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Inter-chain 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

Jaeyoung Song and **Frank Jordan**
Department of Chemistry, Rutgers University, Newark, NJ 07102

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, E2 and E3, each present in multiple copies. The E2 component forms the core of the complexes, each copy consisting of variable numbers of lipoyl domains (LD, lipoic acid covalently amidated onto a lysine residue), peripheral subunit binding domain (PSBD), and catalytic (or core) domain (CD). The reaction starts with a ThDP-dependent decarboxylation on E1 to an enamine/C2α carbanion, followed by oxidation and acetyl transfer to form S-acetyldihydrolipoamide E2, then transfer of this acetyl group from LD to coenzyme A on CD. The dihydrolipoamide E2 is finally re-oxidized by the E3 component. This report investigates whether the acetyl group is passed from the LD to the CD in an intra- or inter-chain reaction. Using an *E. coli* E2 component having a single LD, two types of constructs were prepared: one has a Lys to Ala substitution on LD at the Lys carrying the lipoic acid, making E2 incompetent towards posttranslational ligation of lipoic acid, hence, towards reductive acetylation; the other in which the His believed to catalyze the transthiolacetylation in the CD is substituted to A or C, the absence of His rendering it incompetent towards acetyl-CoA formation. Both kinetic and mass spectrometric evidence support inter-chain 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-coenzymeA with the assistance of three principal protein components E1, E2 and 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 multi-domain E2 component forms the core of the complexes [see domain structure for E2 from *Escheerichia coli* (E2ec) in Scheme 2]. It consists of variable number (1–3) of LD's 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 the 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 LD and CD, and it is plausible that inter-chain acyl transfer would take place between an LD of one chain and the CD of a different one, with mobility of LD afforded by flexible Ala-Pro-rich linkers(8–11, 13–16). The comment in ref. 12, that "A morphological unit

Frank Jordan: frjordan@rutgers.edu; phone, (973) 353-5470; fax, (973) 353-1264.

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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 inter-chain 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 the 1-lip E2ec offers advantages for mechanistic studies(17–19). Two types of constructs of the 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 trans-thiolacetylation in the CD is substituted to A or C, H399C and H399A. The first variant is incompetent towards post-translational ligation of the lipoic acid, hence towards reductive acetylation. The other two variants are potentially incompetent towards acetyl-CoA formation by virtue of the absence of the catalytic histidine residue. These constructs then enable us to carry out a complementation experiment: should acetyl transfer reaction proceed intra-chain, the two types of constructs should each be inactive either together or individually; however, should acetyl transfer proceed inter-chain, addition of the two types of constructs should produce measurable activity. As 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 acetylCoA 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 coenzyme A (CoA) were from USB (Cleveland, OH). *E. coli* BL21(DE3) cells were from Novagen (Novagen, EMD Chemicals, Gibbstown, NJ). Lysozyme, formic acid and methanol for 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, LA). The following primers were used to create the K41A, H399A and H399C variants of E2ec (substituted bases underlined) according to methods published from this laboratory (18,19):

K41A, 5′ GATCACCGTAGAAGGCGAC**GC**AGCTTCTATGGAAGTTCCG 3′;

H399A, 5′ CTCTCTCCTTCGAC**GC**CCGCGTGATCGACG 3′;

H399C, 5′ TCTCTCTCCTTCGAC**TG**CCGCGTGATCGACGG 3′

Creation of 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 to 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 freshly plated cell bank was used to grow the inoculums at 37 °C overnight (5–6 culture tubes each of 10 mL medium + 50 μg/mL ampicillin). Subsequently, it was inoculated in 5–6 shaker flasks containing 500 mL LB + 50 μg/mL ampicillin fortified with 0.1 mM DL-α-lipoic acid and incubated at 37 °C at 250 rpm until 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 (2 200 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 (4 min total time, 10 s 'ON' and 10 s 'OFF'), and 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 HCl, 1 mM DTT, and 0.2 NaCl and dialyzed against 2 L of dialysis buffer. Dialyzed protein was centrifuged at 28 978 g for 20 min and treated with 1000 U DNAse, 500 U Nuclease, and 5 mM $MgCl_2$ and incubated for 1 h on ice followed by centrifugation at 29 000 g for 20 min. Protein was applied to HiPrep 26x60 Sephacryl S-300 High Resolution gel-filtration column pre-equilibrated with three column volumes of 20 mM KH_2PO_4 column buffer (pH 7.2) containing 0.5 mM EDTA, 1 mM benzamidine HCl, 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 0.2 mL/min. Protein was monitored at 280 nm, and by SDS-PAGE. Pure fractions were combined and centrifuged for 4 h at 121 000 g, and then the pellet was resuspended in 1.0 mL of column buffer containing an additional 0.30 M NaCl (total 0.50 M) and kept on ice in the cold room for 15 h. Undissolved protein was removed by centrifugation (29 000 g for 20 min), and final purity was judged by SDS-PAGE.

