Accelerated Publications

Histidine 407, a Phantom Residue in the E1 Subunit of the *Escherichia coli* Pyruvate Dehydrogenase Complex, Activates Reductive Acetylation of Lipoamide on the E2 Subunit. An Explanation for Conservation of Active Sites between the E1 Subunit and Transketolase†

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ABSTRACT: Least squares alignment of the *E. coli* pyruvate dehydrogenase multienzyme complex E1 subunit and yeast transketolase crystal structures indicates a general structural similarity between the two enzymes and provides a plausible location for a short-loop region in the E1 structure that was unobserved due to disorder. The residue H407, located in this region, is shown to be able to penetrate the active site. Suggested by this comparison, the H407A E1 variant was created, and H407 was shown to participate in the reductive acetylation of both an independently expressed lipoyl domain and the intact 1-lipoyl E2 subunit. While the H407A substitution only modestly affected the reaction through pyruvate decarboxylation (ca. 14% activity compared to parental E1), the overall complex has a much impaired activity, at most 0.15% compared to parental E1. Isothermal titration calorimetry measurements show that the binding of the lipoyl domain to the H407A E1 variant is much weaker than that to parental E1. At the same time, mass spectrometric measurements clearly demonstrate much impaired reductive acetylation of the independently expressed lipoyl domain and of the intact 1-lipoyl E2 by the H407A variant compared to the parental E1. A proposal is presented to explain the remarkable conservation of the three-dimensional structure at the active centers of the *E. coli* E1 subunit and transketolase on the basis of the parallels in the ligation-type reactions carried out and the need to protonate a very weak acid, a dithiolane sulfur atom in the former, and a carbonyl oxygen atom in the latter.

The *Escherichia coli* pyruvate dehydrogenase multienzyme complex (PDHc)[1] catalyzes the oxidative decarboxylation of pyruvate in the following overall reaction (*1*):

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

In *Escherichia coli* three different enzyme components are involved in the above reaction: **E1** or pyruvate dehydrogenase, utilizing thiamin diphosphate (ThDP) as a cofactor (EC1.2.4.1; E1); **E2** or dihydrolipoamide acetyltransferase, which contains covalently bound lipoyl groups (EC2.3.1.12; E2); and **E3** or dihydrolipoamide dehydrogenase, containing tightly bound FAD (EC1.8.1.4; E3). The multienzyme complex performs the following series of reactions (*2*−*4*):

$$\text{pyruvate} + \text{E1}-\text{ThDP}-\text{Mg}^{2+} \rightarrow \text{E1}-\text{hydroxyethylidene}-\text{ThDP}-\text{Mg}^{2+} + \text{CO}_2 \quad (2)$$

$$\text{E1}-\text{hydroxyethylidene}-\text{ThDP}-\text{Mg}^{2+} + \text{E2}-\text{lipoamide} \rightarrow \text{E1}-\text{ThDP}-\text{Mg}^{2+} + \text{E2}-\text{acetyldihydrolipoamide} \quad (3)$$

$$\text{E2}-\text{acetyldihydrolipoamide} + \text{CoA} \rightarrow \text{E2}-\text{dihydrolipoamide} + \text{acetyl-CoA} \quad (4)$$

$$\text{E2}-\text{dihydrolipoamide} + \text{E3}-\text{FAD} \rightarrow \text{E2}-\text{lipoamide} + \text{E3}-\text{FADH}_2 \quad (5)$$

$$\text{E3}-\text{FADH}_2 + \text{NAD}^+ \rightarrow \text{E3}-\text{FAD} + \text{NADH} \quad (6)$$

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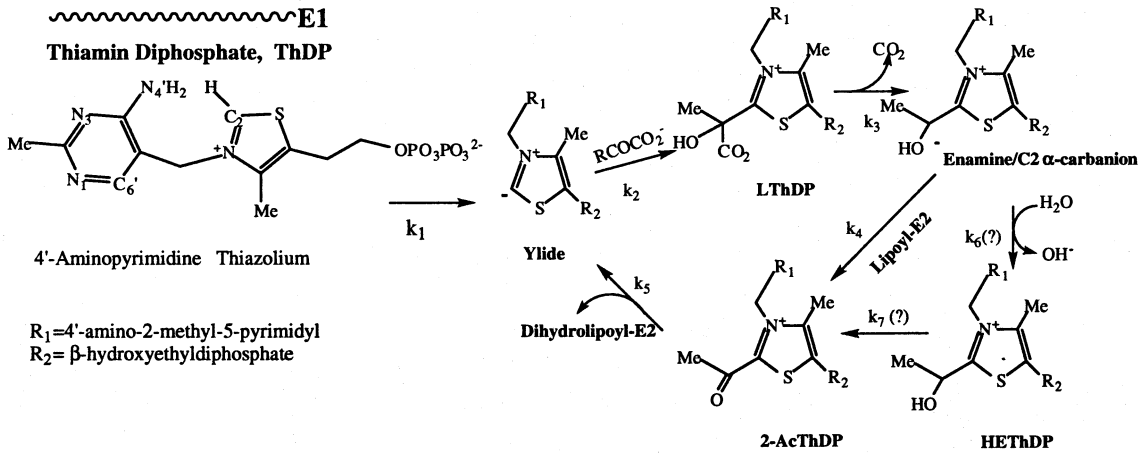
Scheme 1



The complex consists of multiple copies of each component, an ideal polypeptide stoichiometry being as follows: 24 E1, mass of 99 474 Da (*5*); 24 E2, mass of 65 959 Da (*6*); and 12 E3, mass of 50 554 Da (*7*); for a total calculated molecular weight of 4.57×10^6.

