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# Interchain Acetyl Transfer in the E2 Component of Bacterial Pyruvate Dehydrogenase Suggests a Model with Different Roles for Each Chain in a Trimer of the Homooligomeric Component

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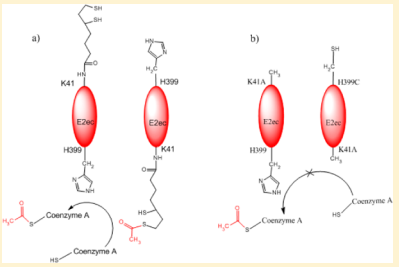
Supporting Information

**ABSTRACT:** The bacterial pyruvate dehydrogenase complex carries out conversion of pyruvate to acetyl-coenzyme A with the assistance of thiamin diphosphate (ThDP), several other cofactors, and three principal protein components, E1−E3, each present in multiple copies. The E2 component forms the core of the complexes, each copy consisting of variable numbers of lipoyl domains (LDs, lipoic acid covalently amidated at a lysine residue), peripheral subunit binding domains (PSBDs), and catalytic (or core) domains (CDs). The reaction starts with a ThDP-dependent decarboxylation on E1 to an enamine/C2$\tilde{\alpha}$ carbanion, followed by oxidation and acetyl transfer to form *S*-acetyldihydrolipoamide E2, and then transfer of this acetyl group from the LD to coenzyme A on the CD. The dihydrolipoamide E2 is finally reoxidized by the E3 component. This report investigates whether the acetyl group is passed from the LD to the CD in an intra- or interchain reaction. Using an *Escherichia coli* E2 component having a single LD, two types of constructs were prepared: one with a Lys to Ala substitution in the LD at the Lys carrying the lipoic acid, making E2 incompetent toward post-translational ligation of lipoic acid and, hence, toward reductive acetylation, and the other in which the His believed to catalyze the transthiolacetylation in the CD is substituted with A or C, the absence of His rendering it incompetent toward acetyl-CoA formation. Both kinetic evidence and mass spectrometric evidence support interchain transfer of the acetyl groups, providing a novel model for the presence of multiples of three chains in all E2 components, and their assembly in bacterial enzymes.




The bacterial pyruvate dehydrogenase complex (PDHc) carries out conversion of pyruvate to acetyl-coenzyme A with the assistance of three principal protein components, E1−E3[1] (Scheme 1). In the mammalian enzyme,[2] there are three additional components mostly responsible for regulation: a kinase, a phosphatase, and an E3-binding protein (E3BP).[3,4] Each component exists in multiple copies with total molecular masses in the range of 4.5−10 MDa. The multidomain E2 component forms the core of the complexes [see the domain structure for E2 from *Escherichia coli* (E2ec) in Scheme 2]. It consists of variable numbers[1−3] of LDs to which the cofactor lipoic acid is covalently attached in a post-translational reaction, a PSBD, and a CD, where acetyl-CoA is produced.[1] Although the number of copies of icosahedral mammalian E2 is still controversial, there is agreement that the sum (E2 + E3BP) is 60, a multiple of three,[5−7] while there are 24 copies of the octahedral *E. coli* E2 (E2ec), again a multiple of three.[1] For the *E. coli* PDHc, a stoichiometry of 12 E1ec dimers, 8 E2ec trimers, and 6 E3ec dimers was proposed.[8−12] All 24 copies of E2ec have the LD and the CD, and it is plausible that interchain acyl transfer would take place between an LD of one chain and the CD of a different one, with the mobility of the LD afforded by flexible Ala- and Pro-rich linkers.[8−11,13−16] The comment in ref 12 that, "A morphological unit consisting of three subunits appears to be important in the assembly of both types of polyhedral forms" (icosahedral or octahedral), prompted us to explore potential mechanistic consequences of this observation.

We here address the question of whether the acetyl group is passed from the LD to the CD in an intra- or interchain reaction by using an *E. coli* E2 component designed with only a single LD (1-lip E2ec), rather than the three LDs in the wild-type enzyme (3-lip E2ec). Earlier, it was shown that the activities of 1-lip E2ec and 3-lip E2ec are virtually the same, while 1-lip E2ec offers advantages for mechanistic studies.[17−19] Two types of constructs of 1-lip E2ec were prepared to test our hypothesis: one in which the lysine on the LD ordinarily carrying the lipoic acid is changed to alanine (K41A) and two others in which the histidine believed to catalyze the transthiolacetylation in the CD is substituted with A or C (H399C or H399A, respectively). The first variant is incompetent toward post-translational ligation of the lipoic acid and, hence, toward reductive acetylation. The other two variants are potentially incompetent toward acetyl-CoA formation by virtue of the absence of the catalytic histidine residue. These constructs then allow us to conduct a complementation experiment: should the acetyl transfer reaction proceed in an intrachain fashion, the two types of constructs should each be inactive either together or individually; however, should acetyl transfer proceed in an interchain fashion, addition of the two types of constructs should produce measurable activity. As a