Measurement of the PDHc activity

Overall activity of the PDHc consisting of 1-lip E2ec and its variants was measured after reconstitution to complex with independently expressed E1ec and E3ec components at 25 °C, and the mass ratio of wild type E1ec: E2ec: E3ec complex was 1:1:1. First, E2-E3 sub-complex was assembled by pre-incubating 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 E2ec-E3ec sub-complex (from the pre-incubation) 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 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 CoA (100 μM final concentration) and 3 μg of E1-E2-E3 complex. The overall activities of K41A E2ec, H399A E2ec and H399C E2ec were monitored with the same mass ratio as 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 inter-chain 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. Since K41A E2ec carries a catalytic His, which may produce overall activity by acetyl transfer from lipoyl lysine on H399C E2ec, the concentration of K41A was fixed. The mass ratio of E2ec variants of K41A:H399C for incubation was 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 in this mixture with a mass ratio of 1:1:1 for E1ec: K41A E2ec, H399C E2ec: E3ec. In a control experiment, the doubly substituted variant, K41A/H399C E2ec, was used, and the overall activity was again measured on reconstitution of complex with E1ec, E3ec, and pyruvate. Then, K41A E2ec and K41A/H399C E2ec were mixed and incubated in the same mass ratio as K41A:H399C above. The concentration of K41A was fixed again to K41A: K41A/H399C = 1:1, 1:1.5, 1:2.

To test whether inter-chain 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 measured 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 components as used in the activity measurements above.

Sample preparation for FTMS experiments—All biochemical experiments were carried out 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 E2ec-E3ec sub-complex (from the previous pre-incubation) 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 16 000 g for 20 min, and then 50 μL of clarified sample was mixed with 50 μL of MS running solvent containing 0.1 % (v/v) formic acid, 50 % MeOH (v/v) in deionized water. Samples were then injected into ESI-FTMS. 20 scans were acquired.

Quantitative analysis of acetyl-CoA production using FTMS

To quantify acetyl-CoA production by the variants, a standard curve was created at a variety of concentrations of acetyl-CoA. Samples were prepared as above.

Standard curve for acetyl-CoA determination using ESI-detected FTMS—50 μL of 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 for FTMS yielding final concentrations of 1, 5, 10,

20, 30, 40, 50, 100, and 200 μM. These samples were then injected into FTMS by syringe pump (rate = 2 μL/min). 20 scans were acquired. After acquiring data, Sigma plot was used to calibrate and plot the data.

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 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, respectively. 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), but recombinant E2ec variants were all overproduced, and the endogenous 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.

On reconstitution with E1ec and E3ec, the K41A E2ec and H399C E2ec displayed diminished activity compared to parental 1-lip E2ec: 2.5% and 2.8%, respectively, indicating they were nearly totally impaired for overall activities. 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 (Supporting Information Fig. S1) at the mass ratios of K41A:H399C=1:1, 1:1.5, 1:2, reconstituted PDHc activities increased with increasing concentration of H399C E2ec up to a maximum of 22.7%, clearly well above the baseline activity measured with the individual variants (Table 1 and Fig. 2 top).

As an important control, we used the K41A/H399C E2ec doubly substituted variant: its pre-incubation with K41A E2ec followed by reconstitution with E1ec and E3ec (Supporting information Fig. S1) in the same ratio as used for K41A:H399C; i.e., K41A:K41A/H399C=1:1, 1:1.5, 1:2, the overall activity (Table 1 and Fig. 2) yielded very low activity once more according to the ΔA_{340}/time (Figure 2, bottom; notice significant noise for these measurements). This indicates that there is little if any inter-chain acetyl transfer from the doubly substituted variant (impaired in both lipoylation and catalytic sites) to the catalytic site of K41A E2ec and ultimately to Coenzyme A. Most importantly, the sparingly little 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, 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 30 min incubation. In contrast, pre-incubation of K41A E2ec with H399C E2ec in a 1:1 molar ratio, then reconstitution with E1ec and E3ec produced 10 % activity from the inactive state almost immediately on mixing (Supporting information Fig. S2), and reached a maximum activity 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, the other from H399C E2ec, is much slower than is 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 inter-chain 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 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 768.123 m/z and 810.133 m/z, respectively (Supporting information Fig. S2). Isotopic patterns of these standards also matched theoretical data. To quantify concentration of acetyl-CoA produced by E1ec, E2ec and pyruvate in the PDHc reaction by FTMS, a standard curve was constructed for acetyl-CoA (1, 5, 10, 20, and 30 μM), generating a linear plot of intensity vs. acetyl-CoA concentration (Supporting information Fig. S3). 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.

The 1-lip E2ec clearly produced acetyl-CoA at 810.13 m/z (Fig. 3, left), while the acetyl-CoA produced by H399A E2ec, H399C E2ec, and K41A E2ec was much less. The scales of 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 Supporting information Fig. S3, the concentration of acetyl-CoA produced by E1ec, E2ec (and its variants), E3ec, and pyruvate was quantified. The reaction was started with 100 μM of CoA, yielding the concentration of acetyl-CoA (in parentheses): 1-lip E2ec (26.0 μM), H399A (12.3 μM), H399C (0.47 μM), K41A (0.03 μM). Again, the H399C, but not H399A, substitution abolished activity.

