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*Accelerated Publications*

# Evidence for Dramatic Acceleration of a C−H Bond Ionization Rate in Thiamin Diphosphate Enzymes by the Protein Environment†

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ABSTRACT: The hypothesis that thiamin diphosphate-dependent enzymes achieve a significant fraction of their catalytic rate acceleration by providing a protein environment that helps to stabilize unstable zwitterionic/dipolar intermediates (including the enamine/C2α-carbanion present on all such enzymes) was tested experimentally using the intermediate C2α-hydroxyethylthiamin diphosphate (HEThDP) with the *Escherichia coli* pyruvate dehydrogenase complex and its E1 subunit (PDHc-E1). Using pre-steady-state and steady-state methods, it was shown that HEThDP is a substrate for this enzyme after ionization of the C2α-H bond. An experiment was then carried out to measure the PDHc-E1 catalyzed pre-steady-state rate constant for the D → H exchange from the C2α position of HEThDP-$d_4$, as an indicator of the formation of the enamine. Importantly, the enzyme accelerates the rate of ionization of this bond by a factor of $10^7$, corresponding to a 10 kcal/mol stabilization of the enamine intermediate by the enzyme. This finding is likely a general feature of thiamin diphosphate enzymes.

Identifying the sources of the enormous catalytic rate accelerations that many enzymes display relative to the corresponding chemical models remains a major goal of the enzymologist. Thiamin diphosphate (ThDP)[1]-dependent enzymes have long served as a paradigm for enzymes, which gain a significant fraction of their catalytic power from a "solvent" or "environmental" factor, provided by the special anhydrous protein milieu. As a typical member of ThDP-dependent 2-oxoacid decarboxylases, the reaction of the pyruvate dehydrogenase multienzyme complex (*1*−*4*) (PDHc, see Scheme 1) comprises several elementary steps, in which there is at least partial charge neutralization in going from the initial state to the transition state.

Ample chemical precedents suggest that a reaction such as the decarboxylation of the first ThDP-bound covalent intermediate with substrate C2α-lactyl-ThDP (LThDP) to the enamine/C2α-carbanion would take place much faster in solvents of lower dielectric constant. In this instance, there is a major reduction in charge separation in going from the zwitterionic LThDP (full positive charge at the thiazolium N3 atom and a full negative one at the carboxylate ion) to the resonance-stabilized enamine with both a neutral and a dipolar (zwitterionic) resonance contribution. The key first step, generation of the ylide from the thiazolium salt by

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[1] Abbreviations: ThDP, thiamin diphosphate; HEThDP, C2α-hydroxyethylthiamin diphosphate; LThDP, C2α-lactylthiamin diphosphate; YPDC, yeast pyruvate decarboxylase; *E. coli*, *Escherichia coli*; PDHc, pyruvate dehydrogenase complex from *E. coli*; PDHc-E1, the first ThDP-dependent subunit of PDHc; PDHc-E2, the second subunit of the complex, in this paper carrying a single lipoyl domain; DCPIP, 2,6-dichlorophenolindophenol; KPi, potassium phosphate at the indicated total concentration; FT-ICR MS, Fourier transform ion cyclotron resonance mass spectrometry; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry.

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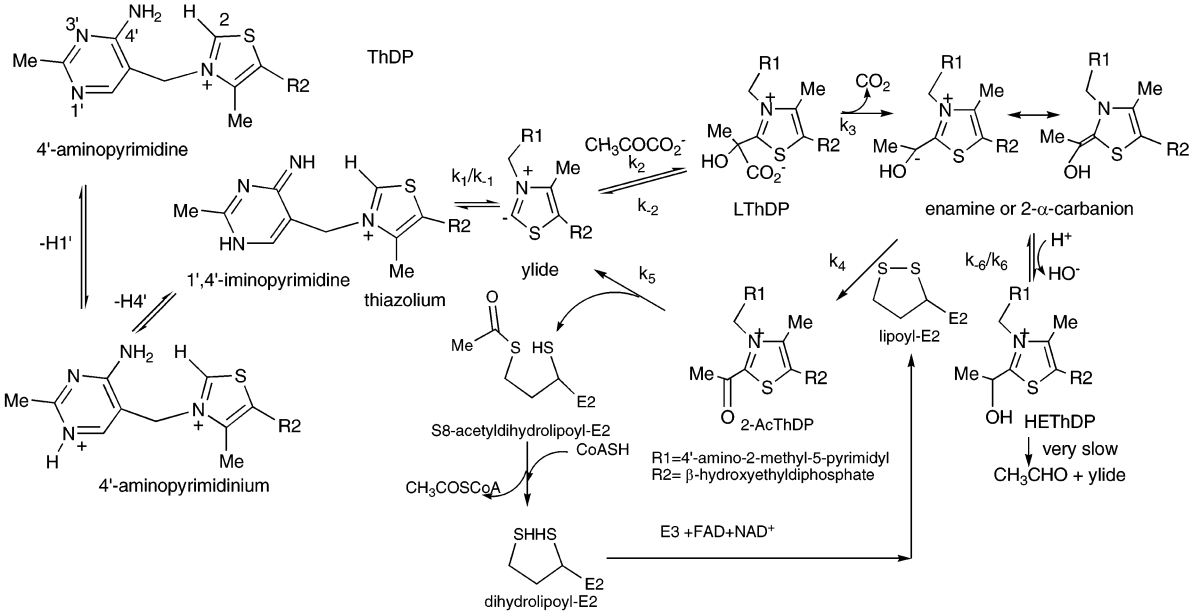
Scheme 1: Mechanism of Pyruvate Dehydrogenase Complex-E1 Subunit




