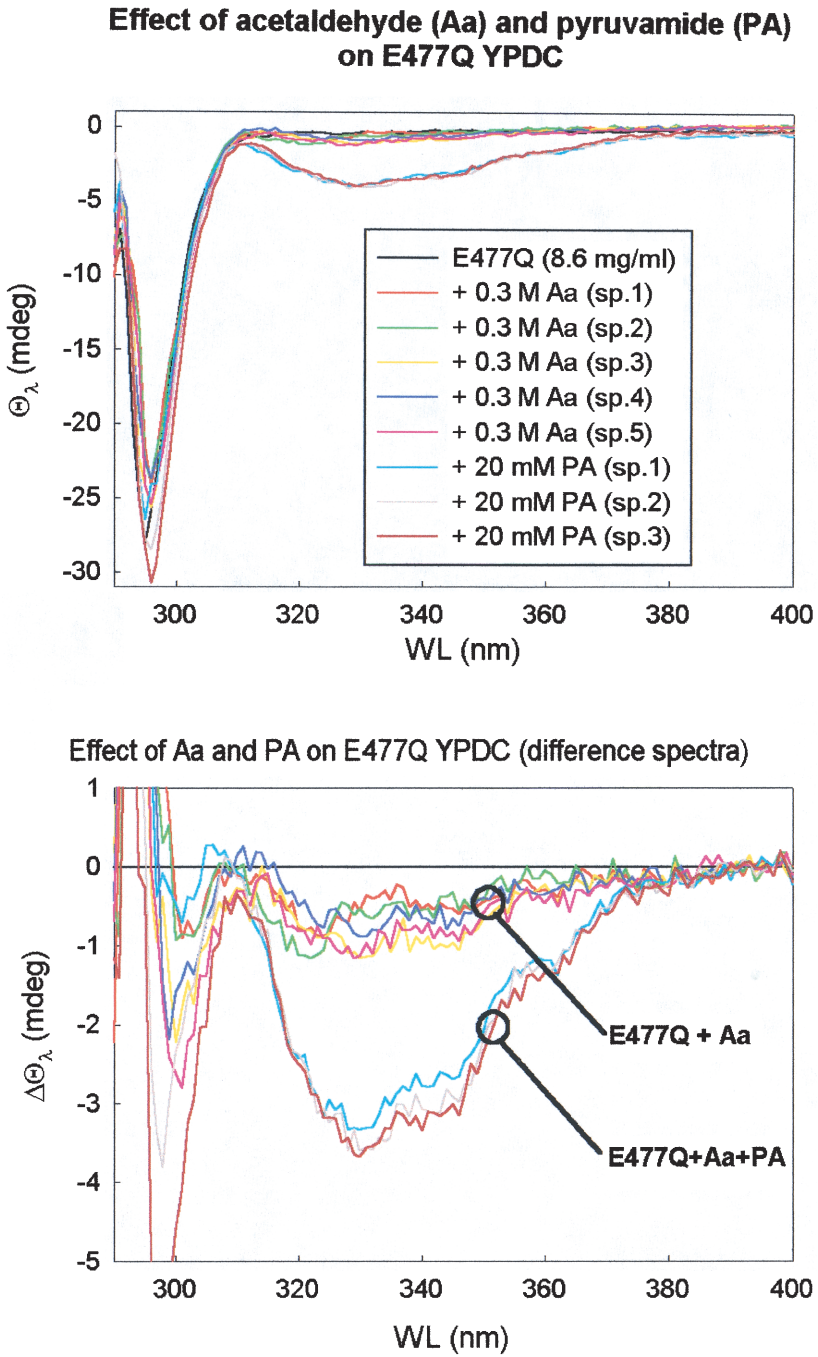


FIG. 6. Circular dichroism spectra of the E477Q YPDC variant in the presence of acetaldehyde in the absence and presence of pyruvamide. Enzyme was dissolved to a concentration 8.6 mg/ml (or 143 μM active sites). Acetaldehyde was added to 0.3 M concentration at 25°C. Five consecutive spectra were recorded at the same temperature. Pyruvamide was added to a final concentration of 20 mM and three more spectra were recorded. Time interval between consecutive spectra in each group is 10 min. Upper panel represents actual spectra, difference spectra are shown in the lower panel.

E477Q YPDC variants reassures us that the observation pertains to the 1′,4′-imino-ThDP, since the λ_{max} in the circular dichroic spectrum is 306 nm (Fig. 5), unchanged compared to the experiment with pyruvate as substrate. Next, we carried out a CD experiment in which we added 0.3 M acetaldehyde (the product of the reaction) to the E477Q variant in the absence and presence of pyruvamide, leading to the spectra in Fig. 6. This time there is no reaction [acetaldehyde will form acetoin but in a very slow reaction (*25*)] and no time dependence. Instead, we now observe two CD signals, a weak positive one with a λ_{max} near 310 nm, and a strong broad negative one with a λ_{max} between 330 and 350 nm and an unsymmetrical shape. The latter is very reminiscent of a CD signature observed on several ThDP enzymes, especially clearly on transketolase (*26*). As discussed in one of our recent papers, YPDC in the presence of acetaldehyde assumes a different conformational state from that in the presence of pyruvate (*27*). This conformational state appears to be responsible for acetoin formation and is characterized by impaired acetaldehyde release. The similarity of the transketolase reaction to the YPDC carboligase side reactions suggests that the negative CD signal between 330 and 350 nm might be a reflection of the functional involvement of the 4′-aminopyrimidine ring, indeed of the entire ThDP in a ligation reaction.

Supported by the results of the model studies, we suggest that both the absorbance with λ_{max} near 300–310 and the positive circular dichroic peak in the same region can be assigned to the 1′,4′-imino-ThDP. Now that an electronic spectral signature has been identified for this (so far only putative) intermediate, it will be easier to design studies to establish conditions under which the 1′,4′-imino-ThDP exists for longer time periods, and to support the thesis that this newly identified intermediate is on the reaction pathway. While the residue E477 appears to be more important in the post-decarboxylation, than in the pre-decarboxylation regime of the reaction in Scheme 1 (*16*), this residue obviously has an influence on the life-time of the 1′,4′-imino-ThDP. Unpublished evidence from this laboratory suggests a role for E477 in the protonation of the enamine (M. Liu, Z. Zhang, and F. Jordan), perhaps with an intervening water molecule between the 4′-nitrogen atom of ThDP and the carboxylic acid group of E477. We also note that the chemical shifts of the imino tautomers in Table 1 provide important guides, should one seek NMR evidence for the imino-ThDP on ThDP enzymes, a challenging task in view of the size of the enzymes.

Finally, the collective evidence is now compelling to take into account the role of the 4′-aminopyrimidine, in addition to that of the thiazolium ring when studying catalysis by ThDP enzymes. Elsewhere, we suggested that the enforced V conformation with its consequences here discussed, including the intramolecular acid-base catalysis by the 4′-aminopyrimidine, and in combination with a solvent effect, accounts for the bulk of the rate acceleration provided by YPDC (*28*). At the same time, the surrounding acid-base side chains provide 3–4 orders of magnitude of rate acceleration at most (*15*). These and other data from this laboratory allowed us to formulate a new mechanism for YPDC using alternating active sites. According to this mechanism, the amino/iminopyrimidine takes on a central role as an acid–base catalyst in the majority of the chemical steps, and as an integral part of a communication pathway between active sites of the functional dimer (*29*).

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