Inter-chain acetyl transfer in the E2 component was next tested by FTMS. The production of acetyl-CoA with a fixed concentration of K41A E2ec complemented with increasing concentrations of H399C was quantified (Fig. 3 right, X and Y axis of all spectra are the same) with mass ratio of K41A E2ec to H399C E2ec 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 mass ratio of K41A E2ec to H399C E2ec, 11.3 μM of acetyl-CoA was produced from 100 μM CoA, while even a 1:5 mass ratio only produced 14.8 μM, indicating that the 1:1 mass ratio was sufficient to confirm inter-chain acetyl transfer between the two E2ec variants. The H399A E2ec provides a very good control and also raises a new mechanistic question. On the one hand, a comparison of the activities and acetyl-CoA production by the H399A E2ec and H399C E2ec supports our use of the latter to demonstrate inter-chain acetyl transfer within the E2ec component. On the other hand, the same comparison also points out our lack of understanding of the trans-thiolacetylation mechanism, transfer of the acetyl group from the acetyldihydrolipoylE2 to CoA at the E2 catalytic center. Should H399 be a crucial catalytic residue, say a general acid-base catalyst, its substitution for alanine should abolish the activity, clearly not the case. The H399C E2ec substitution suggests that this cysteine

diminishes the activity for acetyl-CoA production to base line by a hitherto uncharacterized mechanism.

These results collectively strongly support our hypothesis that inter-chain communication between the lipoyllysine site on H399C and the catalytic site on K41A gave rise to the production of acetyl-CoA on 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 inter-chain, rather than an intra-chain mechanism. Both activity assay and direct mass spectral measurement of the acetyl-CoA support the inter-chain acetyl transfer hypothesis. A comparison of the behavior of the H399A E2ec with that of the H399C E2ec showed that on complementation with K41A E2ec, the H399A E2ec produced significantly higher activity and 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) retained detectable activity(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 inter-chain transfer of acetyl groups and reducing equivalents between LD’s, whereas in this study we demonstrate inter-chain acetyl transfer from a LD to the CD and to coenzyme A. The results prompt us to propose the following tentative 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 it its active center can be reached by the acetyldihydrolipoyl group of chain A so transthiolacetylation to Coenzyme A can take place (Fig. 4). In a single turnover according to this model, chain A would be reductively acetylated (Fig. 4 step 1), and chain B would accept, then transfer the acetyl group to Coenzyme A, not using its lipoyl group at all, while chain A would not use its catalytic center (Fig. 4 step 2). Chain C, with similar orientation to A, would not use its catalytic center either, but it would communicate reducing equivalents between chain A and E3ec (Fig. 4 step 3), and chain C would be re-oxidized by the E3 component leading to NADH as a final product of the catalytic cycle (Fig. 4 step 4). This model also provides an explanation for the finding that E1ec and E3ec compete for overlapping(13–16), in our experience non-identical(20), sites on the PSBD.

It is useful to consider some of the many important contributions prior to this work pointing to the possibility/probability of inter-chain group transfer on the basis of structural and mechanistic information. (1) The trimeric nature of the core domains has been established since publication of X-ray structures some years ago (e.g., ref. 22,23). (2) In the 1-lipoyl E2 construct here used, 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 coworkers (24). 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 on the human E2 cry-electron microscopy suggested flexible N-terminal, but less flexible core domains (26), both papers concluding significant distances among E2 chains, but also 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 inter-chain acetyl transfer for the *E. coli* E2 component. The transfer among chains of acetyl groups and reducing equivalents had been proposed many years ago, albeit their rates need further research (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 'intra-trimer' or 'inter-trimer' path. However, the experiments did rule out transfer to an adjacent complex. Earlier it was shown by others, that E1 chains in this complex exchange at very slow rates ($t_{1/2}$ of many hours) (29,30). For this complex, for purposes of such experiments each complex molecule is 'sequestered' once assembled. Our work addresses, and provides a tool to solve an issue common in enzymes/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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbrevations used

ThDP	thiamin diphosphate
PDHc	pyruvate dehydrogenase complex
E1	the first pyruvate dehydrogenase component of PDHc
E2	the second dihydrolipoylacetyltransferase component of PDHc
1-lip E2	the single lipoyl construct of E2 from *E. coli*
3-lip E2	the wild type three lipoyl E2 from *E. coli*
E3	the 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	coenzymeA

References

1. Reed LJ. Multienzyme complexes. Acc Chem Res. 1974; 7:40–46.
2. Patel, MS.; Roche, TE.; Harris, RA. Alpha-keto acid dehydrogenase complexes. Birkhäuser; Basle, Switzerland: 1996.
3. Gopalakrishnan S, Rahmatullah M, Radke GA, Powers-Greenwood S, Roche TE. Role of protein X in the function of the mammalian pyruvate dehydrogenase complex. Biochem Biophys Res Commun. 1989; 160:715–721. [PubMed: 2719694]