C2−H ionization (proton loss), would also benefit from the same environmental factor. Indeed, Crosby and Lienhard (*5*) and Kemp and O'Brien (*6*), published convincing model chemistry showing very significant rate accelerations when either reaction is transferred to solvents of low dielectric constant. We recently showed that the decarboxylation of compounds related to LThDP can take place with first-order rate constants approaching the turnover number of relevant enzymes even in the absence of the 4′-aminopyrimidine ring, provided a suitable solvent and base are selected (*7*).

Designing experiments to test this hypothesis on the enzymes remains challenging. Model chemistry in this laboratory has dealt with the structure and chemistry of the enamine (*3*, *8*), mostly derived from compounds mimicking the covalent complex between ThDP and acetaldehyde, C2α-hydroxyethylThDP (HEThDP), an intermediate almost certainly on the pathway of the yeast pyruvate decarboxylase (YPDC; *9*). The models allowed us to determine both the rate constants and p$K_a$ for deprotonation/reprotonation at the C2α position in the HEThDP = enamine equilibrium (*10*) as well as for the corresponding reaction relevant to the enzyme benzoylformate decarboxylase, C2α-hydroxybenzylThDP (HBThDP = enamine; refs *11* and *12*). Similar information on ThDP enzymes would provide strong evidence pro or con for the intervention of such an "environmental factor".

Although earlier we reported detection of the enamine derived from HBThDP on YPDC, notwithstanding its very high p$K_a$ for C2αH ionization in water (*13*, *14*), no rate constant could be obtained for its formation on the enzyme. While not on the oxidative pathway, HEThDP has often been used as an alternate substrate on both PDHc (*15*) and pyruvate oxidase (*16*; the latter is an enzyme with both ThDP and flavin at the active center and produces acetate or acetyl phosphate), both of which share with YPDC the mechanism through LThDP decarboxylation.

In this paper, we show that HEThDP is a substrate for PDHc-E1 from *E. coli*. This enzyme consists of multiple copies of three subunits, ThDP-dependent pyruvate dehydrogenase E1 (PDHc-E1), dihydrolipoamide acetyltransferase E2 with covalently bound lipoamide (PDHc-E2), and dihydrolipoamide dehydrogenase (PDHc-E3 with FAD and $NAD^+$) and carries out the series of reactions shown in Scheme 1, and the overall reaction in eq 1:

$$\text{pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (1)$$

The HEThDP must lose the C2αH and produce the enamine (a central intermediate in all ThDP enzymes), and the 2-carbon fragment is then successfully transferred to the PDHc-E2 subunit and ultimately leads to NADH production. Since HEThDP itself is not on the PDHc-E1 reaction pathway, determination of the rate constant for its C2αH ionization to the enamine would provide evidence for the intrinsic ability of this enzyme to enhance this reactivity. We now report this rate constant for the first time. The magnitude of the rate acceleration of this C2αH dissociation step by PDHc-E1 provides dramatic demonstration that ThDP enzymes achieve a significant fraction of their catalytic power from stabilization of this central enamine/C2α carbanion intermediate. The deductions here reached are believed to be of significance to ThDP catalysis in general, hence of potential importance to all ThDP enzymes.