The recently completed 1.85 Å crystal structure of the *E. coli* pyruvate dehydrogenase complex E1 subunit (PDHc−E1) revealed the overall domain structures within subunits, the method of subunit assembly to form functional dimers, and the amino acid side chains near the catalytic ThDP at the active sites situated in the dimer interface (*8*). There were three regions found to have no traceable electron density; residues 1−55, 401−413, and 541−557, such that only about 91% of the structure was observed (*8*). Mass spectrometry on washed and dissolved crystals of E1 clearly showed the full-length protein was indeed present, indicating that no proteolysis had taken place and the unobserved regions therefore must simply be disordered in the crystals. From a structural point of view, *E. coli* PDHc E1 is most similar to the related enzyme transketolase (TK; 17% sequence identity), a ThDP enzyme with very different function, with a role in the pentose shunt in sugar metabolism. The enzyme TK carries out a reaction akin to the classical benzoin condensation catalyzed by ThDP in model systems. The reaction is readily reversible and proceeds via an enamine intermediate similar to that in Scheme 1.

A comparison of the *E. coli* PDHc−E1 and TK active centers suggested that there is a residue H407 on a loop not visible in the structure of the *E. coli* PDHc−E1 (presumably the region is too mobile), that could indeed assist the reductive acetylation once the loop becomes less mobile. This hypothesis appears to be on the right track according to experiments here described and carried out with the parental *E. coli* PDHc−E1 and its H407A variant. Kinetic, isothermal titration calorimetry, and mass spectral experiments suggest that H407 is involved in the communication between E1 and E2. This single substitution not only provides a plausible mechanism for assistance of the reductive acetylation of lipoamide−E2 by some residue(s) on E1, but it also creates a framework for unifying the diverse mechanisms carried out by PDHc and TK. The results have likely relevance to communication between the E1 and E2 subunits in the entire family of 2-oxoacid dehydrogenase multienzyme complexes.

EXPERIMENTAL PROCEDURES

Materials. The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The Wizard 373 DNA purification system was from Promega (Madison, WI), and the ABI Prism dye terminator cycle sequencing-ready reaction kit with AmpliTag DNA polymerase, FS, was from Perkin-Elmer (Applied Biosystems, Forster City, CA).

Bacteria and Plasmids. *E. coli* strain JRG 3456 transformed with pGS 878 was used for overexpression and site-directed mutagenesis of the *aceE* gene encoding the E1 subunit of *E. coli* PDHc (*9*). *E. coli* strain JM 101 carrying the plasmid pGS331 encoding the hybrid lipoyl domain was kindly provided by Professor John Guest of the University of Sheffield. This domain comprises residues 1−33 of the first, and residues 233−289 of the third (innermost) lipoyl domain of the E2 subunit (*10*), and its structure was shown by NMR to be similar to that of the innermost lipoyl domain (*11*). As shown here and elsewhere (*30*), this hybrid lipoyl domain is fully functional. The pET-22b(+)-1-lip E2 vector in *E. coli* BL21(DE3) cells was used for overexpression of 1-lip E2 (*30*). *E. coli* BL21(DE3) cells were from Novagene Inc. (Madison, WI).

Methods. Structural Comparisons. The *E. coli* pyruvate dehydrogenase E1 (*8*) and yeast TK (*12*) crystal structures have been determined at 1.85 and 2.5 Å resolution, respectively, with the coordinates used for both structures as deposited in the Protein Data Bank (access codes 1L8A and 1TRK). Structural alignment of the two enzymes was obtained by least-squares methods using Cα atoms with the program O (*13*). Initially a small set of 21 equivalent Cα core atoms in each structure (residues 75−95 from PDHc

[1] Abbreviations: ThDP, thiamin diphosphate; PDHc, pyruvate dehydrogenase multienzyme complex; *E. coli*, *Escherichia coli*; E1, pyruvate dehydrogenase, the first subunit of PDHc (ThDP-dependent); E2, dihydrolipoamide acetyltransferase, the second subunit of PDHc with three lipoyl domains; 1-lip E2, dihydrolipoamide acetyltransferase with a single lipoyl domain per subunit; E3, dihydrolipoamide dehydrogenase, the third subunit of PDHc; YPDC, yeast pyruvate decarboxylase; TK, transketolase; ThTTDP, thiamin 2-thiothiazolone diphosphate; CD, circular dichroism; DCPIP, 2,6-dichlorophenolindophenol; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; FT-ICR MS, Fourier transform ion cyclotron resonance mass spectrometry; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; Sinapinic acid, 3,5-dimethoxy-4-hydroxycinnamic acid.

E1 with residues 5−25 of TK) produced a rms deviation of 0.746 Å. Upon examining the resulting superimposed structures 541 Cα atoms (80% of the TK structure, 61% of the E1 structure) were then deemed equivalent and the least squares optimization was repeated, this time yielding an rms deviation of 2.0 Å.

Construction of the H407A Variant of E1. Mutagenesis reactions were carried out using double-stranded pGS878 DNA, two synthetic mutagenic primers complementary to opposite stands of DNA, and the reagents supplied with the QuikChange site-directed mutagenesis kit for 16 cycles. The following synthetic oligonucleotide, and its complement were used as mutagenic primers (mismatched bases underlined and mutant codons in boldface type):

5′-GGTAAAAACATCGCG**GCC**CAGGTTAAGAAAATG-3′ (primer)

5′-CATTTTCTAACCTG**GGC**CGCGATGTTTTTACC-3′ (antiprimer)

The success of the H407A mutation was verified by sequencing the entire E1 DNA gene with the specific primers reported previously (*14*), using the ABI Prism Ready-Reaction dye terminator cycle sequencing kit from Perkin-Elmer.

Overexpression of Parental E1 and of the H407A Variant. *E. coli* JRG 3456 cells transformed with the corresponding plasmids were grown for 16 h at 37 °C in LB medium (20 mL) containing 0.1% glucose, 2 mM acetate, and 50 μg/mL ampicillin and were used to inoculate 1000 mL of the same medium. Cells were grown to A_{650} = 0.6−0.7 and then induced with IPTG (1 mM), harvested after 5−6 h, washed with 20 mM KH_2PO_4 (pH 7.0), and stored at −20 °C.