4. Neagle J, De Marcucci O, Dunbar B, Lindsay JG. Component X of mammalian pyruvate dehydrogenase complex: structural and functional relationship to the lipoate acetyltransferase (E2) component. FEBS Lett. 1989; 253:11–15. [PubMed: 2759236]
5. Brautigam CA, Wynn RM, Chuang JL, Chuang DT. Subunit and catalytic component stoichiometries of an in vitro reconstituted human pyruvate dehydrogenase complex. J Biol Chem. 2009; 284:13086–13098. [PubMed: 19240034]
6. Hiromasa Y, Fujisawa T, Aso Y, Roche TE. Organization of the cores of the mammalian pyruvate dehydrogenase complex formed by E2 and E2 plus the E3-binding protein and their capacities to bind the E1 and E3 components. J Biol Chem. 2004; 279:6921–6933. [PubMed: 14638692]
7. Vijayakrishnan S, Kelly SM, Gilbert RJ, Callow P, Bhella D, Forsyth T, Lindsay JG, Byron O. Solution structure and characterisation of the human pyruvate dehydrogenase complex core assembly. J Mol Biol. 2010; 399:71–93. [PubMed: 20361979]
8. Eley MH, Namihira G, Hamilton L, Munk P, Reed LJ. α-Keto acid dehydrogenase complexes. 18 Subunit composition of the Escherichia coli pyruvate dehydrogenase complex. Arch Biochem Biophys. 1972; 152:655–669. [PubMed: 4564450]
9. Bleile DM, Munk P, Oliver RM, Reed LJ. Subunit structure of dihydrolipoyl transacetylase component of pyruvate dehydrogenase complex from Escherichia coli. Proc Natl Acad Sci USA. 1979; 76:4385–4389. [PubMed: 388441]
10. Stephens PE, Darlison MG, Lewis HM, Guest JR. The pyruvate dehydrogenase complex of Escherichia coli K12. Nucleotide sequence encoding the dihydrolipoamide acetyltransferase component. Eur J Biochem. 1983; 133:481–489. [PubMed: 6345153]
11. Angelides KJ, Akiyama SK, Hammes GG. Subunit stoichiometry and molecular weight of the pyruvate dehydrogenase multienzyme complex from Escherichia coli. Proc Natl Acad Sci. 1979; 76:3279–3283. [PubMed: 386335]
12. CaJacob CA, Frey PA, Hainfeld JF, Wall JS, Yang H. Escherichia coli pyruvate dehydrogenase complex: particle masses of the complex and component enzymes measured by scanning transmission electron microscopy. Biochemistry. 1985; 24:2425–2431. [PubMed: 3925985]
13. Reed LJ, Hackert ML. Structure-function relationships in dihydrolipoamide acyltransferases. J Biol Chem. 1990; 265:8971–8974. [PubMed: 2188967]
14. Perham RN. Domains, motifs, and linkers in 2-oxo acid dehydrogenase multienzyme complexes: a paradigm in the design of a multifunctional protein. Biochemistry. 1991; 30:8501–8512. [PubMed: 1888719]
15. Perham RN. Swinging arms and swinging domains in multifunctional enzymes: catalytic machines for multistep reactions. Annu Rev Biochem. 2000; 69:961–1004. [PubMed: 10966480]
16. de Kok A, Hengeveld AF, Martin A, Westphal AH. The pyruvate dehydrogenase multi-enzyme complex from Gram-negative bacteria. Biochim Biophys Acta. 1998; 1385:353–366. [PubMed: 9655933]
17. Russell GC, Machado RS, Guest JR. Overproduction of the pyruvate dehydrogenase multienzyme complex of Escherichia coli and site-directed substitutions in the E1p and E2p subunits. Biochem J. 1992; 287(Pt 2):611–619. [PubMed: 1445221]
18. Yi J, Nemeria N, McNally A, Jordan F, Machado RS, Guest JR. Effect of substitutions in the thiamin diphosphate-magnesium fold on the activation of the pyruvate dehydrogenase complex from Escherichia coli by cofactors and substrate. J Biol Chem. 1996; 271:33192–33200. [PubMed: 8969175]
19. Wei W, Li H, Nemeria N, Jordan F. Expression and purification of the dihydrolipoamide acetyltransferase and dihydrolipoamide dehydrogenase subunits of the Escherichia coli pyruvate dehydrogenase multienzyme complex: a mass spectrometric assay for reductive acetylation of dihydrolipoamide acetyltransferase. Protein Expr Purif. 2003; 28:140–150. [PubMed: 12651118]
20. Park, YH. PhD Dissertation. Rutgers University Graduate Faculty; Newark: 2008.
21. Park YH, Wei W, Zhou L, Nemeria N, Jordan F. Amino-terminal residues 1–45 of the Escherichia coli pyruvate dehydrogenase complex E1 subunit interact with the E2 subunit and are required for activity of the complex but not for reductive acetylation of the E2 subunit. Biochemistry. 2004; 43:14037–14046. [PubMed: 15518552]

22. Mattevi A, Obmolova G, Schulze E, Kalk KH, Westphal AH, de Kok A, Hol WG. Atomic structure of the cubic core of the pyruvate dehydrogenase multienzyme complex. Science. 1992; 255:1544–1550. [PubMed: 1549782]