## EXPERIMENTAL PROCEDURES

*Materials*. ThDP was from USB (Cleveland, OH), acetaldehyde-$d_4$ was from Cambridge Isotope Laboratories (Andover, MA), and all other reagents were from Fisher (Pittsburgh, PA).

*UV−Vis spectra* were recorded on a Varian Cary 300 spectrophotometer at 30 °C.

*Enzyme Activity Measurements with HEThDP as Substrate*. For the E1-specific assay of PDHc-E1, the reduction of DCPIP was monitored at 600 nm ($\epsilon_{600}$ = 15 600 $M^{-1}$ $cm^{-1}$) in the presence of 0.2 mM HEThDP (or as specified) according to ref *17*.

To assay the overall PDHc activity, the production of NADH (the final product of the PDHc) was monitored at 340 nm ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) at 30 °C in the presence of HEThDP (0.2 mM or as specified) according to ref *19*.

*Stopped-flow measurements* were carried out on an Applied Photophysics SM. 18 MV stopped-flow reaction analyzer at 30 °C. The reduction of DCPIP was monitored at 600 nm and water was used for background calibration. Into one syringe was loaded (in parentheses are the final concentrations after mixing) PDHc-E1 (0.5 mg/mL, or as specified) and $MgCl_2$ (20 mM, or as specified) in 20 mM KPi buffer (pH 7.0), and into the other was loaded HEThDP (0.5 mM, or as specified) and DCPIP (0.1 mM) in 20 mM KPi buffer (pH 7.0). A total of 4000 data points were collected in each experiment both for the steady-state phase (0−50 s) and for the pre-steady-state phase (0−5 s). The experimental data were analyzed using the SigmaPlot 2001 (SPSS Inc.). For both the steady-state phase and for the pre-steady-state phase, the rate constants were obtained by linear fit of the data.

*Monitoring the H/D Exchange at the C2α Position of HEThDP Catalyzed by PDHc-E1*. (*a*) *Nuclear Magnetic Resonance*. Spectra were recorded on a Varian 500 MHz INOVA spectrometer at 25 °C. The solution in a 5 mm tube (0.8 mL, 90% $D_2O$) contained HEThDP (2.5 mM), $MgCl_2$ (2 mM), and PDHc-E1 (1.0 or 2.0 mg/mL) in KPi buffer (20 mM, pH 7.0). The water peak was suppressed using the PRESAT method. Each spectrum represents the accumulation of 256 scans.

(*b*) *Fourier Transform Ion Cyclotron Resonance Mass Spectrometry* (*FT-ICR MS*). We wished to determine the rate of D → H exchange at the C2α position of HEThDP in $H_2O$ under pre-steady conditions, where HEThDP-$d_4$ carries deuterons at the C2α ($d_1$) and C2$\beta$ ($d_3$) positions, these being derived from acetaldehyde-$d_4$:

$$\text{HEThDP-}d_4 + \text{PDHc-E1} \rightarrow \text{HEThDP-}d_3$$

The D → H exchange experiments were carried out on a Chemical-Quench-Flow model RQF-3 (KinTek Corporation, TX) at 30 °C. Into syringe A was loaded PDHc-E1 (5.0 mg/mL) and 10 mM $MgCl_2$ in 50 mM KPi buffer (pH 7.0). Into syringe B was loaded HEThDP-$d_4$ (2 mM) and 10 mM $MgCl_2$ in 20 mM KPi buffer (pH 7.0). Equal volumes (15 $\mu$L) from each syringe were mixed. At 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 3.0, and 5.0 s after mixing, the reaction was quenched with 1 M HCl containing 10% HCOOH (v/v). The PDHc-E1 was removed by centrifugation (14000 rpm, 30 min), and the sample was freeze-dried overnight. Prior to the MS experiments, the residue was dissolved in 50 $\mu$L of Milli-Q water and purified by HPLC to remove salts from the sample.

To obtain the rate constant for H/D exchange, the percentage of HEThDP-$d_3$ formed at different time points was determined from the relative intensity of the respective monoisotopic ions of HEThDP-$d_4$ and HEThDP-$d_3$, specifically from the change in concentration of the latter. The mass spectra were acquired on a Bruker Apex II 4.7 T FT-ICR MS using electrospray in the positive ionization mode. The sample solutions were introduced into the mass spectrometer through a Cole-Parmer syringe pump at a flow rate of 60 $\mu$L/h. Each mass spectrum (taking about 1 s) was the result of the average of 10 scans.