Overexpression of Lipoyl Domain. *E. coli* cells (JM101) transformed with pGS331 plasmid were used for expression of lipoyl domain. Cells were grown at 37 °C in 4000 mL of LB medium supplemented with 0.1% glucose and 100 μg/mL ampicillin until an A_{650} of 0.5−0.7 was achieved. Then IPTG (0.042 mM) and DL-α-lipoic acid (0.5 mM) were added to induce the expression and generate the lipoylated form of the lipoyl domain and cells were grown at 37 °C for 16 h. The harvested bacteria were washed with 20 mM KH_2PO_4 (pH 7.0) containing EDTA (2 mM), NaN_3 (0.02%), PMSF (1 mM), and benzamidine·HCl (1 mM) and were stored at −20 °C.

Overexpression of 1-lip E2. *E. coli* BL21(DE3) cells transformed with the pET-22b(+)-1-lip-E2 plasmid were grown for 16 h at 37 °C on an LB plate containing 50 μg/mL ampicillin. A single colony was used to inoculate 1000 mL of LB medium containing 50 μg/mL of ampicillin and 0.1 mM of DL-α-lipoic acid. Cells were grown to A_{650} = 0.4−0.6 and then induced with IPTG (1 mM), harvested after 3−4 h, washed with 20 mM KH_2PO_4 (pH 7.0) containing 1 mM EDTA, and stored at −20 °C.

Purification of E1, H407A E1, Lipoyl Domain, and 1-lip E2. Purification of parental E1 and the H407A variant of E1 was carried out following the protocol described previously (*9*); that of the lipoyl domain according to Ali et al. (*10*) and of 1-lip E2 will be presented elsewhere (*30*).

Activity and Related Measurements. The E1 activity was measured by reconstituting PDHc activity with added E2−E3 subcomplex monitoring the pyruvate-dependent reduction of NAD^+ at 340 nm as published previously (*9*). The E1-specific activity was measured in the model reaction monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm (*14*).

Reductive Acetylation of the Lipoyl Domain and of 1-lip E2 by E1 or H407A E1 Monitored by MALDI-TOF Mass Spectrometry. For the reductive acetylation of lipoyl domain, the E1 subunit or its H407A variant (0.1 μM or 0.5 μM) was incubated in 20 mM HEPES (pH 7.0) with 2 mM $MgCl_2$, 0.2 mM ThDP, 2.0 mM pyruvate, and 0.6 mM lipoyl domain in a total volume 0.10 mL at 25 °C. Aliquots (1 μL) were withdrawn at different times and were mixed with 1 μL of sinapinic acid as matrix on the target plate. For the reductive acetylation of the 1-lip E2, the E1 subunit or its H407A variant (0.1 μM) was incubated in 50 mM Tris−HCl (pH 8.2), containing 0.3 M NaCl, 2 mM $MgCl_2$, 0.2 mM ThDP, 2.0 mM pyruvate, and the 1-lip E2 (2 mg/mL) in a total volume 0.1 mL at 25 °C. After 30 min, trypsin was added to digest the 1-lip E2 at 25 °C (wt/wt ratio of 1-lip E2:trypsin = 200:1). After 2 h, benzamidine·HCl (0.15 mg/mL) was added to terminate the reaction. Samples were desalted with $ZipTip_{C18}$ (Millipore Corp. Bedford, MA), and 1 μL was mixed with 1 μL of sinapinic acid on the target plate. Mass spectra were acquired on a Voyager-DE PRO MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser to desorb and ionize samples. The accelerating voltage used was 25 kV. The spectrometer was calibrated using ubiquitin (mass of 8566 Da) or bovine α-lactalbumin (mass of 14 179 Da) for mass determination of the lipoyl domain.

Isothermal Titration Calorimetry. Isothermal titration calorimetry experiments were carried out on a VP-ITC MicroCalorimeter (MicroCal Inc., Northampton, MA). The E1 (15 μM) or its H407A variant (15 μM) placed in the calorimetric cell (cell volume = 1.436 mL) was titrated with lipoyl domain in the injection syringe (at an initial concentration of 1.5 mM). The titration experiments were performed at 25 °C in 20 mM KH_2PO_4 (pH 7.0) containing 5 mM $MgCl_2$ and 0.2 mM ThDP. The lipoyl domain was injected into the calorimetric cell in 10 μL increments with a total of 29 injections and at a speed of 310 rpm. The reference experiment for heat release resulting from dilution of lipoyl domain into the calorimetric cell was performed under the same conditions. The reference experiment was subtracted before fitting the binding isoterm using the Origin (version 5.0) program (MicroCal, Northampton, MA). The binding parameters n, $\Delta H°$, $\Delta S°$, and K (the association constant) were determined directly from the titration experiment. The $\Delta G°$ was calculated from the equation: $\Delta G° = -RT \ln K = \Delta H° - T\Delta S°$. Proteins and buffer were degassed prior to the experiment by stirring under vacuum.

Fluorescence Spectroscopy. Fluorescence spectra of E1 and its H407A variant were recorded at 25 °C on an SLM8100 spectrofluorometer in 3 mL quartz cuvettes with an excitation wavelength of 290 nm and emission in the 300−450 nm range. The concentration of E1 and H407A E1 was 0.05 mg/mL in 20 mM KH_2PO_4 (pH 7.0). The value of K_d was calculated as published previously (*14*).