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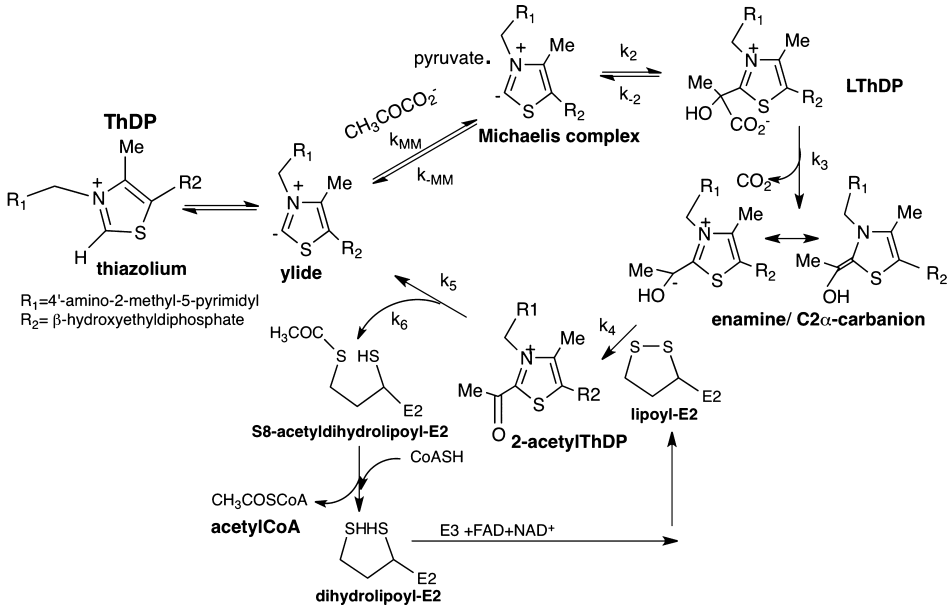
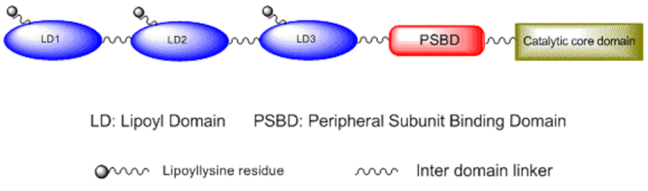
**Scheme 1. Mechanism of the Pyruvate Dehydrogenase Complex**




**Scheme 2. Domain Architecture of Wild-Type E2ec**




control, the K41A/H399C doubly substituted variant was also constructed, carrying neither a lipoylation site nor the active center His. The experimental design for this complementation of E2ec variants is illustrated in Figure 1. We confirmed the results of the kinetics experiments by ascertaining acetyl-CoA formation from CoA using mass spectrometric analysis. The results suggest a plausible model for utilization of multiples of three chains present in E2 components, and for their assembly in the bacterial class of such complexes.

## EXPERIMENTAL PROCEDURES

**Materials.** Thiamin diphosphate (ThDP), $NAD^+$, dithiothreitol (DTT), isopropyl β-D-1-thiogalactopyranoside (IPTG), micrococcal nuclease, deoxyribonuclease I, acetylcoenzyme A (acetyl-CoA), phenylmethanesulfonyl fluoride (PMSF), and

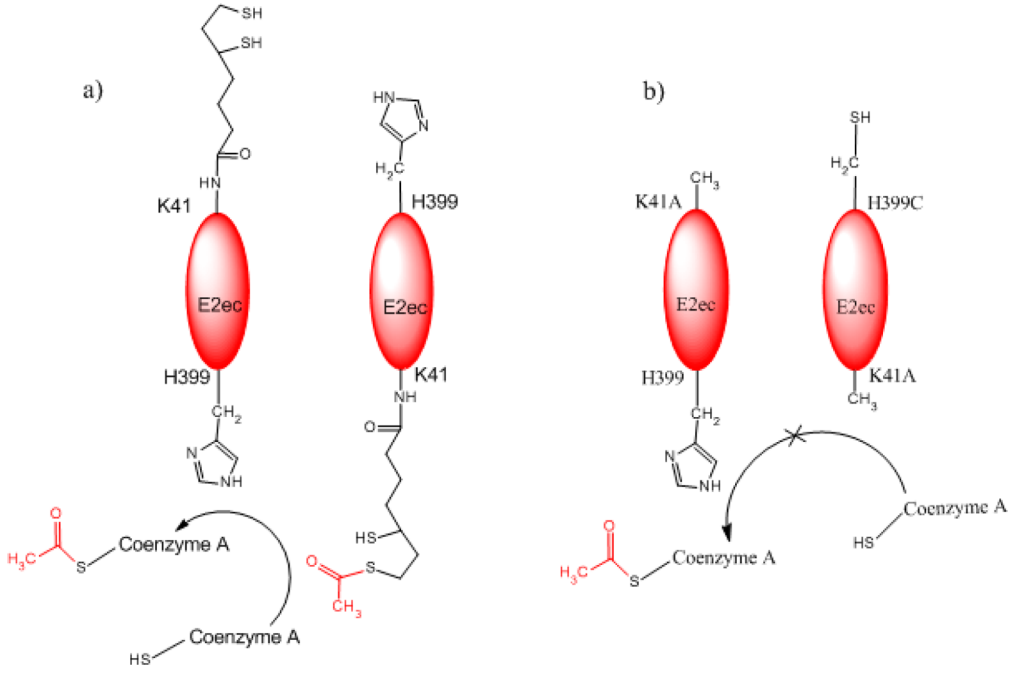



**Figure 1.** Complementation experiments designed to test for interchain acetyl transfer on E2ec. (a) Scheme for monitoring intersubunit acetyl transfer between K41A E2ec and H399C E2ec. (b) Control experiment with the doubly substituted variant (K41A/H399C E2ec). Both the lipoylation site and the transthiolesterification site were modified simultaneously.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coenzyme A (CoA) were from USB (Cleveland, OH). *E. coli* BL21(DE3) cells were from Novagen (EMD Chemicals, Gibbstown, NJ). Lysozyme, formic acid, and methanol as the solvent of mass spectrometry were from Sigma-Aldrich (St. Louis, MO). Protease inhibitor cocktail tablets supplied in glass vials were from Roche Applied Science (Indianapolis, IN)

**Plasmid Purification and Site-Directed Mutagenesis.** The Wizard Plus Minipreps DNA purification system was used for purification of DNA (Promega, Madison, WI). The QuikChange site-directed mutagenesis kit was used for single-site substitution (Stratagene, La Jolla, CA). Mutagenic primers were from IDTdna (Coralville, IA). The following primers were used to create the K41A, H399A, and H399C variants of E2ec (substituted bases underlined) according to methods published by this laboratory:[18,19] K41A, 5′ GATCACCGTAGAAGGCGAC**GC**AGCTTCTATGGAAGTTCCG 3′; H399A, 5′ CTCTCTCCTTCGAC**GC**CCGCGTGATCGACG 3′; H399C, 5′ TCTCTCTCCTTCGAC**TG**CCGCGTGATCGACGG 3′.

