A Dynamic Loop at the Active Center of the *Escherichia coli* Pyruvate Dehydrogenase Complex E1 Component Modulates Substrate Utilization and Chemical Communication with the E2 Component

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Our crystallographic studies have shown that two active center loops (an inner loop formed by residues 401–413 and outer loop formed by residues 541–557) of the E1 component of the *Escherichia coli* pyruvate dehydrogenase complex become organized only on binding a substrate analog that is capable of forming a stable thiamin diphosphate-bound covalent intermediate. We showed that residue His-407 on the inner loop has a key role in the mechanism, especially in the reductive acetylation of the *E. coli* dihydrolipoamide transacetylase component, whereas crystallographic results showed a result of this residue in a disorder-order transition of these two loops, and the ordered conformation gives rise to numerous new contacts between the inner loop and the active center. We present mapping of the conserved residues on the inner loop. Kinetic, spectroscopic, and crystallographic studies on some inner loop variants led us to conclude that sites residues flanking His-407 are important for stabilization/ordering of the inner loop thereby facilitating completion of the active site. The results further suggest that a disorder to order transition of the dynamic inner loop is essential for substrate entry to the active site, for sequestering active site chemistry from undesirable side reactions, as well as for communication between the E1 and E2 components of the *E. coli* pyruvate dehydrogenase multienzyme complex.

The *Escherichia coli* pyruvate dehydrogenase multienzyme complex is comprised of the following three enzyme components: E1ec2 (24 copies, 99,474 Da), E2ec (24 copies, 65,959 Da), and E3ec (12 copies, 50,554 Da) (1–3). The complex catalyzes the oxidative decarboxylation of pyruvate according to Equation 1 (4),

\[
\text{CH}_3\text{COCOO}^- + \text{NAD}^+ + \text{CoASH} \rightarrow \text{CH}_3\text{COSCoA} + \text{CO}_2 + \text{NADH} \quad (\text{Eq. 1})
\]

The components catalyze parts of the overall reaction (5–7) as shown in Equations 2–6,

\[
\begin{align*}
\text{pyruvate} + \text{E1ec-ThDP-Mg}^{2+} & \rightarrow \\
\text{E1ec-hydroxyethylidene-ThDP-Mg}^{2+} + \text{CO}_2 & \quad (\text{Eq. 2}) \\
\text{E1ec-hydroxyethylidene-ThDP-Mg}^{2+} + \text{E2ec-lipoamide} & \rightarrow \\
\text{E1ec-ThDP-Mg}^{2+} + \text{E2ec-acetyl-dihydrolipoamide} & \quad (\text{Eq. 3}) \\
\text{E2ec-acetyl-dihydrolipoamide} + \text{CoA} & \rightarrow \\
\text{E2ec-dihydrolipoamide} + \text{acyl-CoA} & \quad (\text{Eq. 4}) \\
\text{E2ec-dihydrolipoamide} + \text{E3ec-FAD} & \rightarrow \\
\text{E2ec-lipoamide} + \text{E3-FADH}_2 & \quad (\text{Eq. 5}) \\
\text{E3-FADH}_2 + \text{NAD}^+ & \rightarrow \text{E3ec-FAD} + \text{NADH} + \text{H}^+ & \quad (\text{Eq. 6})
\end{align*}
\]