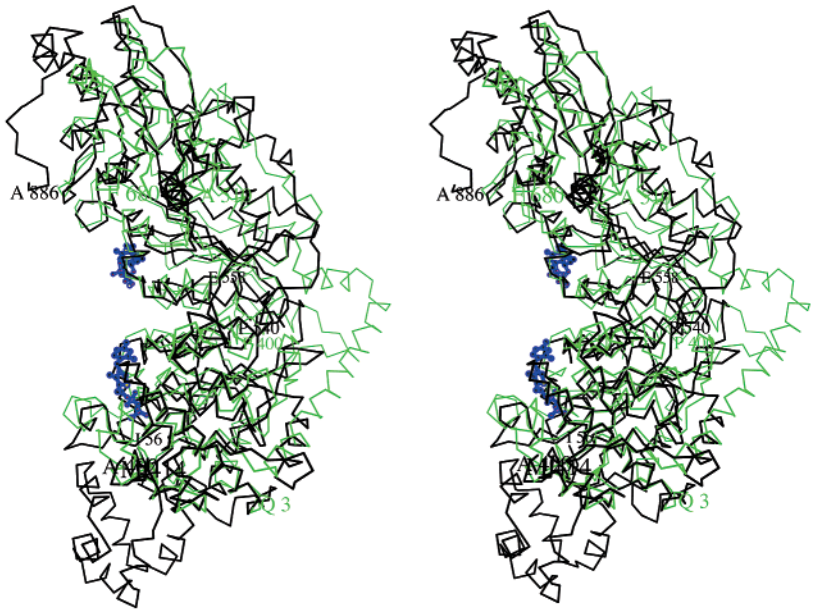


FIGURE 1: Stereo drawing of the superposition of the *E. coli* PDHc E1 subunit with the *S. cerevisiae* TK subunit after least squares alignment. Colors are black and green for the E1 and TK structures, respectively. In blue are shown two molecules of ThDP in the active sites of PDHc−E1. The figure was created with the program MOLSCRIPT (*28*).

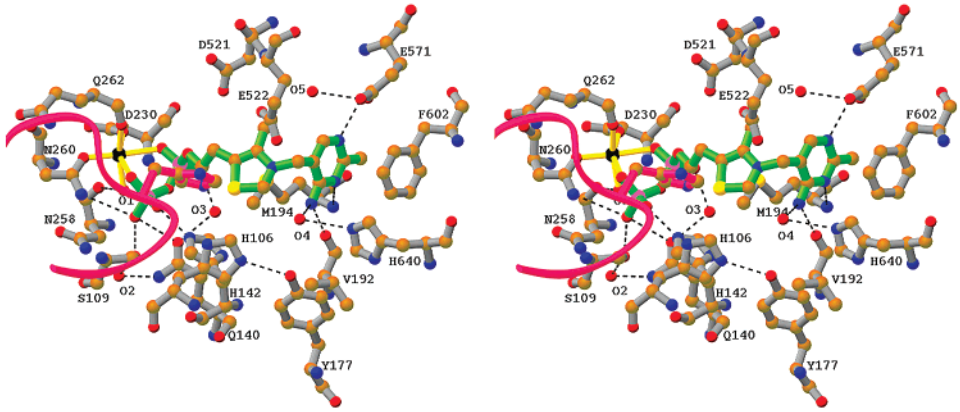


FIGURE 2: Stereo drawing of the *E. coli* PDHc E1 active site environment with the TK H263 (and hence E1 H407) residue superimposed as indicated from least-squares alignment of the two enzymes. E1 residues with numbers less than 471 are from the N-terminal domain of one subunit, whereas those numbered greater than 470 are from the middle domain of the "other" subunit across the dimer interface. The main chain and histidine residue unobserved in E1 but positioned via least squares alignment with TK is shown colored in magenta. Several water molecules are included in the drawing. The figure was created with the program RIBBONS (*29*). Thiamin diphosphate is depicted in green.

RESULTS

Structural Comparison of E. coli PDHc E1 and Yeast TK. The structural alignment of the *E. coli* PDHc E1 and yeast TK showed 541 Cα atoms to be structurally equivalent with a rms deviation of 2.0 Å, indicating that subunits are indeed structurally similar in the two proteins (Figure 1). The binding mode and the conformation of the ThDP in the active sites of these enzymes is also similar (*8*, *15*). Subsequent independent least squares alignment of the corresponding individual domains gave rms deviations of 1.58, 1.65, and 1.71 Å for the N-terminal, middle, and C-terminal domains, respectively, indicating small shifts in the relative organization of domains which form the intact subunits. On the basis of the structural alignment and overall agreement, one might expect the second missing region in PDHc E1 (residues 401−413) to be structurally similar to corresponding TK loop residues 257−269. In TK the loop residues 257−269 are near the ThDP binding region, and the side chain of the invariant residue H263 is hydrogen bonded to one of the β-phosphoryl oxygen atoms of the diphosphate moiety of ThDP (*15*). The amino acid sequence alignment of the above-mentioned regions of the yeast TK and *E. coli* PDHc E1 is

PDHc	E1	401	EGKNIA**H**QVKKMN	413
	TK	257	AGSHSV**H**GAPLRA	269

According to a BLAST search, the residue H407 in PDHc−E1 is highly conserved in the family of bacterial E1's, as is the corresponding H263 in the family of transketolases. The conserved nature of these histidines, along with the overall structural agreement, indicates that the potentially reactive residue H407 in E1 may indeed enter the E1 active site and participate in catalysis at some point(s) in the sequence of reactions. A stereo diagram illustrating the

Table 1: Overall and E1-Specific Activities and Binding Parameters for Thiamin Diphosphate by Parental E1 and Its H407A Variant

enzyme	pyruvate:NAD^+ oxidoreductase activity[a,c] (u/mg E1)	k_{cat} (s^{-1})	DCPIP reaction, starting with pyruvate[c] (u/mg E1)	DCPIP reaction, starting with HEThDP[c] (u/mg E1)	K_d, ThDP (μM)[b]
E1 parental	16.36 ± 1.97 (100%)	54.6 ± 6.8	0.385 ± 0.11 (100%)	0.036 ± 0.010 (100%)	1.84
H407A E1	0.025 ± 0.002 (0.15%)	0.081 ± 0.007	0.047 ± 0.017 (12%)	0.0062 ± 0.0002 (17%)	1.83

[a] For assaying the overall PDHc activity via NADH production, the H407A E1 variant was reconstituted with E2−E3 subcomplex for 1 h at 25 °C at a mass ratio of 1:2. [b] The K_d values for ThDP binding were determined by quenching of fluorescence of the parental E1 or its H407A variant with ThDP. [c] One unit of activity (u) is defined as the amount of NADH produced (or DCPIP reduced in the E1-specific reaction), in μmol min^{-1} mg^{-1} of E1.