**Creation of the K41A/H339C Doubly Substituted Variant.** For a control experiment, the K41A/H399C doubly substituted variant was created. The plasmid of H399C was purified and used for the substitution of K41 with alanine (using the primer from above) to create K41A/H399C, which carries neither the lipoylation site nor the catalytic histidine.

**Protein Expression and Purification.** The procedure for expression and purification of 1-lip E2ec was modified from our previous publication.[18,19] A single colony from a freshly plated cell bank was used to grow the inoculums at 37 °C overnight (five or six culture tubes each with 10 mL of medium and 50 μg/mL ampicillin). Subsequently, it was inoculated in five or six shaker flasks containing 500 mL of LB and 50 μg/mL ampicillin fortified with 0.1 mM DL-α-lipoic acid and incubated at 37 °C and 250 rpm until the $A_{650}$ reached 0.6−0.8. To the culture was then added 1.0 mM IPTG, and the mixture was incubated for an additional 4 h. The cells were harvested by centrifugation (2200*g* for 5 min) and washed with 20 mM $KH_2PO_4$ (pH 7.5) containing 0.10 M NaCl and 0.25 mM EDTA. The washed pellet could be stored below −20 °C until further use. Cells were thawed and resuspended in 50 mL of 20 mM $KH_2PO_4$ buffer (pH 7.5) containing one protease inhibitor cocktail tablet, which also supplies 2 mM EDTA and 0.1 M NaCl. Lysozyme (0.60 mg/mL) and 1 mM PMSF were added, and the cell suspension was incubated for 20 min on ice with intermittent stirring. Cells were then sonicated to disrupt the membrane (total time of 4 min, 10 s on and 10 s off); the lysate was fractionated with $(NH_4)SO_4$ (0−25% and 25−50%), and the 25−50% pellet fraction was collected. After fractionation with $(NH_4)SO_4$, the pellet was dissolved in 20 mM $KH_2PO_4$ dialysis buffer (pH 7.5) containing 1 mM EDTA, 1 mM benzamidine hydrochloride, 1 mM DTT, and 0.2 NaCl and dialyzed against 2 L of dialysis buffer. Dialyzed protein was centrifuged at 28978*g* for 20 min and treated with 1000 units of DNase, 500 units of nuclease, and 5 mM $MgCl_2$ and incubated for 1 h on ice followed by centrifugation at 29000*g* for 20 min. Protein was applied to HiPrep 26 × 60 Sephacryl S-300 high-resolution gel-filtration column pre-equilibrated with 3 column volumes of 20 mM $KH_2PO_4$ column buffer (pH 7.2) containing 0.5 mM EDTA, 1 mM benzamidine hydrochloride, 1 mM DTT, and 0.2 M NaCl at a flow rate of 1.5 mL/min, and the column was run overnight in the cold room at a rate of 0.2 mL/min. Protein was monitored at 280 nm and by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Pure fractions were combined and centrifuged for 4 h at 121000*g*, and then the pellet was resuspended in 1.0 mL of column buffer containing an additional 0.30 M NaCl (total of 0.50 M) and kept on ice in the cold room for 15 h. Undissolved protein was removed by centrifugation (29000*g* for 20 min), and the final purity was judged by SDS−PAGE.

**Measurement of PDHc Activity.** The overall activity of the PDHc consisting of 1-lip E2ec and its variants was measured after reconstitution to form a complex with independently expressed E1ec and E3ec components at 25 °C, and the wild-type E1ec:E2ec:E3ec complex mass ratio was 1:1:1. First, the E2−E3 subcomplex was assembled by preincubating E2ec (100 μg) and E3ec (100 μg) in 1 mL of 20 mM $KH_2PO_4$ (pH 7.5) for 1 h. Next, E1 (10 μg) was added to the mixture of 20 μg of the E2ec−E3ec subcomplex (from the preincubation) in 200 μL of 20 mM $KH_2PO_4$ (pH 7.5), and the mixture was incubated for 10 min. The ovarall activity was measured in 980 μL of reaction medium containing 100 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 2 mM sodium pyruvate, 2.5 mM $NAD^+$, 0.2 mM ThDP, and 2.6 mM DTT, initiating the reaction by addition of 20 μL of CoA (final concentration of 100 μM) and 3 μg of the E1−E2−E3 complex. The overall activities of K41A E2ec, H399A E2ec, and H399C E2ec were monitored with the same mass ratio that was used for the wild-type complex. The pyruvate-dependent reduction of $NAD^+$ was monitored at 340 nm on a Varian DMS 300 spectrophotometer.

To test for interchain acetyl transfer, K41A E2ec and H399C E2ec were incubated at various mass ratios for 30 min prior to the reconstitution with E1ec, E3ec, and pyruvate. Because K41A E2ec carries a catalytic His, which may produce overall activity by the transfer of an acetyl group from lipoyllysine on H399C E2ec, the concentration of K41A was fixed. The K41A E2ec:H399C E2ec mass ratios for incubation were 1:1, 1:1.5, and 1:2. After incubation for 30 min in 20 mM $KH_2PO_4$ (pH 7.0), E1ec and E3ec were introduced into this mixture with a 1:1:1 E1ec:K41A E2ec, H399C E2ec:E3ec mass ratio. In a control experiment, the doubly substituted variant, K41A/H399C E2ec, was used, and the overall activity was again measured upon reconstitution of the complex with E1ec, E3ec, and pyruvate. Then, K41A E2ec and K41A/H399C E2ec were mixed and incubated at the same mass ratio used for K41A and H399C above. The concentration of K41A was fixed again to K41A:K41A/H399C ratios of 1:1, 1:1.5, and 1:2.

To test whether interchain acetyl transfer takes place via E2 chain communication within one (holo)PDHc molecule or communication between E2 chains located on different PDHc molecules, two PDH complexes were prepared, one from K41A E2ec and the other from H399C E2ec. These two types of complexes were then mixed in a 1:1 molar ratio, and NADH production was monitored for 30 min.