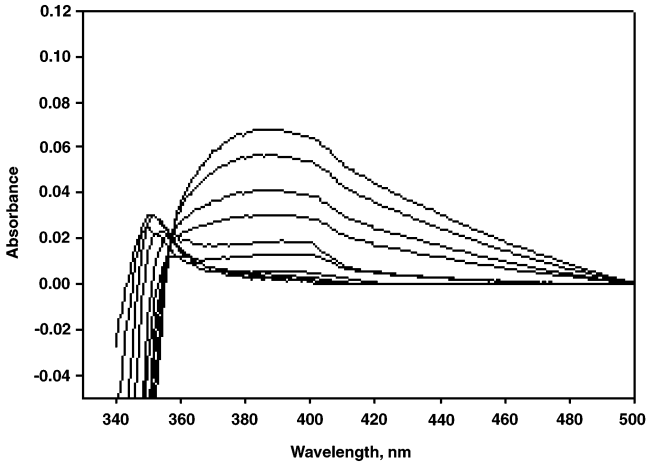



FIGURE 1: Spectroscopic evidence for formation of the enamine from HEThDP on the E91D YPDC by oxidative trapping. The E91D YPDC (0.5 mg/mL) was incubated with 10 mM HEThDP at pH 6.1 (100 mM phosphate buffer containing 2 mM $MgCl_2$) at 10 °C. The reaction was initiated by adding 4-thiopyridine and 4,4′-dithiodipyridine (0.125 mM each). Under these conditions, the enamine is oxidized and produces *N*-acetyl-4-thiopyridine with $\lambda_{max}$ at 382 nm; repetitive scans are shown for 5 min intervals.

A pair of tertiary amines, only differing in one having a $-CD_3$ group rather than a $-CH_3$ group, was used to establish the linearity range of the ion intensity signals. A series of solutions were made in which the concentration ratio of undeuterated compound relative to the deuterated one was 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, and 0.2, and the mass spectra were acquired with the conditions described above. The intensities of the monoisotopic ions of the undeuterated and deuterated compounds were measured. The concentration ratio of the pair of model compounds, nondeuterated to the deuterated species, is listed in column one of Table S1. The intensity ratio of the monoisotopic ions of this pair of compounds is in column two of Table S1. A plot of the ion intensity ratio versus the concentration ratio results in a linear relationship (Figure S1) with a correlation coefficient of $R^2 = 0.9998$. It is therefore concluded that the ionization responses of this pair of compounds is linear over a concentration ratio ranging from 0.002 to 0.2.

*Reductive acetylation of independently expressed lipoyl domain and PDHc-E2* was monitored via MALDI-TOF mass spectrometry by the procedure recently developed in our laboratory (*17*, *19*).

## RESULTS AND DISCUSSION

Evidence had been presented earlier that the enamine could indeed be produced from HBThDP (p$K_a$ off the enzyme is near 15−16; see refs *11* and *12*) on YPDC, an enzyme endowed with a spacious active center (*13*, *14*). That the HEThDP (p$K_a$ estimated near 18 in water; ref *10*) could also lose the C2α proton to form the enamine had to be demonstrated indirectly, by oxidative trapping using a method developed in our laboratory (Figure 1, ref *14*). Although these experiments inform regarding the ability of YPDC to generate the enamine, they do not provide a quantitative yardstick for rate measurements.

The PDHc is a more complex system and both steady-state and pre-steady-state methods were used to establish how

Scheme 2

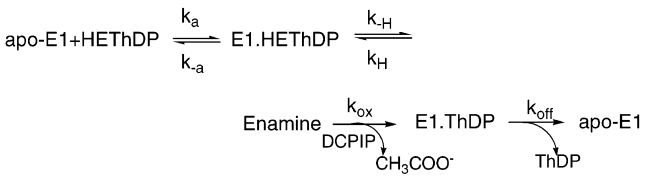



Table 1: Kinetic Isotope Effect for C2αH Ionization from HEThDP and HEThDP-$d_4$ Assaying for Enamine Formation by Reduction of DCPIP[a]

| substrate | $k_{cat}$ ($s^{-1}$) steady state | $k_{cat}$ ($s^{-1}$) pre-steady state |
|---|---|---|
| HEThDP | $0.037 \pm 0.005$ | $0.67 \pm 0.12$ |
| HEThDP-$d_4$ | $0.037 \pm 0.011$ | $0.46 \pm 0.06$ |
| | isotope effect: 1.0 | isotope effect: 1.5 |

[a] All experiments carried out in triplicates at 30 °C with 10 mM final $Mg^{2+}$ present. For the pre-steady-state HEThDP and HEThDP-$d_4$ at 0.25 mM.

it utilizes HEThDP as substrate. Once HEThDP carries out its substrate-like reactions, the resulting ThDP must leave the enzyme prior to occupancy by the next molecule of HEThDP. Hence, under steady-state conditions, the rates are mostly controlled by the dissociation rate constant for ThDP from the enzyme.