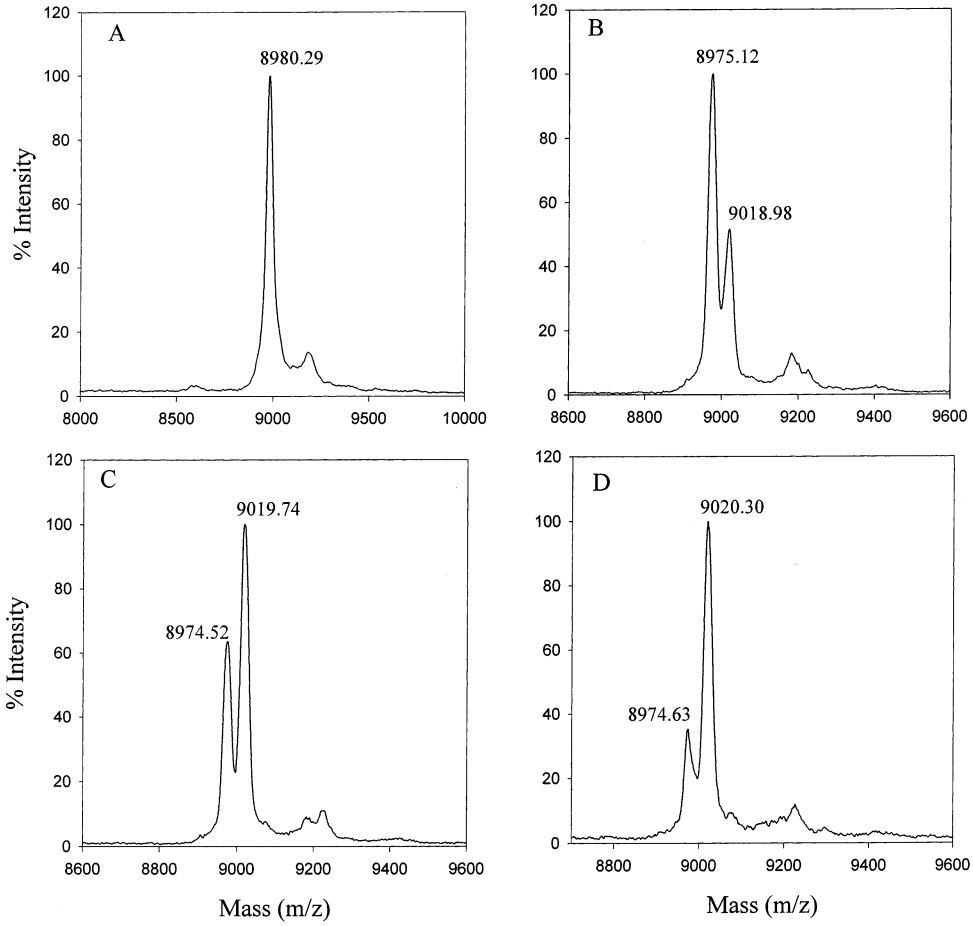


FIGURE 3: Time-course of reductive acetylation of the lipoyl domain by pyruvate and the H407A E1 variant monitored by MALDI-TOF mass spectrometry. Spectra show the unacetylated and acetylated forms of the lipoyl domain at 30 s (A), 3 min (B), 5 min (C), and 30 min (D) of incubation with pyruvate and the H407A E1 variant. The conditions are described under Experimental Procedures.

position of TK H263 (and hence E1 H407) in the E1 active site after structural alignment is given in Figure 2. On the basis of these findings, we undertook characterization of the behavior of E1 with the H407A substitution to probe whether H407, though unobserved due to disorder in the E1 crystal structure, may play a role in the reaction mechanism.

Characterization of the H407A Variant of E1. The H407A E1 variant was overexpressed from the IPTG-inducible expression plasmid pGS878. Typical yield of purified protein was 14 mg/L of induced culture, similar to the yield of the parental E1. The activity measured in the overall PDHc reaction after reconstitution with E2−E3 subcomplex was hardly detectable under the conditions used for parental E1 (*9*). However, preincubation with E2−E3 subcomplex for 1 h improved the overall activity to about 0.15% relative to the parental E1 (Table 1). This activity was calculated once steady state was reached, since the progress curve for NADH production in the overall PDHc assay using H407A E1 exhibited a prolonged lag phase not seen with the parental E1. We used an external artificial oxidizing agent DCPIP to trap the enamine being formed either in the forward reaction starting with pyruvate (where it demonstrates possible impairment of reactions through decarboxylation) or from HEThDP (showing whether the C2α-H can be ionized for the subsequent oxidation/reduction process). According to the DCPIP assay, the specific activity of H407A E1 was about 12% starting with pyruvate and 17% starting with HEThDP as substrates compared to the parental E1, indicating that the pathway through decarboxylation is only modestly affected by the H407A substitution in the E1 subunit (Table 1). Addition of ThDP to the H407A E1 variant quenched 14.8% of the fluorescence compared with 16% for parental E1 (*14*) and gave a K_d of 1.83 μM for ThDP, similar to the value of 1.84 μM obtained with the parental E1 (Table