The E1ec catalyzes thiamin diphosphate (ThDP)-dependent decarboxylation of pyruvate whose product reductively acetylates the lipoamide group of E2ec (supplemental Scheme I). The crystal structure of E1ec with ThDP bound revealed the absence of defined electron density for regions encompassing residues 1–55, 401–413, and 541–557 (8). The first of these regions, the N-terminal one, was shown to interact with E2ec (9). A BLAST search and structure alignment of E1ec with transketolase suggested that a highly conserved residue His-407 of the 401–413 disordered loop (henceforth the “inner” loop)
occupies space similar to that proposed for the catalytically important residue His-263 in transketolase. Guided by these modeling studies, we subsequently showed that the residue His-407 has an important role in the reductive acetylation of E2ec, i.e. in inter-component communication (10). It was exciting to discover that in the crystal structure of E1ec in complex with phosphonolactyl-ThDP (PLThDP), a stable analog of LThDP in supplemental Scheme I, both the inner and the outer loop (residues 541–557) become organized (11). From this latter structure many interactions between loop residues and between bound PLThDP and loop residues are seen (Fig. 1A). This study gives structural insights into the role of the His-407 residue and of the inner loop bearing it, not seen in the E1ec-ThDP complex because of disorder of the loops. A BLAST search and sequence alignment showed that residues on the inner loop are among conserved residues in E1ec and the E1 component of other bacterial 2-oxoacid dehydrogenase complexes with octahedral symmetry (Fig. 2). On the basis of these findings, we set out to investigate potential roles (if any) of conserved loop residues, particularly their effect on loop dynamics, catalysis, and E1ec:E2ec active center communication. Site-directed mutagenesis and various spectroscopic approaches revealed that charged residues on both termini of the inner loop contribute to its stability. Whereas Asn-404 has an important role in outer loop stability (11), Gln-408 might contribute toward intermediate stability. The studies provide strong biochemical evidence regarding the role of the inner loop in covalent addition of substrate to ThDP and specificity and in sequestering the active site from solvent. The experiments also provided an opportunity to test our own hypothesis regarding active center communication between E1ec and E2ec in the complex (8, 10, 11).

EXPERIMENTAL PROCEDURES

Materials—Thiamin diphosphate, NAD⁺, coenzyme A, dithiothreitol, and isopropyl 1-thio-β-D-galactopyranoside were from U. S. Biochemical Corp., and DCPIP was from Sigma. The QuikChange® site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Wizard® Plus Minipreps DNA purification system was from Promega (Madison WI).

Bacteria and Plasmids—E. coli strain JRG 3456 deficient in native E1(ec) gene was transformed with pGS878 plasmid containing aceE gene encoding the E1ec and was used for overexpression and site-directed mutagenesis (12). The pET-22b (+)-1-lipooyl E2ec vector transformed in E. coli BL21(DE3) cells was used for overexpression of 1-lipooyl domain E2ec (1-lip E2ec) (13). This single lipooyl E2ec construct is virtually indistinguishable in its biochemical behavior from the three-lipooyl wild type E2ec and is mechanistically useful.

Plasmid Isolation and Site-directed Mutagenesis—Plasmid purification was carried out as per protocol for Wizard® Plus Minipreps DNA purification system. Mutagenesis was per-
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I] ODP1_ECOLI 394 YGMGDAAGKNTAHDQHKMN 413
ODP1_MYCTU 395 YLGKHEGKNATHQHKLLT 414
ODP1_HAEIN 393 YKIE-EASHQHKDQKES 411
Q8EJ25_PSEPK 388 YGTG-AGEAATNTKVD 406
A1ECF5_VIBCH 393 YGMDAAGKHSAHQQKMMD 412
II] A1FM88_PSEPU 388 YGTG-AGEAATNTKVD 408
Q1B615_MYCSS 419 YTGKHEGKNATHQKEL 438
Q4IUH0_AZOV1 396 YGTG-AGEAATNTKVD 416

**FIGURE 2.** Representative sequence alignments of homologous (αj) (I) pyruvate dehydrogenase complex E1 components and (II) 2-oxo acid dehydrogenase complex E1 components from various sources with *E. coli* E1ec active site inner loop. Sequence identities for the sequences used are as follows: ODP1_ECOLI (P0AFGB8 E. coli); ODP1_MYCTU (Q10504) Mycobacterium tuberculosis; ODP1_HAEIN (P45119) Haemophilus influenzae; Q8QZ25_PSEPK Pseudomonas putida (strain KT2440); A1ECPC_VIBCH Vibrio cholerae (V52); A1FM88_PSEPU Pseudomonas putida (W619); Q1B6LS5_MYCSS Mycobacterium sp. (strain MCS); and Q4IUH0_AZOV1 A. vinelandii AvOP. Conserved residues from the loop are shown in boldface type.