23. Knapp JE, Carroll D, Lawson JE, Ernst SR, Reed LJ, Hackert ML. Expression, Purification, and Structural Analysis of the Trimeric Form of the Catalytic Domain of the Escherichia coli Dihydrolipoamide Succinyltransferase. Protein Science. 2000; 9:37–48. [PubMed: 10739245]

24. Miles JS, Guest JR, Radford SE, Perham RN. Investgation of the Mechanism of Active Site Coupling in the Pyruvate Dehydrogenase Complex of *Escherichia coli* by Protein Engineering. J Mol Biol. 1988; 202:97–106. [PubMed: 3050122]

25. Lengyel JS, Stott KM, Wu X, Brooks BR, Balbo A, Schuck P, Perham RN, Subramaniam S, Milne JL. Extended polypeptide linkers establish the spatial architecture of a pyruvate dehydrogenase multienzyme complex. Structure. 2008; 16:93–103. [PubMed: 18184587]

26. Yu X, Hiromasa Y, Tsen H, Chiu W, Stoops JK, Roche TE, Zhou ZH. Structures of the human yruvate dehydrogenase complex cores: a highly conserved catalytic center with flexible N-terminal domains. Structure. 2008; 16:104–114. [PubMed: 18184588]

27. Stepp LR, Bleile DM, McRorie DK, Pettit FH, Reed LJ. Use of trypsin and lipoamidase to study the role of lipoic acid moieties in the pyruvate and α-ketoglutarate dehydrogenase complexes of *Escherichia coli*. Biochemistry. 1981; 20:4555–4560. [PubMed: 6794598]

28. Rahmatullah M, Radke GA, Andrews PC, Roche TE. Changes in the Core of the Mammalian-Pyruvate Dehydrogenase Complex upon Selective Removal of the Lipoyl Domain from the Transacetylase Component But Not from the Protein X Component. J Biol Chem. 1990; 265:14512–14517. [PubMed: 2167319]

29. Collins JH, Reed LJ. Acyl group and electron pair relay system: A network of interacting lipoyl moieties in the pyruvate and α-ketoglutarate dehydrogenase complexes from Escherichia coli. Proc Natl Acad Sci USA. 1977; 74:4223–4227. [PubMed: 200908]

30. Hale G, Bates DJ, sand Perham RN. Subunit exchange in the pyruvate dehydrogenase complex of *Escherichia coli*. FEBS Lett. 1979; 104:343–346. [PubMed: 383510]

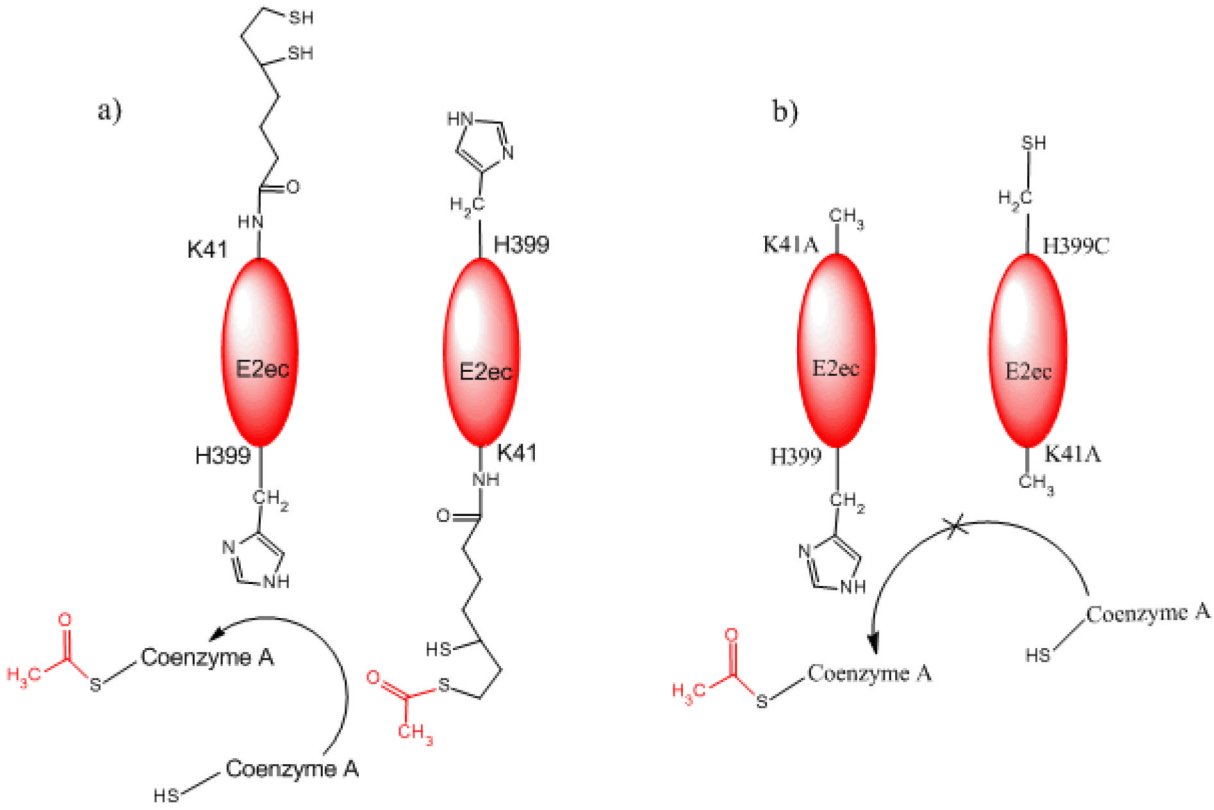


Figure 1.