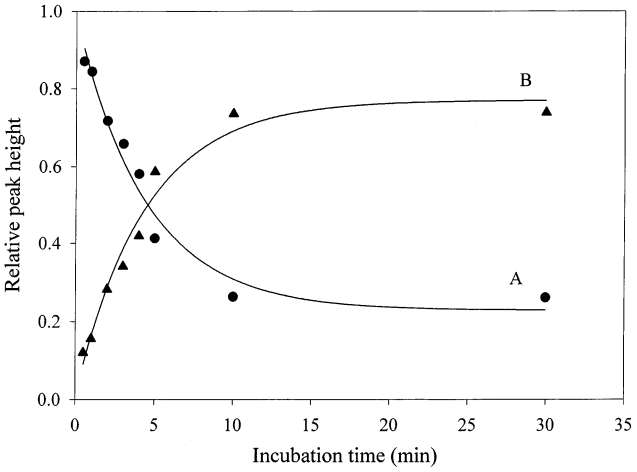


FIGURE 4: Curves of the time-dependent reductive acetylation of the lipoyl domain by pyruvate and the H407A E1 variant based on the relative peak height determined for each form. Curve A, exponential depletion of the unacetylated lipoyl domain; curve B, formation of the reductively acetylated lipoyl domain. Conditions as described for Figure 3 and under Experimental Procedures.

1). In addition, the near-UV CD spectrum of the H407A E1 exhibited the positive band centered at 330 nm on addition of thiamin 2-thiothiazolone diphosphate (ThTTDP), similar to that observed with the parental E1 (data not shown) (*14*). This indicates that binding of neither ThDP nor of ThTTDP is affected by the H407A substitution in the E1 subunit.

Reductive Acetylation of the Lipoyl Domain and of 1-lip E2 by the H407A Variant of E1. It is evident from the results presented above that the reductive acetylation reaction of E2 is affected by the H407A substitution in the E1 subunit. To confirm this hypothesis, the reductive acetylation of the independently expressed lipoyl domain was analyzed by MALDI-TOF MS. It was shown that after 1 h of incubation with H407A E1 variant and pyruvate, the lipoyl domain was fully acetylated, similarly to parental E1. MALDI-TOF MS gave a mass of 9019.67 ± 0.66 Da for acetylated lipoyl domain compared with the theoretical mass of 9019 Da. In addition, FT-ICR mass spectrometry (data not shown) gave a mass of 9019 Da, identical to the theoretical value. At shorter incubation times, the time course for reductive acetylation as monitored by MALDI-TOF MS was significantly different for the parental E1 and the H407A E1 variant (Figures 3A-3D present data for H407A E1). Within 30 s, the lipoyl domain incubated with the parental E1 and pyruvate was fully acetylated (data not shown). However, with the H407A E1 variant, we could monitor the time course of interconversion of the unacetylated and acetylated forms of the lipoyl domain (Figure 3A−D). With increasing incubation time, the peak for the acetylated form of lipoyl domain (9019.67 ± 0.66 Da) increases, while that corresponding to the unacetylated form (8974.75 ± 0.32 Da) diminished in size. However, even after 30 min of incubation with H407A E1 variant and pyruvate, the unacetylated form is still observed (Figure 3D). From the data in Figure 4, a pseudo-first-order rate constant of 0.0038 s^{-1} could be calculated for reductive acetylation of the lipoyl domain, significantly smaller than the k_{cat} of 0.8 s^{-1} reported for *E. coli* E1 (*16*) and *Azotobacter vinelandii* E1 (*17*). This indicates that the H407A substitution in the E1 subunit dramatically affects the rate of the reductive acetylation reaction.