**Mass Spectrometric Analysis of Acetyl-CoA Formation.** As an independent measure of the complementation ability of the K41A and H399C E2ec variants, the formation of acetyl-CoA from CoA, starting the reaction with pyruvate, was assessed using an Apex-ultra 7.0 T 70 hybrid FTMS from Bruker Daltonics (Billerica, MA) with an ESI source. Both positive and negative ion modes were tested, and the positive ion mode provided better separation and resolution for acetyl-CoA. For detecting acetyl-CoA formation in the overall PDHc reaction, we used the mass ratios of wild-type and variant E2

**Table 1. Overall PDHc Activities of E2ec Variants with Various Mass Ratios**[a]

| type | E2ec variant | E1ec:E2ec:E3ec mass ratio in overall reaction | overall PDHc activity (%)[b] |
|---|---|---|---|
| single variant | Lys41Ala | 1:1:1 | 2.5 ± 0.2 |
| | His399Cys | 1:1:1 | 2.8 ± 0.2 |
| | His399Ala | 1:1:1 | 5.6 ± 0.3 |
| | Lys41Ala/His399Cys (doubly substituted) | 1:1:1 | 0.9 ± 0.1 |
| mixture of two variants | Lys41Ala:His399Cys (separate variants) | 1:(1:1):1 | 10.3 ± 0.2 |
| | | 1:(1:1.5):1 | 18.3 ± 0.3 |
| | | 1:(1:2):1 | 22.7 ± 0.3 |
| | Lys41Ala:Lys41Ala/His399Cys (singly and doubly substituted) | 1:(1:1):1 | 2.1 ± 0.1 |
| | | 1:(1:1.5):1 | 3.0 ± 0.1 |
| | | 1:(1:2):1 | 3.0 ± 0.1 |

[a]The production of NADH was monitored at 340 nm. All experiments were performed at 25 °C; each experiment performed more than 20 times. [b]The full activity of PDHc assembled from the E1, E2, and E3 components is in the range of 14−17 μmol of NADH produced $min^{-1}$ (mg of complex)$^{-1}$.

components as used in the activity measurements described above.

**Sample Preparation for Fourier Transfer Mass Spectrometry Experiments.** All biochemical experiments were conducted at 25 °C. For the experiment with 1-lip E2ec, a mixture of 100 μg each of E2ec and E3ec was first incubated in 1 mL of 20 mM $KH_2PO_4$ (pH 7.0) for 1 h. Subsequently, 1 μg of E1ec was added to the mixture of 2 μg of the E2ec−E3ec subcomplex (from the previous preincubation) in 980 μL of 0.1 M Tris-HCl (pH 8.0) containing 1 mM $MgCl_2$, 2 mM sodium pyruvate, 2.5 mM $NAD^+$, 0.2 mM ThDP, and 2.6 mM DTT, and the mixture was incubated for 10 min. The reaction was initiated by adding CoA (100 μM), and the mixture was incubated for 10 min. After 10 min, the reaction was quenched by adding a solution of 12.5% trichloroacetic acid in 1 M HCl. [It is important to note that this is not a kinetic experiment; rather, we are measuring the amount of acetyl-CoA formed under the same conditions by different E2 constructs or their mixtures during a fixed time. The reaction in fact is over in 1 min according to the steady state assay, after which the absorbance no longer increases.] Next, protein was removed by centrifugation at 16000*g* for 20 min, and then 50 μL of the clarified sample was mixed with 50 μL of MS running solvent containing 0.1% (v/v) formic acid and 50% MeOH (v/v) in deionized water. Samples were then injected into the ESI-FTMS. Twenty scans were acquired.

**Quantitative Analysis of Acetyl-CoA Production Using the FTMS.** To quantify acetyl-CoA production by the variants, a standard curve was created at a variety of acetyl-CoA concentrations. Samples were prepared as described above.

**Standard Curve for Acetyl-CoA Determination using the ESI-Detected FTMS.** A sample (50 μL) was mixed with 50 μL of MS running solvent containing 0.1% (v/v) formic acid and 50% MeOH (v/v) in deionized water for the FTMS, yielding final concentrations of 1, 5, 10, 20, 30, 40, 50, 100, and 200 μM. These samples were then injected into the FTMS with a syringe pump (rate of 2 μL/min). Twenty scans were acquired. After data had been acquired, Sigmaplot was used to calibrate and plot the data.