formed using the QuikChange® site-directed mutagenesis kit. PCR was carried out for 16 cycles using an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany). The following mutagenic primers were used to substitute single amino acids on wild type E1ec (substituted bases underlined):

- Q408A, 5’-GATAAAACATCGCGACCCGCGTTAAGAAAATGACATGG-3’; E401A, 5’-GACGCCGCTCAGGTTAACATCGCGC3’-3’; E401K, 5’-GACGCCGCTAAGGTTAACATCGCGC-3’; K403E, 5’-GCGGCTGAAGGTTAACATCGCGC-3’; K404E, 5’-GCGGCTGAAGGTTAACATCGCGC-3’; N404A, 5’-GGCTGAAGGTTAACATCGCGC-3’; K410A, 5’-GGCCACCCAGTTGCGAATGAAATGACATGGACGG-3’; K410E, 5’-GGCCACCCAGTTGCGAATGAAATGACATGGACGG-3’; K411A, 5’-GGCCACCCAGTTGCGAATGAAATGACATGGACGG-3’; and K411E, 5’-GGCCACCCAGTTGCGAATGAAATGACATGGACGG-3’.

**Protein Expression and Purification**—Procedure for expression and purification of wild type E1ec and all its singly substituted variants was described elsewhere (9). Additional purification to homogeneity, if required, was performed by applying the protein on a TSK DEAE-5PW HPLC column and eluting with a linear gradient (0.075–0.5M) of NaCl in 20 mM KH2PO4 buffer (pH 7.5). All proteins used were >98% pure as judged by SDS-PAGE and densitometry.

**Activity Measurements**—Activities of wild type E1ec and variants were measured either by reconstitution with independently expressed E2ec and E3ec subunits (12) or with 3-lipoyl domain E2ec obtained from National BioResource Project (National Institute of Genetics, Shizukuoka, Japan) (14) and E3ec. The E1ec subunit-specific activity was determined by monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm (15). Percent activities reported for variants are compared with similarly treated E1ec. All activity measurements were carried out at 30 °C.

**Circular Dichroism Spectroscopy**—CD spectroscopy was used to determine the dissociation constants of substrate analog methyl acetyl phosphate (MAP) (Kd(MAP)) for E1ec and loop variants. Spectra were recorded on an Aviv model 202 CD spectrometer or Chirascan CD spectrometer (Applied Photochemistry) at 30 °C as described previously (15) with minor modifications. Briefly, a 1-cm path length quartz cuvette was used for the near-UV region (250–400 nm). The instrument was calibrated using (+)-10-camphorsulfonic acid as a reference standard. ThDP was removed prior to the experiment by chromatographing E1ec and its variants on a Sephadex G-25 column equilibrated with 10 mM KH2PO4, pH 7.0. For recording CD spectra, 1.0–1.8 mg/ml of E1ec and the variants were dissolved in 10 mM KH2PO4 (pH 7.0) containing 1.0 mM MgCl2, 0.2 mM ThDP and titrated with variable concentrations of MAP. Data were collected at a wavelength step of 1.0 nm, an integration time of 1 s, and a bandwidth of 1 nm. Data preparation, fitting, and calculations were done using Sigmaplot (Systat Software) as described previously (15). Carboligation products were analyzed according to an NMR method described elsewhere (16).

Temperature dependence of Kd (Kd = 1/Kd(MAP)) was used to calculate thermodynamic parameters for substrate analog binding. Nonlinear fitting was done using nonlinear integrated van’t Hoff equation (17).