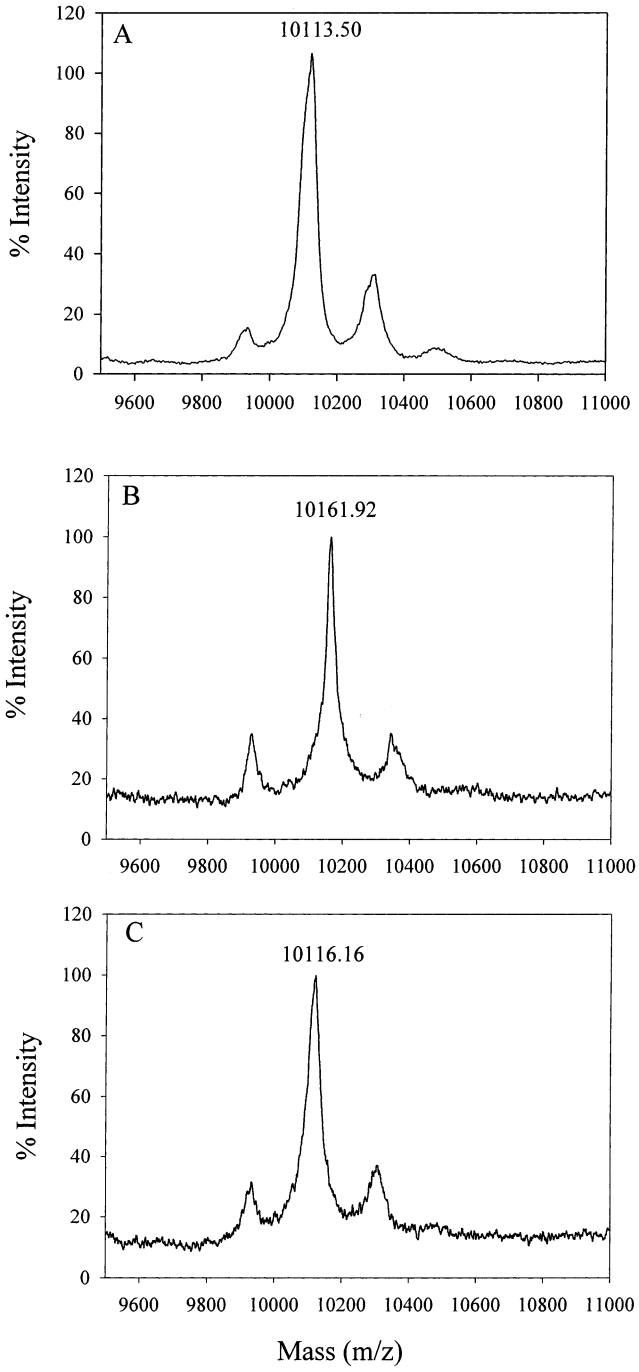
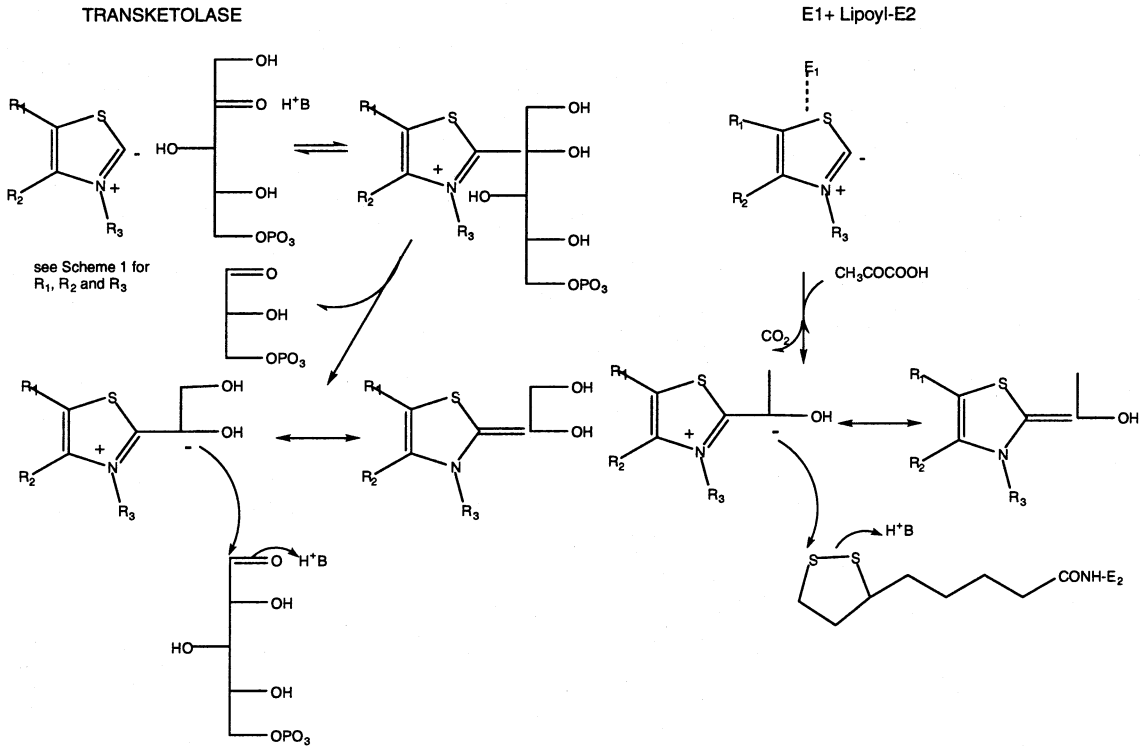


FIGURE 5: MALDI-TOF mass spectra of the lipoyl domain excised from the 1-lip E2 by tryptic digestion. A, prior to reductive acetyl transfer; theoretical mass = 10 109.5 Da. B, after reductive acetylation with pyruvate and parental E1; theoretical mass = 10 153.5 Da. C, after attempted reductive acetylation with pyruvate and the H407A E1 variant. The conditions for the reductive acetylation of the1-lip E2 with parental E1 or its H407A variant and pyruvate and for the tryptic digestion are described under Experimental Procedures.

The results above were obtained with an independently expressed lipoyl domain. We also constructed a plasmid for expression of the 1-lip E2 subunit with a single lipoyl domain per E2 subunit (*30*). Incubation of the 1-lip E2 with E1 and pyruvate for 30 min at 25 °C followed by tryptic digestion led to the appearance of the lipoyl domain with mass of

Scheme 2



10 159 ± 3 Da, clearly signaling acetylated lipoyl domain [Figures 5A and 5B present data for the unacetylated (theoretical mass = 10,109.5 Da) and acetylated (theoretical mass = 10,153.5 Da) forms of lipoyl domain excised from the 1-lip E2 subunit]. When the H407A E1 variant was preincubated with 1-lip E2 and pyruvate, only the unacetylated form of the lipoyl domain was detected in the MS spectrum under the same conditions (Figure 5C), affirming that H407 in the E1 subunit is important for reductive acetylation of the 1-lip E2 subunit.

Isothermal Titration Calorimetry. A calorimetric titration of parental E1 and H407A E1 with lipoyl domain was performed. Analysis of the data after subtraction of the reference experiment yields an association constant of 6.8×10^4 M^{-1} (K_d = 15 μM), similar in magnitude to the K_m for reductive acetylation of the *E. coli* lipoyl domain (33 μM) and smaller than the K_d for binding the lipoyl domain to the E1 (0.3 mM) published previously (*18*). Binding of the lipoyl domain to E1 is exergonic ($\Delta G°$ = −6.6 kcal/mol) with a small but favorable enthalpy of binding ($\Delta H°$ = −0.62 kcal/mol). The entropy of binding ($T\Delta S°$) was large (5.98 kcal/mol), indicating that the interaction of the lipoyl domain with E1 is entropy-driven. These binding parameters are in the range of $\Delta G°$ and $\Delta H°$ published previously for protein−protein and protein−peptide interactions (*19*). Titration of the H407A E1 with lipoyl domain revealed only very weak binding. The heat released was very small ($\Delta H° \sim -0.06$ kcal/mol) and originated primarily from the dilution of lipoyl domain into the calorimetric cell. Apparently, the residue H407 also affects the free energy of binding between E1 and E2.