*Steady-State Kinetic Analysis of HEThDP Utilization by PDHc and Its E1 Subunit.* To check for reaction of HEThDP with the PDHc-E1 subunit per se, the enamine was oxidatively trapped by the external oxidizing agent 2,6-dichlorophenolindophenol (DCPIP) in the absence of E2 and E3 (Scheme 1). This is a suitable method for detection of the enamine on both YPDC and PDHc-E1 with the caveat that the external oxidizing agent DCPIP may not access the active site hence the rate of the redox process could be limited by the long-distance nature of the electron transfer(s). The rate of reduction of DCPIP by HEThDP in the absence of PDHc-E1 is negligible, and the $k_{cat}$ with the enzyme was 0.147 $s^{-1}$, lower than the $1.28 \pm 0.37$ $s^{-1}$ (*17*) measured when starting with ThDP and pyruvate, but showing that PDHc-E1 could utilize HEThDP as sole substrate ($K_{m,HEThDP} = 28 \pm 3$ $\mu$M, and a Hill coefficient $n$ of $0.92 \pm 0.06$).

On the basis of this experiment, we hypothesize the mechanism in Scheme 2, where the rate constants have the following meaning: $k_a/k_{-a}$ for Michaelis complex formation; $k_{-H}/k_H$ for deprotonation and reprotonation at the C2α position; $k_{ox}$ for oxidation of the enamine, and $k_{off}$ for loss of ThDP, and regeneration of apo-E1 (apo-E1 is the E1 subunit devoid of ThDP). A priori, any of the steps could be rate limiting.

The primary deuterium kinetic isotope effect (KIE) was measured for the reaction, using PDHc-E1 (100 $\mu$g) with HEThDP (0.2 to 0.5 mM) or HEThDP-$d_4$ (0.25 mM), along with $Mg^{2+}$ (10 mM) and DCPIP (0.1 mM). Under steady-state conditions, there is no isotope effect at the C2α position when HEThDP is used as substrate with DCPIP as oxidant (Table 1), hence the C2αH ionization is not rate limiting.

Next, we examined whether the entire PDHc could also utilize HEThDP as substrate by monitoring NADH production in the overall reaction. Parallel experiments were carried out with (a) ThDP as cofactor and pyruvate as substrate and (b) HEThDP as substrate in the absence of ThDP and pyruvate. It was shown that the PDHc indeed could use

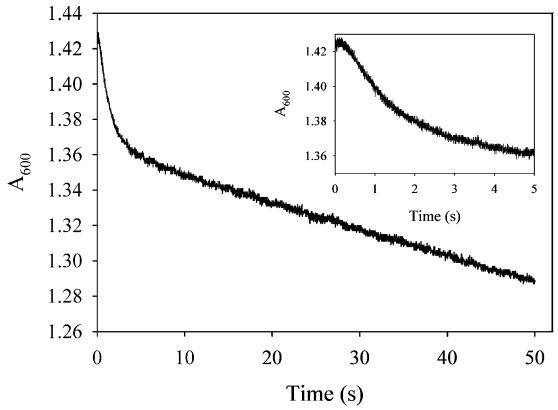



FIGURE 2: Reaction progress curve for the pre-steady-state utilization of HEThDP by PDHc-E1. In the stopped-flow instrument at 30 °C into one syringe was loaded PDHc-E1 (final concentration 0.5 mg/mL) and $Mg^{2+}$ (final concentration 20 mM) in 20 mM potassium phosphate (KPi) buffer (pH 7.0); into the other syringe was loaded DCPIP (final concentration 0.1 mM) and HEThDP (final concentration 0.5 mM) in 20 mM KPi buffer (pH 7.0).

HEThDP as substrate, albeit at a very slow rate ($k_{cat} = 0.067$ $s^{-1}$ as compared with $k_{cat} = 60$ $s^{-1}$ when starting with ThDP and pyruvate), consistent with the release of ThDP from the PDHc being the rate-limiting step.