The apparent values of the free energy ΔG, enthalpy ΔH, entropy ΔS, and the heat capacity change ΔCp of the binding reaction were calculated using Equations 7–11, respectively.

\[
\ln K_d = a + b (1/T) + c \ln T \quad \text{(Eq. 7)}
\]

\[
\Delta G = -RT \ln K_d \quad \text{(Eq. 8)}
\]

\[
\Delta H = R (cT - b) \quad \text{(Eq. 9)}
\]

\[
\Delta S = (\Delta H - \Delta G)/T \quad \text{(Eq. 10)}
\]

\[
\Delta C_p = Rc \quad \text{(Eq. 11)}
\]

**Fluorescence Measurements**—The binding affinity of ThDP to E1ec and variants was measured by quenching of the intrinsic protein fluorescence by the coenzyme as described previously (15).

**Mass Spectrometric Determination of Reductive Acetylation**—Reductive acetylation of independently expressed lipoyl domain by the E1ec or its variants and pyruvate was monitored by incubating the protein (0.1 or 0.5 μM) in 20 mM HEPES (pH 7.5) containing 2.0 mM MgCl2, 2.0 mM pyruvate, and 0.6 mM lipoyl domain in a total volume of 0.10 ml at 25 °C. Aliquots (0.5 μl) were withdrawn at different times and were mixed with 0.5 μM of α-cyano-4-hydroxy-cinnamic acid matrix on the target plate. Mass spectra were acquired on a 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA). The spectrometer was calibrated using ubiquitin (8566 Da) and lipoyl domain (8975 Da) as mass standards.

**Chiral Gas Chromatography**—To analyze the carboligation side products and their enantiomeric excess, chiral gas chromatography (GC) was used (18). The carboligation reaction was
initiated by adding 8.0 mM pyruvate to 10.0 mM KH₂PO₄ (pH 7.0) containing 0.2 mM ThDP, 1.0 mM Mg²⁺, and 30.0 μM E1ec or its variants and incubated overnight at 4 °C. Acetoin from the supernatant of carboligation reaction was extracted with chloroform and injected into the Chiladex B-DM chiral column on an HP 5950 gas chromatograph (Astece, Advanced Separation Technologies, Inc.) equipped with a flame ionization detector at a flow rate of 1.2 ml/min. Analysis was carried out at 70 °C. A 20-μl aliquot of sample was injected with a 100:1 split ratio.

Because this column interacts more favorably with the (S)-enantiotomer thus delaying its retention time, we could readily assign the enantiomers. Acetolactate, however, could not be analyzed with this method because it would be decarboxylated under GC conditions.

**Crystallographic and Data Collection**—The apo-E401K variant of E1ec was co-crystallized separately with ThDP and PLThDP in the presence of Mg²⁺ by the sitting drop vapor diffusion method as described previously for E1ec (8). The best crystals were obtained at a reservoir solution with 15–20% PEG2000 monomethyl ether, 10% propanol, 0.2% Na₂HPO₄ in 60 mM HEPES buffer (pH 7.05). Drops were 6–10 μl consisting of equal parts of reservoir and protein solution. The crystals grew within 4–6 weeks at 22 °C and were isomorphous to crystals of E1ec complexed with ThDP (8). Synchrotron x-ray data sets for E401K-ThDP and E401K-PLThDP complexes were collected from single crystals flash-cooled at -180 °C at SERCAT (sector 22, ID line, Advanced Photon Source, Argonne National Laboratory). All data sets were processed with the HKL2000 package (19). The data sets were truncated at 1.85 and 1.77 Å resolution for the E401K-ThDP and E401K-PLThDP crystals, respectively.