Complementation experiments designed to test for inter-chain acetyl transfer on E2ec. (a) The experiment to monitor inter-subunit acetyl transfer between K41A E2ec and H399C E2ec. (b) Control experiment with doubly substituted variant (K41A/H399C E2ec). Both the lipoylation site and the transthiolesterification site were modified simultaneously.

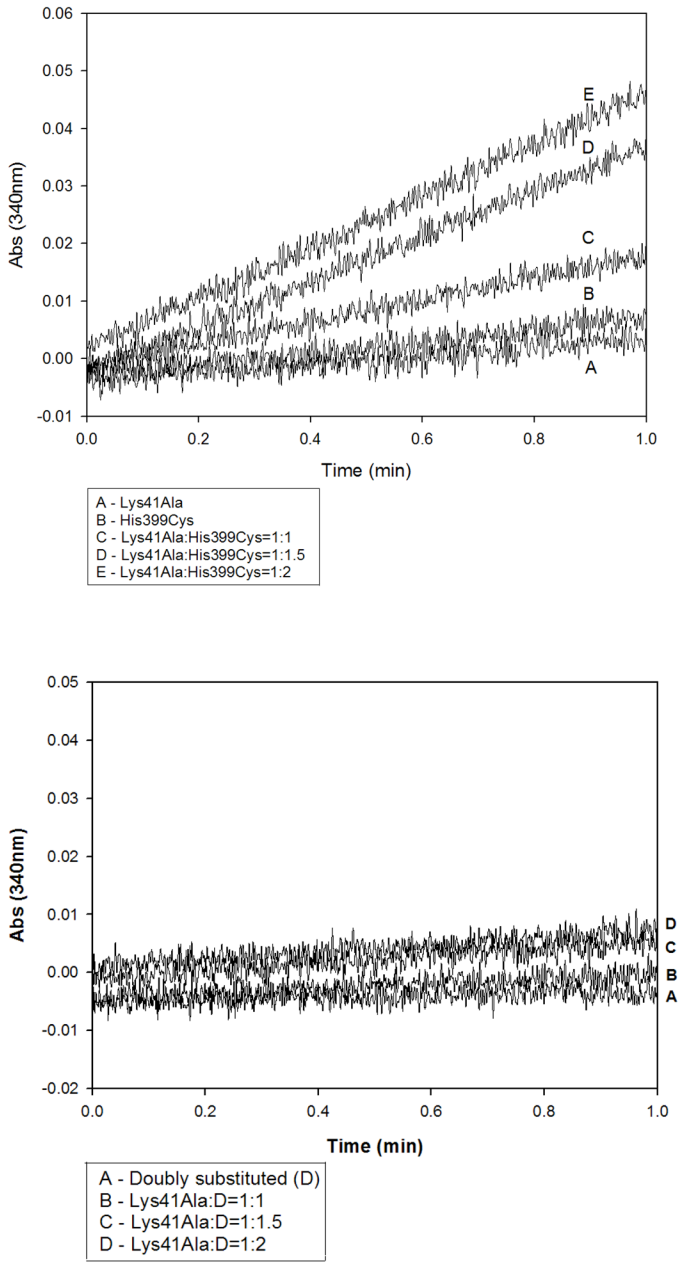


Figure 2.

Activity assay for E2ec variants reconstituted with E1ec and E3ec. Top. Mixtures of K41A E2ec and H399C E2ec in various mass ratios were incubated, then E1ec+E3ec were added enabling use of the NADH assay for overall activity of the complex. Bottom. Fixed concentration of K41A E2ec was incubated with increasing concentrations of K41A/H399C E2ec. Next, E1ec and E3ec were added to assess enable PDHc activity.