*Pre-Steady-State Analysis of HEThDP Utilization by PDHc-E1.* Pre-steady-state experiments were carried out for oxidative trapping of the enamine by DCPIP. Progress curves for the first 50 s resulting from mixing HEThDP with PDHc-E1 in the presence of 0.1 mM DCPIP and 20 mM $MgCl_2$ on a stopped-flow instrument (Figure 2) displayed two kinetic phases: a pre-steady-state burst (the first 5 s) and a steady state (linear part between 10 and 50 s). The total change in absorbance during the initial burst corresponds to 4.5 $\mu$M DCPIP being reduced, and, in turn, to 4.5 $\mu$M concentration of active centers, in good agreement with the 5 $\mu$M of active centers calculated from the protein content. These results signal that both active centers on the PDHc-E1 dimer bind HEThDP and catalyze the model redox reaction. A first-order rate constant of 0.67 $s^{-1}$ was determined for the pre-steady-state phase, compared to a $k_{cat} = 1.28 \pm 0.37$ $s^{-1}$ observed with pyruvate and ThDP when using DCPIP in the E1-specific assay under steady-state conditions. The pre-steady-state reaction rate was found to be directly proportional to the concentration of $Mg^{2+}$, reaching saturation at 10 mM $Mg^{2+}$ concentration, suggesting that $Mg^{2+}$ increases the rate of binding of HEThDP to PDHc-E1 (details on this and other kinetics experiments are presented in ref *18*). Using HEThDP and HEThDP-$d_4$ in parallel experiments, the KIE was determined at saturating (3 mM) concentration for each, with 10 mM $Mg^{2+}$ also present, yielding a H/D KIE of 1.5 at the C2α position (Table 1). The KIE value suggests that the release of the proton (deuteron) is partially rate limiting; presumably, oxidation of the enamine by DCPIP is comparable in rate or slower, hence the ionization of the C2αH could be even faster than 0.67 $s^{-1}$.

A $^1$H NMR study (see below) indicated that PDHc-E1 catalyzed the formation of ThDP from HEThDP, suggesting that acetaldehyde could be released. Therefore, the alcohol dehydrogenase/NADH coupled assay was employed to monitor the formation of acetaldehyde, monitoring the depletion of NADH at 340 nm with time, yielding a $k_{cat}$ of

0.0061 $s^{-1}$ for the conversion of HEThDP to ThDP. Hence, acetaldehyde formation is a minor side reaction of E1.

*Reductive Acetylation of Lipoyl Domain and Intact PDHc-E2 by HEThDP and PDHc-E1.* It was important to demonstrate that the two-carbon moiety derived from HEThDP was indeed transferred to the lipoyl domain (independently expressed) and especially to intact PDHc-E2, i.e., that we could reductively acetylate these proteins starting with HEThDP. We have shown that MALDI-TOF MS is well suited for this purpose (*17*, *19*). On incubation of the lipoyl domain (theoretical mass 8,975 Da) with PDHc-E1 and HEThDP for different times, after 3 min both the unacetylated and the acetylated forms of lipoyl domain were in evidence (observed mass = 9019.33 ± 1.80 Da, theoretical mass of reductively acetylated lipoyl domain is 9019 Da). The ratio of unacetylated to acetylated lipoyl domain calculated from the relative peak heights was 0.80:0.20 at 3 min and 0.50:0.50 after 40 min of incubation, indicating a slow rate of reductive acetylation when HEThDP is used as substrate (Figure S2) and yielded a pseudo-first-order rate constant of 0.0018 $s^{-1}$. For sake of comparison, with the parental PDHc-E1 and pyruvate as substrates, within 30 s (the first time point in our experiments), only the reductively acetylated form of lipoyl domain could be detected, while with the H407A active center PDHc-E1 variant even after 80 min of incubation with HEThDP, only the unacetylated form of lipoyl domain could be detected. The HEThDP carries out reductive acetylation much more slowly than pyruvate and ThDP, for which $k_{cat}$ = 0.8 $s^{-1}$ was reported for the enzymes from *Azotobacter vinelandii* (*20*) and *E. coli* (*21*).