**Model Building and Refinement**—The previous E1ec-ThDP structure, refined to 1.85 Å resolution (8), was used as the starting model for both structure determinations. The initial model included 1602 amino acids without ThDP. Both variant structures were refined using the program CNS (20). Refinements were carried out first keeping the entire α₂ homodimer as a rigid body, and later simulated annealing was performed with a random subset of all data (~5%) set aside for calculation of R_free without imposing any noncrystallographic symmetry. After refinements of the protein parts were complete, 2Fo − Fc composite-simulated annealing omit maps were calculated. The maps revealed the locations of the cofactors ThDP in the E401K-ThDP complex and intermediate analogs PLThDP in the E401K-PLThDP complex. After including the ligands, the models were refined by simulated annealing. Further model building, water-molecule picking, and refinement cycles resulted in R factors of 21.8% for the E401K-ThDP complex and 23.2% for the E401K-PLThDP complex. Inspection of electron density maps, model building, and structural comparisons were carried out using the program O (21). Graphical representations of protein models were generated by using the program RIBBONS (22). Data collection and refinement statistics are given in Table 1. Coordinates and structure factors have been deposited in the Protein Data Bank with the access codes 2QTA and 2QTC for the E401K-ThDP and E401K-PLThDP complexes, respectively.

**RESULTS**

Substitutions of Inner Loop Residues Affect Activities to Varying Degrees—Substitution of the amino acids of the inner loop to alanine did not alter the overall activity of the complex significantly, with the exception of His-407 discussed earlier (10, 11) (Table 2). Substitution with charge-reversed residues, however, led to appreciable reduction in overall activity, especially toward the N-terminal end of the loop. The E1-specific (DCPIP) activities followed a similar trend; charge reversal reduced the activity more than alanine substitution and especially toward lower numbered amino acids. The reduction in E1-specific activity is always smaller than in the overall activity for all variants. A similar change in activity was observed in H407A and H407C (on the cysteineless variants of E1ec; data not shown). Although His-407 and Gln-408 form hydrogen bonds with PLThDP (the latter indirectly through water), and both of them are also in the active site cleft (11), the Q408A substitution has very little effect on overall activity and none on DCPIP activity. The k_cat values followed the same trend as the specific activity, decreasing by as much as 177-fold in charge reversal variants.

K_m and K_d Values for ThDP and Pyruvate Binding—The K_m(ThDP) values (Table 2) were not very different from that for E1ec except for some charge-reversed variants. The latter could not be analyzed by this method because of their low activity. Binding of ThDP according to fluorescence quenching experiments yielded similar K_m(ThDP) values for E1ec and all inner loop variants. However, curve fitting to a Hill equation resulted in a Hill coefficient (n_H) ≥ 2 for K410A (n_H = 2.08), K411A (n_H = 2.39) and K410E (n_H = 2.16), K411E (n_H = 2.68) variants (compared with n_H = 1.06 for E1ec) indicating that these substitutions induced strong positive cooperativity for ThDP binding. The K_m(pyruvate) increased slightly in the E401A and K403A variants, but k_cat/K_m(pyruvate) decreased signif-

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**TABLE 1**

| Crystallographic data and refinement statistics | Apo-E401K + ThDP | Apo-E401K + PLThDP |
|-----------------------------------------------|-----------------|--------------------|
| **Wavelength (Å)**                            | 1.00            | 1.00               |
| **Space group**                               | P2_1            | P2_1               |
| **Unit cell**                                 |                 |                    |
| a                                             | 81.81 Å         | 81.61 Å            |
| b                                             | 141.83 Å        | 142.11 Å           |
| c                                             | 82.56 Å         | 82.14 Å            |
| β                                             | 102.01°         | 102.68°            |
| Resolution                                    | 1.85 Å          | 1.77 Å             |
| Completeness (last shell)                     | 97.1 (91.5)     | 90.6 (76.4)        |
| Total reflections                             | 151,931         | 160,246            |
| Rmerge                                        | 0.075           | 0.100              |
| **Refinement statistics**                     |                 |                    |
| Resolution range                              | 50–1.85 Å       | 50–1.77 Å          |
| R-factor (last shell)                         | 0.218 (0.255)   | 0.232 (0.308)      |
| R_free (last shell)                           | 0.242 (0.281)   | 0.264 (0.339)      |
| No. of residues                               | 1602            | 1602               |
| No. of waters                                 | 413             | 394                |
| **Average β factor (Å)**                      |                 |                    |
| Main chain                                    | 18.61           | 20.65              |
| Side chain                                    | 18.90           | 20.88              |
| Solvent atoms                                 | 18.70           | 21.34              |
| **Root mean square deviations**               |                 |                    |
| Bond lengths                                  | 0.010 Å         | 0.010 Å            |
| Bond angles                                   | 1.5°            | 1.6°               |
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TABLE 2