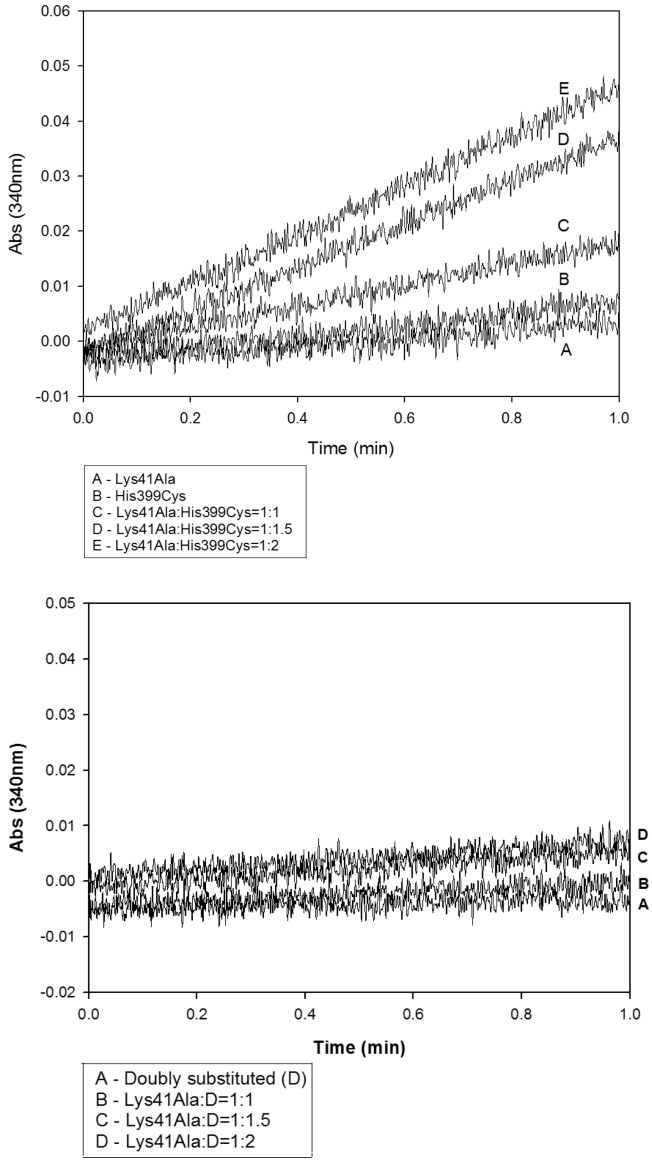


**Figure 2.** Activity assay for E2ec variants reconstituted with E1ec and E3ec. The top panel shows data for mixtures of K41A E2ec and H399C E2ec incubated at various mass ratios; then E1ec and E3ec were added, allowing use of the NADH assay for the overall activity of the complex. In the bottom panel, a fixed concentration of K41A E2ec was incubated with increasing concentrations of K41A/H399C E2ec. Next, E1ec and E3ec were added to assess PDHc activity.

## RESULTS AND DISCUSSION

Substitutions of lipoyl-bearing lysine and the putative catalytic histidine affected the overall PDHc activity. The overall PDHc activity of K41A E2ec was diminished to baseline, while surprisingly, that of H399A E2ec was not completely abolished (Table 1). In contrast to that of H399A E2ec, the overall activity of H399C E2ec was reduced to baseline. The activity was never reduced to zero even with impairment of the lipoylation site and the catalytic His. This could be the result of some endogenous wild-type E2 component present in the *E. coli* cells (the gene for E2 was not knocked out); however, recombinant E2ec variants were all overproduced, and the endogenous


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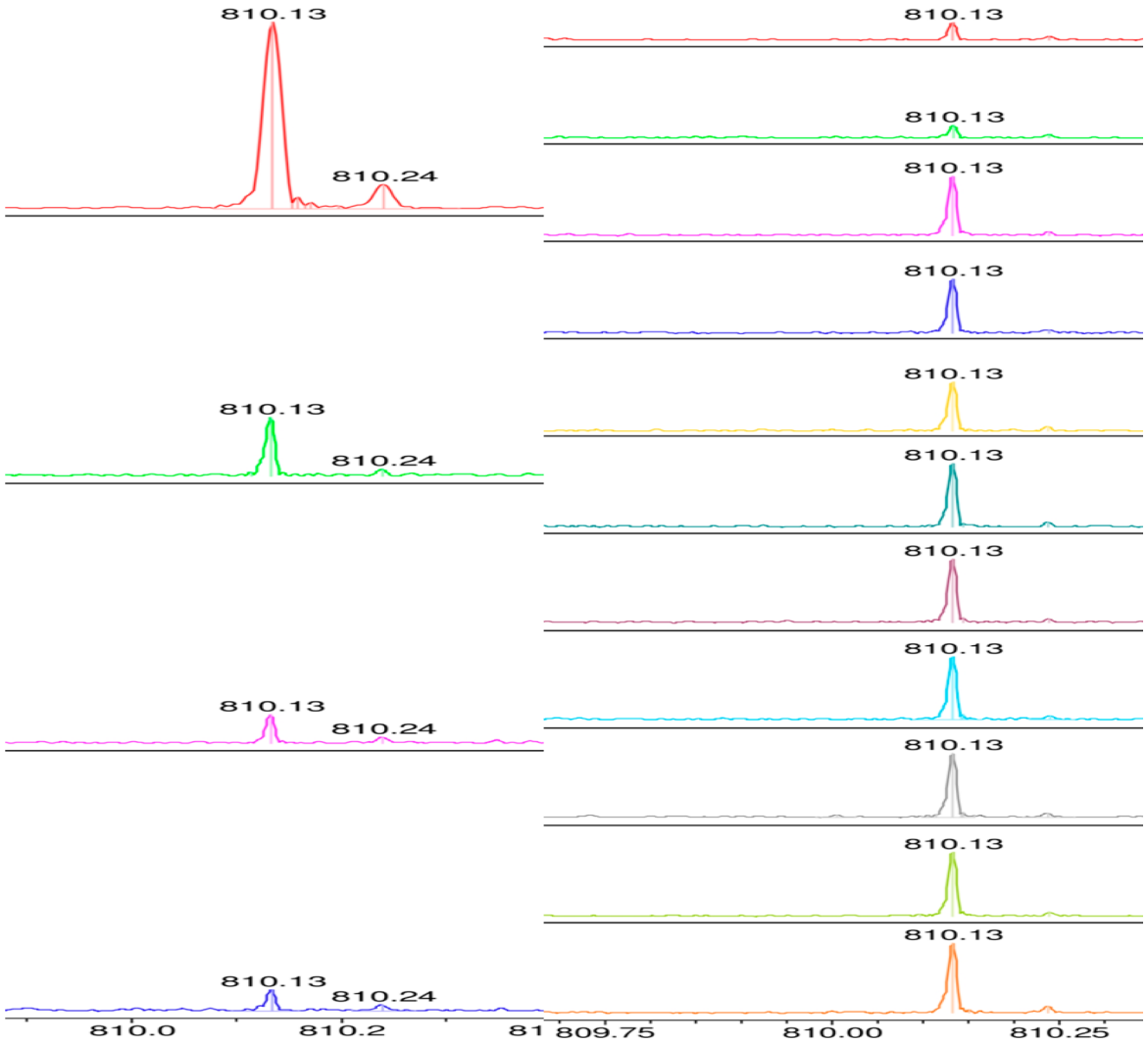


**Figure 3.** Acetyl-CoA produced by E2ec variants detected by the FTMS (left). Acetyl-CoA was observed at $m/z$ 810.13 in different reactions from top to bottom; 1-lip E2ec, H399A E2ec, H399C E2ec, and K41A E2ec. Acetyl-CoA produced by complementation of K41A E2ec and H399C E2ec (right). The amount of acetyl-CoA detected at $m/z$ 810.13 is clearly enhanced by complementation of a fixed K41A E2ec concentration with an increasing concentration of H399C E2ec. From top to bottom: K41A, H399C, and a mixture of K41A and H399C at the indicated mass ratios of 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, and 1:5. The $Y$ axis is arbitrary intensity units, on the same scale on each side.

activity is unlikely to account for a significant fraction of the activity observed. It is also possible that during expression and purification of enzymes, endogenous substances are introduced from the expression host.[20] Nevertheless, the controls below clearly eliminated this issue from consideration.