Similar experiments were then carried out by incubating intact PDHc-E2 (a fully functional special construct with a single lipoyl domain) with PDHc-E1 and HEThDP for 10 min. Subsequent trypsinolysis gives a mass of the prominent amino-terminal PDHc-E2 fragment containing the lipoyl domain and signaling full reductive acetylation (mass = 10,155.32 ± 0.813 Da). As a control, a similar experiment carried out with the E571A variant of PDHc-E1 (a highly conserved residue forming a strong hydrogen bond with the N1′ atom of ThDP), showed no reductive acetylation of PDHc-E2 even after 60 min of incubation with HEThDP. Since with ThDP, pyruvate and the E571A variant, complete reductive acetylation of PDHc-E2 resulted within 1 min, similar to the observations with parental PDHc-E1, we conclude that the residue E571 participates in the sequence of reactions from HEThDP to *S*-acetyldihydrolipoylE2 (see Scheme 1).

*Direct Determination of the Rate of Proton Dissociation from the C2α of HEThDP.* As shown in Schemes 1 and 2, once HEThDP is bound to PDHc-E1, deprotonation at the C2α position generates the enamine, and the back reaction in $D_2O$ solvent would lead to incorporation of deuterons. We monitored this exchange under steady-state (NMR) and pre-steady state (MS) conditions.

The $^1H$ NMR spectrum of HEThDP incubated with PDHc-E1 and $Mg^{2+}$ for 30 min displayed a chemical shift $\delta$ of 5.40 ppm (quartet) for the C2αH and $\delta$ of 1.64 ppm (doublet) for the C2$\beta$Hs. After 15 h, the C2αH resonance at 5.40 ppm is smaller, suggesting that H/D exchange at the C2α position had taken place. Because of the spectral overlap of the ThDP resonance due to the bridge methylene protons at 5.42 ppm and the quartet due to the C2αH proton, we quantified the H/D exchange by integrating the resonance corresponding to the C2$\beta$Hs at 1.64 ppm. The progress curve for H/D exchange at the C2α position of HEThDP, when incubated with PDHc-E1 (the experiment is outlined in the legend to Figure S3) confirmed the H/D exchange at the C2α position, providing direct evidence for formation of the enamine intermediate in the E1 reaction and that PDHc-E1 was required for the observations.

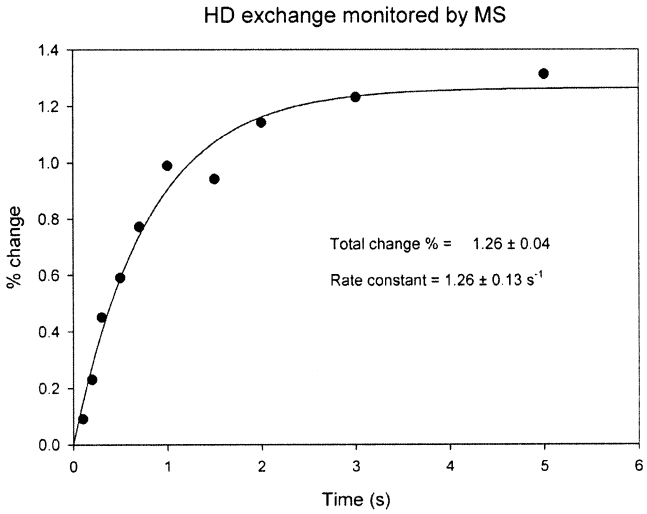



FIGURE 3: Time−course of D → H exchange at the C2α position of HEThDP-$d_4$ monitored by FT-ICR MS. Final concentrations of PDHc-E1 and HEThDP-$d_4$ were 0.025 mM active sites and 1 mM, respectively; hence a 1.26% conversion of HEThDP-$d_4$ to HEThDP-$d_3$ represents a plateau concentration of 0.0126 mM, approximately one-half of the available active centers being utilized during the sampling.

To quantify the rate of H/D exchange in the pre-steady-state, we took advantage of the superior precision of the FT-ICR MS method. We measured the "washout" in $H_2O$ of deuterons from HEThDP-$d_4$, as it is converted to HEThDP-$d_3$, thereby reducing the mass by one unit. This direction for studying exchange was selected since an increased mass would overlap with a busy region due to the myriad of isotopic species. The progress curve for this D → H exchange at the C2α position of HEThDP-$d_4$, when it is rapidly mixed with PDHc-E1, then quenched into acid at the times indicated in Figure 3 gave a rate constant of 1.26 $s^{-1}$, providing direct evidence for very fast formation of the enamine once HEThDP binds to E1. The precision of the FT-ICR MS experiment is noteworthy in view of the fact that only ~1% change in mass is being monitored (calibration is shown under Experimental Procedures). The 1.26% conversion to the HEThDP-$d_3$ (corresponding to 0.0126 mM HEThDP-$d_4$ converted to HEThDP-$d_3$) suggests that only one-half of the active sites is catalytically active in this exchange reaction (legend to Figure 3). The rate constant of 1.26 $s^{-1}$ constitutes a lower limit, since we are breaking a C−D bond rather than a C−H bond. In model studies the KIE for this reaction is 4.7 (*10*); hence, the rate constant for C2α−H ionization may be 5−6 $s^{-1}$, only 10 times slower than the turnover number/active site.