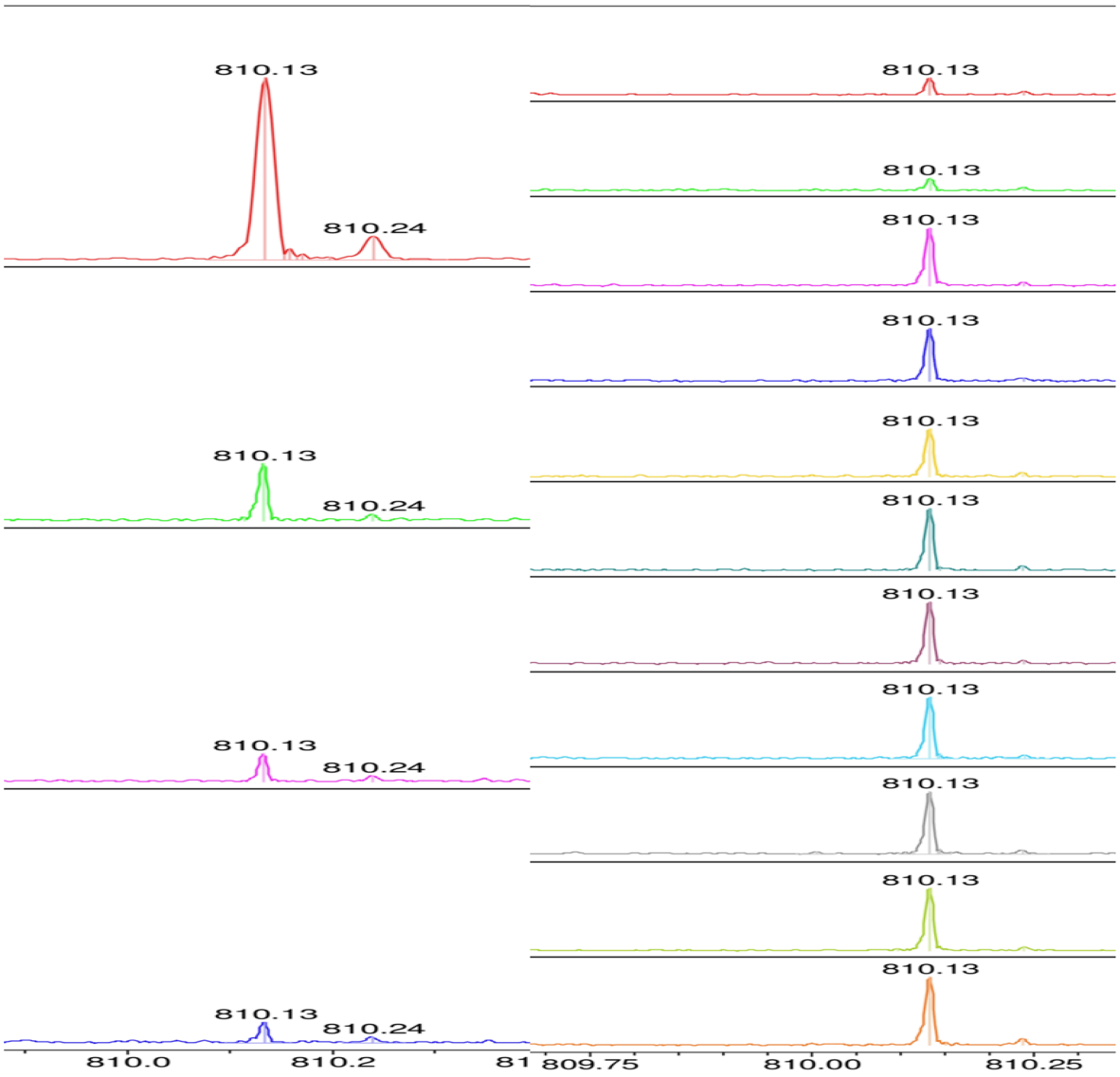


Figure 3.