Upon reconstitution with E1ec and E3ec, K41A E2ec and H399C E2ec displayed diminished activity compared to that of parental 1-lip E2ec (2.5 and 2.8%, respectively), indicating they were nearly totally impaired in terms of overall activity. Elsewhere, extensive mutagenesis studies on E2ec[20,21] also indicated that 2−3% activity is the minimum we can achieve with the strain used for single substitutions, with more activity reduction with multiple substitutions, but when K41A E2ec and H399C E2ec were mixed and incubated (Figure S1 of the Supporting Information) at K41A:H399C mass ratios of 1:1, 1:1.5, and 1:2, reconstituted PDHc activities increased with an increasing concentration of H399C E2ec to a maximum of 22.7%, clearly well above the baseline activity measured with the individual variants (Table 1 and Figure 2, top).

As an important control, we used the K41A/H399C E2ec doubly substituted variant: its preincubation with K41A E2ec followed by reconstitution with E1ec and E3ec (Figure S1 of the Supporting Information) at the same ratio that was used for K41A and H399C; i.e., at K41A:K41A/H399C mass ratios of 1:1, 1:1.5, and 1:2, the overall activity (Table 1 and Figure 2) yielded very low activity once more according to $\Delta A_{340}$/time (Figure 2, bottom; notice significant noise for these measurements). This indicates that there is little if any interchain acetyl transfer from the doubly substituted variant (impaired in both lipoylation and catalytic sites) to the catalytic site of K41A E2ec and ultimately to CoA. Most importantly, the sparingly small activity of this doubly substituted variant provides excellent support for the conclusions drawn regarding enhanced activity observed on complementation of the two singly substituted variants.

Given that each E2ec variant probably exists as a 24-mer, their ability to mix chains had to be established. The overall activity (NADH production) of the mixture of two PDH complexes, one reconstituted from K41A E2ec and the other from H399C E2ec, was nearly zero (2.6%), which is nearly the same as the overall PDHc activity for K41A E2ec by itself (Table 1) after incubation for 30 min. In contrast, preincubation of K41A


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E2ec with H399C E2ec in a 1:1 molar ratio and then reconstitution with E1ec and E3ec produced 10% activity from the inactive state almost immediately upon mixing (Figure S2 of the Supporting Information), and the activity reached a maximum in 25−30 min (data not shown). This result indicated that exchange of E2 chains between preformed PDH complexes, one formed exclusively from K41A E2ec and the other from H399C E2ec, is much slower than exchange of chains between the two E2 oligomers, one formed from K41A E2ec and the other from H399C. Presumably, these 24-mers are produced as soon as the protein is released from the ribosome. Given the central position of the E2 chains in PDHc, the result is perhaps not surprising but needed to be experimentally demonstrated. This experiment is important in the interpretation of the results: it appears that the E2 chains (eight trimers for a total of 24 at the vertexes of a cube according to the octahedral symmetry accepted for this PDHc) are in essence "sequestered" in each complex molecule once assembled from the component enzymes.

Mass spectrometric data provided dramatic independent support for the production of acetyl-CoA in the PDHc reaction by interchain acetyl transfer, in accord with the kinetic activity assay, showing that considerably more NADH was produced on complementation of K41A E2ec and H399C E2ec than with either variant alone. The MS experiments report the amount of acetyl-CoA produced by each variant or a mixture thereof under the same conditions, but not the kinetics of acetyl-CoA formation (see Experimental Procedures). Standard solutions of CoA and acetyl-CoA were prepared and observed at $m/z$ 768.123 and 810.133, respectively (Figure S2 of the Supporting Information). Isotopic patterns of these standards also matched theoretical data. To quantify the concentration of acetyl-CoA produced by E1ec, E2ec, and pyruvate in the PDHc reaction by the FTMS, a standard curve was constructed for acetyl-CoA (1, 5, 10, 20, and 30 $\mu$M), generating a linear plot of intensity versus acetyl-CoA concentration (Figure S3 of the Supporting Information). Excellent goodness of fit of the linear regression assured that it could be used to quantify the acetyl-CoA being produced in the PDHc reaction of all single-site variants (K41A, H399A, and H399C) and mixtures of two variants, K41A and H399C.

1-lip E2ec clearly produced acetyl-CoA at $m/z$ 810.13 (Figure 3, left), while the amount of acetyl-CoA produced by H399A E2ec, H399C E2ec, and K41A E2ec was much smaller. The scales of the $X$ and $Y$ axes are the same in these figures to allow comparison of the acetyl-CoA being produced. Consistent with the NADH assay for PDHc, the H399A E2ec produced much more acetyl-CoA than H399C E2ec or K41A E2ec. Using the standard plot in Figure S3 of the Supporting Information, the concentration of acetyl-CoA produced by E1ec, E2ec (and its variants), E3ec, and pyruvate was quantified. The reaction was started with 100 $\mu$M CoA, yielding the concentrations of acetyl-CoA in parentheses: 1-lip E2ec (26.0 $\mu$M), H399A (12.3 $\mu$M), H399C (0.47 $\mu$M), and K41A (0.03 $\mu$M). Again, the H399C, but not H399A, substitution abolished activity.