DISCUSSION

Structural comparisons of *E. coli* PDHc E1 with yeast TK suggested that the residue H407 in the former might have a role in PDHc catalysis. The subsequent studies clearly showed that the residue H407 is indeed important in catalysis according to the following criteria: (1) The overall PDHc activity (NADH production) of the H407A E1 variant is only about 0.15% of that of the parental E1 when both are reconstituted with E2−E3 subcomplex; (2) the DCPIP assay for trapping the enamine produced from pyruvate indicates that the reactions through decarboxylation are little impaired by the H407A substitution; and (3) according to the quenching of fluorescence by added ThDP, the parental and H407A E1 variant bind ThDP equally well.

In comparison with data obtained in our laboratories, the H263A substitution in TK resulted in 0.5% activity as compared with that of the wild-type enzyme (*20*). The substitution did not affect the K_m for ThDP or the K_m for donor and acceptor substrates, indicating that H263 is involved in catalysis (*20*). The overall structure of the H263A variant of TK was also similar to that of the wild-type enzyme (*20*).

According to thermodynamic measurements with an isothermal titration calorimeter, the formation of the complex between the E1 and the lipoyl domain has a $\Delta G°$ = −6.6 kcal/mol, with only a modest contribution from enthalpy ($\Delta H°$ = −0.62 kcal/mol), and is indeed entropy driven. The same experiment employing the H407A E1 variant with lipoyl domain revealed a $\Delta H°$ of only −0.06 kcal/mol, originating from dilution of the lipoyl domain into the calorimetric cell. We conclude that the residue H407 affects the $\Delta G°$, the free energy of binding between E1 and E2.

The MS experiments with the parental E1 and pyruvate yielded fully reductively acetylated lipoyl domain by the first time point taken at 30 s. Similar experiments with the H407A E1 variant clearly indicated that this reaction was very much slowed (by at least a factor of 30; for the parental E1, we estimate a lower limit of 0.11 s^{-1} for this reaction, but this

number could be much larger). A similar experiment carried out with the 1-lip E2, followed by tryptic digest, indicated no reductive acetylation of this E2 construct under the reaction conditions by the H407A variant and pyruvate, thereby affirming that residue H407 is indeed essential to intersubunit communication. As mentioned under Results, the specific activity of this variant is no more than about 0.15% of that of the parental enzyme; hence, these results are consistent. We conclude that the residue H407 is an important contributor to the reductive acetylation of the E2 subunit by E1.

These results raise two very exciting further issues: (1) What is the mechanism of reductive acetylation of the lipoamide covalently attached to E2 involving H407 of the E1 subunit, and (2) what is the relationship of the reactions of PDHc and transketolase that give rise to the observed conservation of active centers in these two enzymes with seemingly unrelated functions?

In chemical model studies at Rutgers, where we generated a model for the enamine and then attempted to react it with lipoic acid derivatives, we noted that the dithiolane ring of lipoic acid was remarkably unreactive as an electrophile until it was itself activated by an electrophile (*21*, *22*). Very modest electrophilic activation could be achieved using mercury compounds (*21*), but truly dramatic rate accelerations resulted by methylating either one of the sulfur atoms, the methylation serving as a surrogate for the mobile proton (*22*). Under the latter conditions, we could demonstrate formation of a tetrahedral intermediate between the sulfur end of lipoamide and the C2α atom of the enamine and, most importantly, *S*-methylation of lipoamide accelerates the reaction with the enamine by at least a factor of 10^8 (*22*). We therefore proposed that activation of the lipoyl group takes place on the E1 active site, rather than on E2. Drawing on this information, we further hypothesized that there should be a general acid−base residue on the E1 active site that is responsible for assisting the "ligation"-like reaction in eq 3. On the basis of the evidence presented here, we now suggest that the residue H407 is a major player in this activation.

As to the second question, why there should be strong active center conservation between these two enzymes with such seemingly different functions, we suggest an explanation sketched in Scheme 2. A plausible answer is that both TK and E1 + E2 carry out ligation reactions, and more importantly from the point of view of catalysis, both must solve the difficult problem of electrophilic activation of a very weak base as seen in Scheme 2. In TK this takes the form of protonation of a carbonyl functional group (either a ketone or aldehyde), while on E1−E2 it is the protonation of the dithiolane ring. This ligation reaction is only an artifact in the simpler YPDC that we have studied jointly for many years (*23*, *24*), but is the very essence of the TK and PDHc reactions. This explains why there is but little acid−base chemistry conserved in the active centers between YPDC and E1, with the notable exception of E51 on YPDC and E571 on E1, a residue conserved in all ThDP enzymes studied so far and located at the N1′ atom of the 4′-aminopyrimidine ring. The loop with H263 in TK is then predicted to have importance in the ligation step, just as H407 in E1 is important in the reductive acetylation step. We further speculate that other residues nearby which appear to be located at corresponding positions on the two enzymes are charged with creating an electric field to assist the protonation of the weak bases, the carbonyl oxygen in TK and the dithiolane sulfur atom in E1.

There is supporting evidence for this hypothesis concerning the possible role of acid−base catalysis on TK. Residues H30 and H263 are perfectly positioned in the active site of TK to abstract the proton from the fructose-6-phosphate donor substrate modeled into the active site of yeast TK (*25*). The residue H263 is poised to hydrogen bond simultaneously to the diphosphate of ThDP and to the hydroxyl group of the donor molecule (*25*). In the same studies, the two conserved residues H30 and H263 are within hydrogen-bonding distance of the carbonyl oxygen atom of the acceptor substrate erythrose-4-phosphate (*26*). The implications of the reports by Schneider and co-workers is that H263 may also have a role as a proton donor to the aldehyde carbonyl oxygen atom of the acceptor substrate, as suggested in Scheme 2 (*25*, *27*).

Finally, we propose that the conserved features here seen will be common to the entire ThDP superfamily of enzymes charged with such "ligation" mechanisms.

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