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

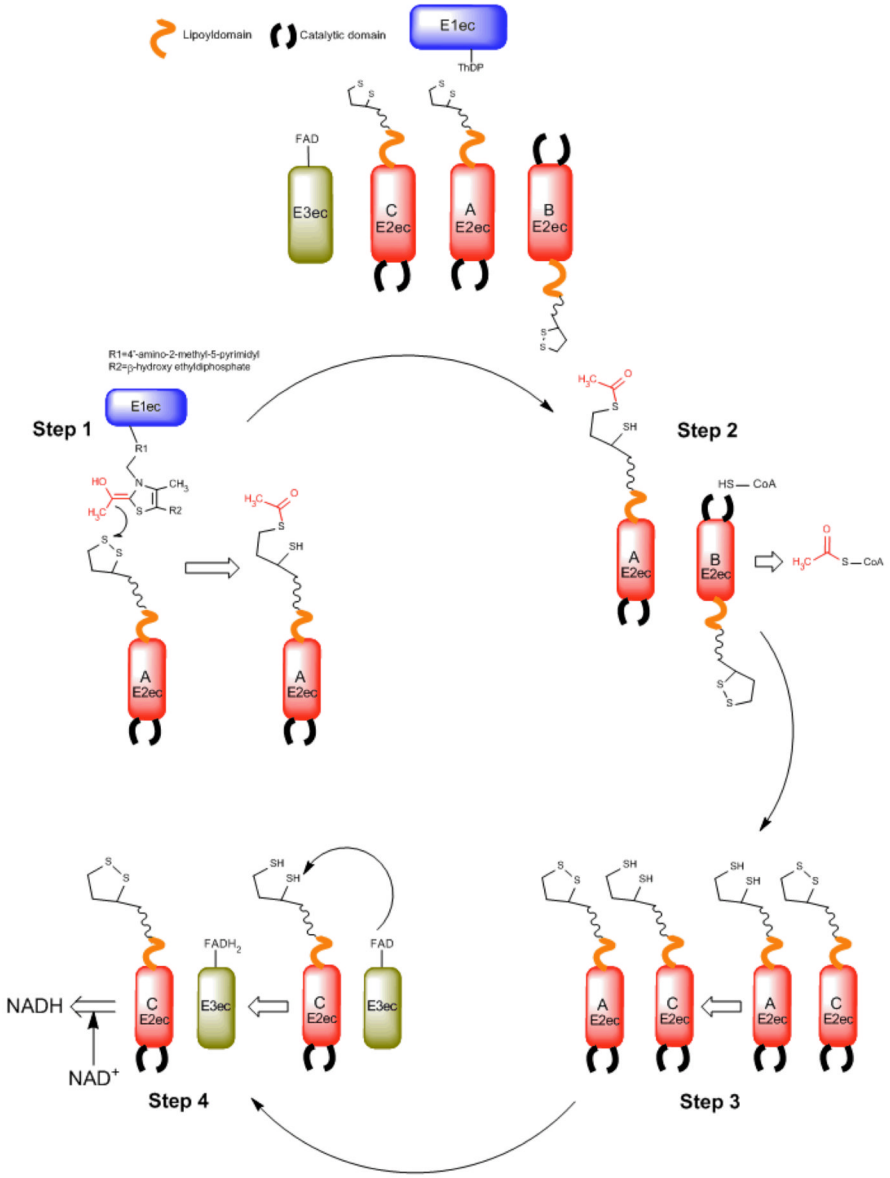
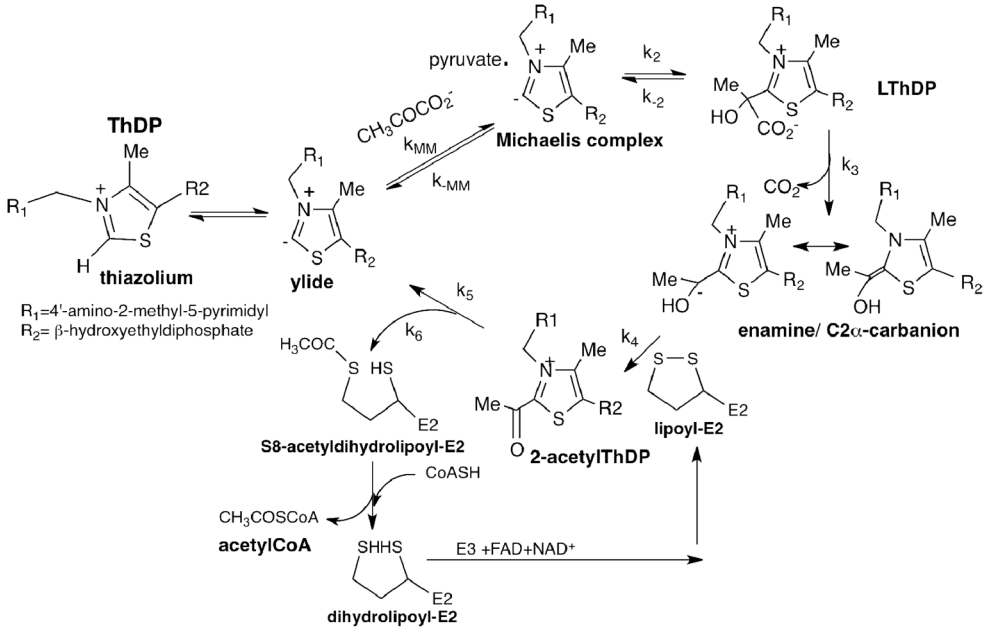
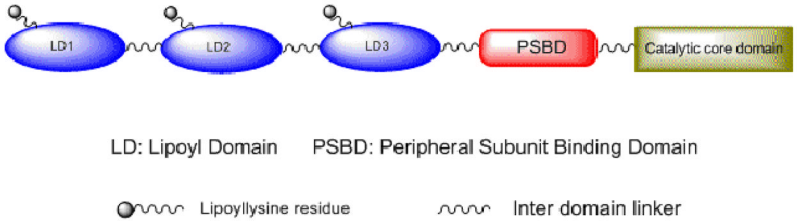


Figure 4.
Model for utilization and assembly of multiples of three E2ec chains in reductive acetylation and acetylCoA formation.



Scheme 1.
Mechanism of pyruvate dehydrogenase complex



Scheme 2.
Domain architecture of wild-type E2ec.

Table 1

Overall PDHc activity of E2ec variants with various mass ratios. The production of NADH was monitored at 340 nm. All experiments were performed at 25 °C, each experiment performed more than 20 times.

Types	E2ec variants	E1ec:E2ec:E3ec mass ratios in overall reaction	Overall PDHc activity (%)[a]
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
Mixtures 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 (single + 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]Full activity of PDHc assembled from the E1, E2 and E3 components is in the range of 14–17 μmoles of NADH produced per min per mg complex.

Table 2

Estimated quantity of acetylCoA obtained by single and complemented variants by FTMS. In all experiments, 100 μM coenzyme A was used, and all reactions (after reconstitution) were incubated for 10 min.

Types	Substitutions of E2ec	E1ec:E2ec:E3ec mass ratios	AcetylCoA produced (μM)
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
Mixtures of two variants	Lys41Ala:His399Cys* (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

*Two variants of E2ec were mixed and incubated to monitor the production of acetylCoA. Quantitative FTMS data have been averaged using 10 independent measurements.