Interchain acetyl transfer in the E2 component was next tested by the FTMS. The production of acetyl-CoA with a fixed concentration of K41A E2ec complemented with increasing concentrations of H399C was quantified (Figure 3 right, $X$ and $Y$ axes of all spectra are the same) with K41A E2ec:H399C E2ec mass ratios of 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, and 1:5. Up to 14.8% conversion to acetyl-CoA is in evidence, exceeding the amount produced by the respective variants by themselves by numbers well in excess of the experimental error (Table 2). Under the experimental conditions, at a 1:1 K41A E2ec:H399C E2ec mass ratio, 11.3 $\mu$M acetyl-CoA was produced from 100 $\mu$M CoA, while even a 1:5 mass ratio only produced 14.8 $\mu$M, indicating that the 1:1 mass ratio was sufficient to confirm interchain acetyl transfer between the two E2ec variants. H399A E2ec provides a very good control and also raises a new mechanistic question. On one hand, a comparison of the activities and acetyl-CoA production by H399A E2ec and H399C E2ec supports our use of the latter to demonstrate interchain acetyl transfer within the E2ec component. On the other hand, the same comparison also points out our lack of understanding of the transthiolacetylation mechanism, transfer of the acetyl group from the acetyldihydrolipoyl E2 to CoA at the E2 catalytic center. Should H399 be a crucial catalytic residue, say a general acid−base catalyst, its substitution with alanine should abolish the activity, which is clearly not the case. The H399C E2ec substitution suggests that this cysteine diminishes the activity for acetyl-CoA production to baseline by a hitherto uncharacterized mechanism.

**Table 2. Estimated Quantities of Acetyl-CoA Obtained by Single and Complemented Variants by the FTMS[a]**

| type | substitutions of E2ec | E1ec:E2ec:E3ec mass ratio | acetyl-CoA produced ($\mu$M) |
|---|---|---|---|
| 1-lip E2ec | none | 1:1:1 | 26.0 ± 0.3 |
| single variant | Lys41Ala | 1:1:1 | 0.03 ± 0.01 |
| | His399Cys | 1:1:1 | 0.47 ± 0.02 |
| | His399Ala | 1:1:1 | 12.3 ± 0.2 |
| mixture of two variants | Lys41Ala:His399Cys[b] (separate variants) | 1:(1:1):1 | 11.3 ± 0.03 |
| | | 1:(1:1.5):1 | 11.8 ± 0.05 |
| | | 1:(1:2):1 | 12.1 ± 0.03 |
| | | 1:(1:2.5):1 | 12.7 ± 0.04 |
| | | 1:(1:3):1 | 13.1 ± 0.02 |
| | | 1:(1:3.5):1 | 13.2 ± 0.01 |
| | | 1:(1:4):1 | 13.3 ± 0.03 |
| | | 1:(1:4.5):1 | 13.4 ± 0.02 |
| | | 1:(1:5):1 | 14.8 ± 0.08 |

[a]In all experiments, 100 $\mu$M coenzyme A was used, and all reaction mixtures (after reconstitution) were incubated for 10 min. Quantitative Fourier transform mass spectrometry data have been averaged using 10 independent measurements. [b]Two variants of E2ec were mixed and incubated to monitor the production of acetyl-CoA.

These results collectively strongly support our hypothesis that interchain communication between the lipoyllysine site on H399C and the catalytic site on K41A gave rise to the production of acetyl-CoA upon reconstitution with E1ec, E3ec, and pyruvate.

## CONCLUSION

A complementation experiment was designed to test the hypothesis that acetyl transfer between acetyldihydrolipoyl-E2 on the LD and coenzyme A, presumably at the CD of E2, takes place by an interchain rather than an intrachain mechanism. Both an activity assay and the direct mass spectral measurement of the acetyl-CoA support the interchain acetyl transfer hypothesis. A comparison of the behavior of H399A E2ec with that of H399C E2ec showed that upon complementation