Activity and binding kinetics of substrate pyruvate and coenzyme ThDP for 401–413 loop variants

For DCPIP activity, monitoring the reduction of 2,6-dichlorophenolindophenol was at 600 nm. 1 unit of activity = 1 μmol of DCPIP reduced per min/mg E1ec. For overall activity (pyruvate:NAD\(^{+}\) oxidoreductase activity), monitoring the production of NADH was at 340 nm. 1 unit of activity = 1 μmol of NAD\(^{+}\) reduced per min/mg E1ec. The DCPIP and overall activities for E1ec were 21.93 ± 2.13 and 0.52 ± 0.032 units, respectively.

| Substitution | Activity* | Activity | Activity | Activity | Activity | Activity |
|--------------|-----------|----------|----------|----------|----------|----------|
|              | DCPIP     | Overall  | DCPIP    | Overall  | DCPIP    | Overall  |
| E1ec         | 100       | 100      | 37.9 ± 1.00 | 260.9 ± 19.3 | 0.145 | 4.62 ± 0.16 | 8.2 | 1.36 ± 0.11 | 1.02 ± 0.012 |
| E401A        | 23.4      | 9.56     | 3.64 ± 0.24 | 628.6 ± 51.2 | 0.0058 | 3.13 ± 0.28 | 1.16 | 3.15 ± 0.23 | 9.08 ± 0.25 |
| E401K        | 4.63      | 1.04     | 0.39 ± 0.05 | 700.8 ± 81.0 | 0.0063 | 4.45 ± 0.99 | 0.99 | 2.66 ± 0.23 | 12.53 ± 0.53 |
| K403A        | 98.0      | 11.6     | 4.41 ± 0.46 | 1304 ± 10.6 | 0.146 | 10.3 ± 0.5 | 1.85 | 3.18 ± 0.31 | 1.31 ± 0.23 |
| K403E        | 5.46      | 0.56     | 0.21 ± 0.04 | 171.4 ± 14.4 | 0.0048 | 8.33 ± 0.73 | 0.92 | 1.88 ± 0.06 | 1.10 ± 0.17 |
| N404A        | 45.1      | 1.8†     | 3.07 ± 0.72 | ND‡       | ND       | ND       | ND       | ND       | ND       |
| H407A†        | 12.0     | 0.15     | 0.081 ± 0.01 | ND       | ND       | ND       | ND       | ND       | ND       |
| Q408A        | 112.1    | 31.5     | 19.03 ± 0.94 | 233.6 ± 19.7 | 0.154 | 4.47 ± 0.04 | 7.963 | 1.75 ± 0.10 | 1.33 ± 0.32 |
| K410A        | 31.6      | 23.0     | 7.68 ± 0.09 | 201.2 ± 22.1 | 0.125 | 14.0 ± 0.98 | 1.79 | 1.20 ± 0.05 | 0.92 ± 0.003 |
| K410E        | 26.1      | 3.70     | 1.53 ± 0.20 | ND       | ND       | ND       | ND       | ND       | ND       |
| K411A        | 77.5      | 68.2     | 35.13 ± 1.49 | 233.6 ± 19.7 | 0.154 | 4.47 ± 0.04 | 7.963 | 1.75 ± 0.10 | 1.33 ± 0.32 |
| K411E        | 88.0      | 38.0     | 25.12 ± 0.67 | 201.2 ± 22.1 | 0.125 | 14.0 ± 0.98 | 1.79 | 1.20 ± 0.05 | 0.92 ± 0.003 |