We present the results of two model studies for comparison. Mieyal et al. (*22*) reported $^1H$ NMR H/D exchange half-

life of 6.5 h for HEThDP (pD 8.5, 50 °C), which, extrapolated to our conditions (pH 7.0, 30 °C) is $<3 \times 10^{-7}$ $s^{-1}$. In this laboratory, Zou obtained $k_{-H}$ (Scheme 2) of 142.6 $M^{-1}$ $s^{-1}$ (37 mol % $Me_2SO$) and 71.4 $M^{-1}$ $s^{-1}$ (32 mol % DMSO) for hydroxyl ion catalyzed abstraction of the C2αH from 2-(α-methoxyhexyl)thiazolium ion (*10*; using experiments similar to those reported in refs *11* and *12*). Given that these concentrations of $Me_2SO$ lead to $pK_w$ of 17.1 and 16.6 (*23*), respectively, extrapolation to pH 7.0, converts the second-order rate constant to $10^{-7}-10^{-8}$ $s^{-1}$, in reasonable agreement with estimates of Mieyal et al. (*22*).

We conclude that the PDHc-E1 accelerates enamine formation by at least a factor of $10^7$. This factor corresponds to a 10 kcal/mol lowering of the activation energy barrier, and we suggest that it is a common feature of all ThDP enzymes. Since HEThDP is not on the pathway of PDHc-E1, we believe that our results reflect the intrinsic ability of the PDHc-E1 active center environment to stabilize the enamine. Although there are a number of potential acid−base groups in the active centers of PDHc-E1 structures (*24*, *25*) that could assist formation of the enamine, we do not believe that any of these residues specifically evolved for this purpose on this enzyme. The experiments showing that the E571A substitution did not allow reductive acetylation of PDHc-E2 (see above) starting with HEThDP, but did allow the same reaction to proceed when starting with pyruvate, suggests that the 1′,4′-iminoThDP is responsible for the C2αH ionization.

An important question is whether the rate constant for enamine formation from HEThDP (1.26 $s^{-1}$ measured, or 5−6 $s^{-1}$ estimated when accounting for the isotope effect) is comparable to its rate of formation from pyruvate. In single-turnover experiments mixing pyruvate with PDHc-E1, a first-order rate constant of $11.7 \pm 2.5$ $s^{-1}$ was obtained for the rate-limiting step,[2] very similar to the value here obtained for HEThDP from a very different experiment.

Consistent with our conclusion, there are reports of the existence of the enamine intermediate in crystals of two different ThDP enzymes (*26*, *27*). On YPDC we have earlier reported that its apolar environment resembles that between 1-hexanol and 1-pentanol interpolating to an effective dielectric constant near 13, and suggesting that this behavior endows the active center with the ability to stabilize zwitterionic/dipolar intermediates (*13*). The environment-induced rate acceleration here demonstrated and the intramolecular acid−base function mediated via the 1′,4′-iminoThDP tautomer (*28*, *29*) enforced by the highly conserved V-coenzyme conformation and the conserved glutamate residue at the N1′ atom (*30*) account for the bulk of the catalytic power of ThDP enzymes, over and above that provided by the coenzyme itself.

## SUPPORTING INFORMATION AVAILABLE

Table S1 and Figure S1, with data demonstrating the ability of the FT-ICR MS method to accurately integrate signals of compounds differing only in isotopic composition; Figure S2 showing the MALDI-TOF MS studies on reductive acetylation of independently expressed lipoyl domain by PDHc-E1 and HEThDP; Figure S3 of NMR results showing the time course of HEThDP-C2α-$d_1$ formation from HEThDP catalyzed by PDHc-E1 under steady-state conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

[2] Nemeria, N., Joseph, E., Zhou, L., Tittmann, K., Hübner, G., and Jordan, F. unpublished observations.

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