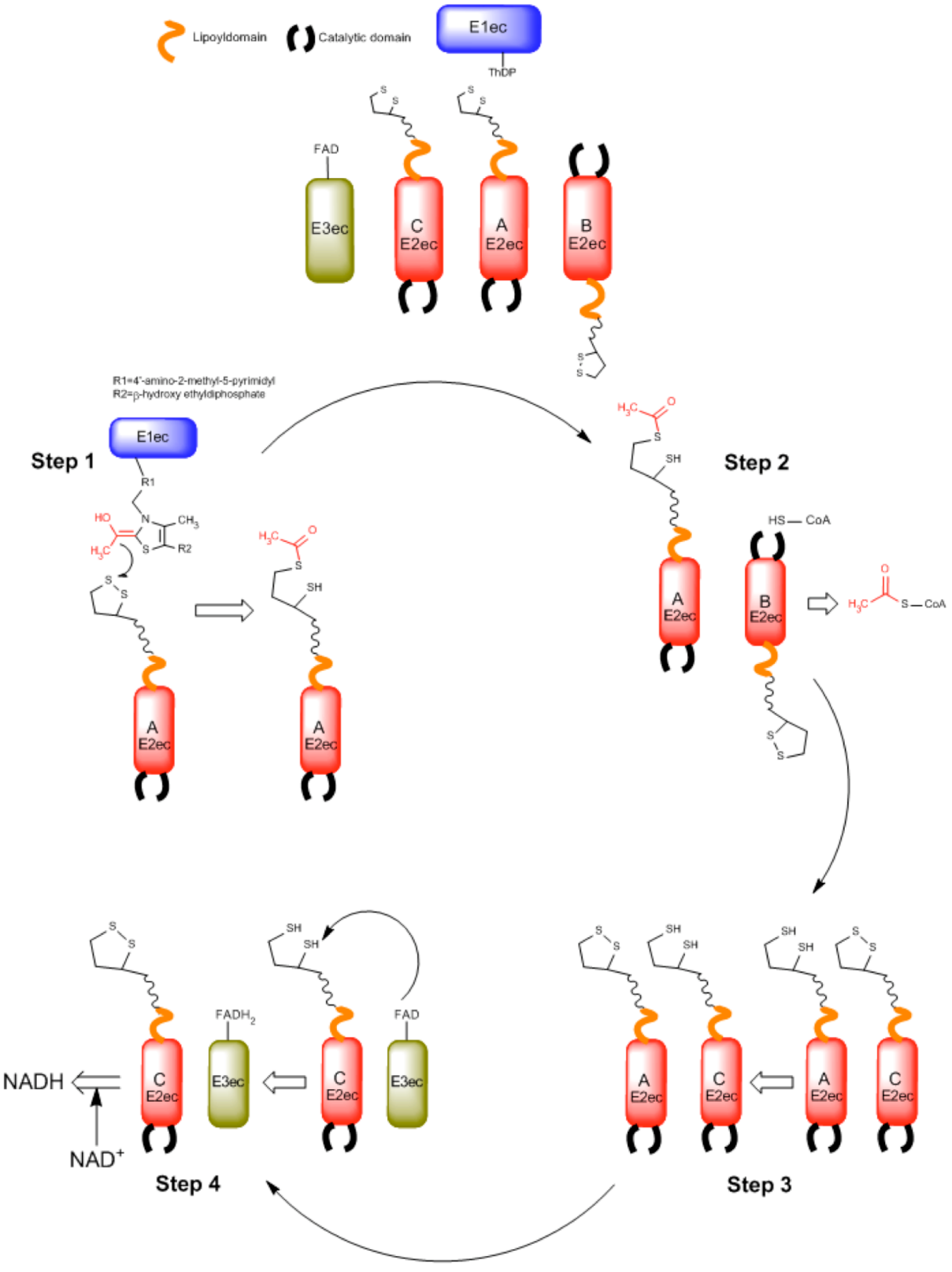


**Figure 4.** Model for utilization and assembly of multiples of three E2ec chains in reductive acetylation and acetyl-CoA formation.

with K41A E2ec, H399A E2ec produced a significantly higher activity and level of acetyl-CoA than did complementation with H399C E2ec. This is consistent with the report that the activity of H602A in 3-lip E2ec (corresponding to H399 in 1-lip E2ec) remained detectable,[16,17] also suggesting the need for further work to elucidate this mechanism.

While the notion of "active site coupling" in such systems has been well substantiated and accepted,[1,13−17] it typically refers to interchain transfer of acetyl groups and reducing equivalents between LDs, whereas in this study, we demonstrate interchain acetyl transfer from a LD to the CD and to coenzyme A. The results prompt us to propose the following tentative


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explanation for the fact that bacterial E2 components exist as multiples of three chains. Let us assume that of the three chains in E2, say chains A, B, and C, chains A and C are in parallel while chain B has an altered orientation such that its active center can be reached by the acetyldihydrolipoyl group of chain A so transthiolacetylation to CoA can take place (Figure 4). In a single turnover according to this model, chain A would be reductively acetylated (Figure 4 step 1) and chain B would accept and then transfer the acetyl group to CoA, not using its lipoyl group at all, while chain A would not use its catalytic center (Figure 4, step 2). Chain C, with an orientation similar to that of A, would not use its catalytic center either; however, it would communicate reducing equivalents between chain A and E3ec (Figure 4, step 3), and chain C would be reoxidized by the E3 component leading to NADH as a final product of the catalytic cycle (Figure 4, step 4). This model also provides an explanation for the finding that E1ec and E3ec compete for overlapping,[13−16] in our experience nonidentical,[20] sites on the PSBD.

It is useful to consider some of the many important contributions prior to this work pointing to the possibility or probability of interchain group transfer on the basis of structural and mechanistic information. (1) The trimeric nature of the core domains has been established since the publication of X-ray structures some years ago (e.g., refs 22 and 23). (2) In the 1-lipoyl E2 construct used here, the importance of the length of the linker connecting the lipoyl domain to the PSBD for efficient active center coupling (hence flexibility vis-à-vis other chains) was shown by Guest and Perham and co-workers.[24] The stiffness of the inner linker regions was implied by NMR studies of peptides corresponding to the linker sequence, suggesting an "extended yet flexible" E2 structure,[25] while for human E2, cryo-electron microscopy suggested flexible N-terminal but less flexible core domains,[26] both papers concluding there are significant distances among E2 chains but also a significant span for reaching the E1 and E3 components. Active site coupling was shown in the *E. coli* and other complexes:[1,13−17,24,27] for example, PDHc activity was retained after release of as much as 50% of either lipoyl domains (by trypsin) or lipoate (by lipoamidase) in the *E. coli* complex,[27] while release of as much as 50% of the lipoyl domains in the human complex had little or no effect on the activity.[28] Different functions for different chains of E2 (one chain engaged with E1, one with E3, and the third with undefined function) were explicitly suggested in Figure 14 of ref 15.

Our work has shown explicitly such interchain acetyl transfer for the *E. coli* E2 component. The transfer among chains of acetyl groups and reducing equivalents had been proposed many years ago, but their rates need further examination.[29] What our results could not do is to clarify whether the acetyl transfer from a dihydrolipoyl group of one chain produces acetyl-CoA at a core domain of a different chain according to an "intratrimer" or "intertrimer" path. However, the experiments did rule out transfer to an adjacent complex. Previously, it was shown by others that E1 chains in this complex exchange at very slow rates ($t_{1/2}$ values of many hours).[29,30] For this complex, for the purposes of such experiments, each complex molecule is sequestered once it is assembled. Our work addresses and provides a tool for solving an issue common in enzymes and proteins: when there are multiple copies of a component, they may serve mechanistically distinct functions in each turnover. We present testable models for further work.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Three figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ABBREVIATIONS

ThDP, thiamin diphosphate; PDHc, pyruvate dehydrogenase complex; E1, first pyruvate dehydrogenase component of PDHc; E2, second dihydrolipoylacetyltransferase component of PDHc; 1-lip E2, single-lipoyl construct of E2 from *E. coli*; 3-lip E2, wild-type three-lipoyl E2 from *E. coli*; E3, third dihydrolipoamide dehydrogenase component of PDHc; LD, lipoyl domain of E2; PSBD, peripheral subunit binding domain of E2; CD, core or catalytic domain of E2; FTMS, Fourier transform mass spectrometer; CoA, coenzyme A.

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