* Overall activity determined by reconstituting with 3-lipoyl domain E2ec (3-lipE2ec) as opposed to 1-lipE2ec used with all other variants. For comparison, the K\(_{\text{cat}}\) of E1ec with 3-lipE2ec is 166.7 ± 1.
† ND indicates not determined due to low activity.
‡ Data were from Ref. 10. The apparent dissociation constant for MAP (K\(_{\text{d(MAP)}}\)) was determined using the circular dichroism signal at 300–305 nm for 401–413 loop variants.

Spectra were scanned at 30 °C by adding appropriate amounts of MAP each time. Protein and buffer background were subtracted from each spectrum, and maxima thus obtained were plotted against MAP concentration to get a value of K\(_{\text{d(MAP)}}\). The numbers in Table 2 are the means ± S.E.

significant (~23- and 25-fold). This indicates that steps from substrate binding through decarboxylation (supplemental Scheme I) are impaired. Because charge reversal of these residues yielded very low activity, they could not be treated in a similar fashion.

Covalent Addition of Substrate to ThDP Is Greatly Impaired in the Loop Variants—All the loop variants that titrated with substrate analog MAP to check the formation of PLThDP (stable analog of pre-decarboxylation intermediate LTThDP (supplemental Scheme II)) gave a CD band with \(\lambda_{\text{max}}\) ~300–305 nm, assigned to the Y′,A′-imino tautomer of PLThDP (16, 23). Variants with very low E1ec-specific activity (E401K and K403E) showed time dependence in formation of this CD signal. Given sufficient time for full development of the signal at each point in the titration, the amplitude of the CD signal will be directly proportional to the concentration of substrate analog, enabling us to obtain the apparent dissociation constant for MAP (K\(_{\text{d(MAP)}}\)). The K\(_{\text{d(MAP)}}\) consists of two dissociation constants (supplemental Scheme II), the first for forming a Michaelis-type noncovalent complex (K\(_{2,\text{MM}}\)) and the second for reversible formation of the covalent PLThDP (or LTThDP) confirm impairment of rates through the first irreversible step, decarboxylation. Because the H407A substitution obliterates loop disorder/order transformation in the presence of PLThDP (11), it is likely that this process is also responsible for the high K\(_{\text{d(MAP)}}\) values. Similar observations with other loop residue substitutions could be explained by a steric hindrance to loop disorder/order transformation by charge reversal at that position.

Substrate Addition to E1ec and Loop Variants Is Entropically Driven—The plot of lnK\(_{\text{cat}}\) versus 1/T was nonlinear for binding of MAP to E1ec indicating that the binding enthalpy (\(\Delta H\)) changes with temperature (Fig. 3). These data were therefore fitted to a nonlinear integrated van’t Hoff equation (see Equation 7). Van’t Hoff plots gave a \(\Delta C_p\) value of ~245.73 cal mol\(^{-1}\) K\(^{-1}\) suggesting coupling of a conformational change with ligand binding. Thermodynamic parameters derived from fitting these data indicate that the binding process is entropically driven within accessible temperature range, and \(\Delta G\) increases with temperature. However, with the loop variants, \(\Delta H\) did not change with temperature resulting in \(\Delta C_p = 0\).
Therefore, the binding process resembles rigid body binding, as compared with binding coupled to a conformational change in E1ec, indicated by a negative $\Delta C\mu$. Nevertheless, the binding of MAP to loop variants was still entropically driven (Table 3).

Crystal Structure Shows Disordered Loops upon Charge Reversal at Residue Glu-401—To understand the structural rationale for the biochemical observations with the loop variants, we crystallized and solved the structures of the E401K variant in complex with the cofactor ThDP and the reaction intermediate analog PLThDP in the active site. Both structures contain two subunits in the asymmetric unit. The two independent molecules are, nevertheless, almost identical and similar to the E1ec-ThDP structure (8). In addition, a more detailed comparison of the variant structures in the active site region revealed that the topology of the active site remains unchanged in both complexes. Least squares fitting of 801 $\alpha$-carbon atoms of the E401K-ThDP and E401K-PLThDP structures results in a root mean square (r.m.s) deviation of 0.22 Å. Except for some side chains at the surface of the protein, the electron density over the entire molecule is generally very well defined in both structures, but as in the native E1ec-ThDP structure there is no interpretable electron density for N-terminal residues Ser-1 to Gly-55 as well as for the two loop regions Glu-401 to Asn-413 and Asn-541 to Lys-557 present in the active site channel. For the PLThDP-containing complex, this is in sharp contrast to its counterpart with the native enzyme, in which ordering of the latter two loop regions is induced by the presence of the substrate analog.

The active sites in both complex structures with the variant are similar to that in the E1ec-ThDP structure and the cofactors are held in the V-conformation. The observed active site cavity includes the residues His-106, Ser-109, Gln-140, Tyr-177, Glu-235, Leu-264, and Lys-392 from one subunit and Asp-521, Glu-522, Ile-569, Tyr-599, Glu-636 and His-640 from the other subunit. A stereo view of the active site environment around the cofactor binding dimer interface is shown in Fig. 4. The positions of the active site residues in both complexes of E401K are similar to those observed for the E1ec-ThDP structure (8). In the E401K-PLThDP complex, the PLThDP conformation is stabilized by hydrogen-bonding interactions with Glu-522, Tyr-599, and His-640. As we have reported for the E1ec-PLThDP structure (11), the E401K-PLThDP structure also revealed a significant distortion in planarity of the C2–C2α bond connecting the substrate analog to the planar thiazolium ring. Apparently, this distortion is independent of the interactions with the inner loop.

Ordering of the Inner Loop Protects the Active Center from Undesirable "Carboligation" Side Reactions—The carboligation products acetolactate and acetoin are formed on E1ec, when instead of being oxidized by lipoxy-E2, the enamine intermediate adds to a second substrate pyruvate or the (rare) product acetaldehyde, yielding acetolactate or acetoin, respectively (Fig. 5I). In E1ec according to NMR data (Table 4) and CD analysis (Fig. 5V), the ratio of acetolactate to acetoin is 1.25 and yields perhaps 10% (S)-acetoin as a major product and a very small amount of (R)-acetolactate, both detectable by their characteristic CD signals ((S)-acetoin gives a positive band with $\lambda_{\max}$ near 280 nm, whereas (R)-acetolactate gives rise to a negative band with $\lambda_{\max}$ at 301–302 nm (16)). If active site loops do not sequester the intermediate because of disorder caused by substitutions, then we would expect this ratio to become larger than obtained with E1ec. Indeed the NMR analysis of carboligation reactions revealed that the molar ratio of acetolactate/acetoin varied in direct proportion with activity, i.e. the lower the activity of the variant, the higher the ratio of acetolactate relative to acetoin.

Some Inner Loop Substitutions Stabilize an Otherwise Transient Michaelis Complex—Earlier, we had reported that addition of excess pyruvate to a solution of E1ec complexed with ThDP and preincubated with 0.2 mM pyruvate reveals the presence of a transient Michaelis complex, characterized by a broad negative CD band centered at 327 nm (24). We hypothesized that under these conditions the Michaelis complex was stabilized by the slow turnover of the substrate because of the presence of a dead-end intermediate, C2–$\alpha$-hydroxyethyl-ThDP, in one of the two active sites (supplemental Scheme I). In the absence of preincubation with 0.20 mM pyruvate, the turnover is very fast, and no Michaelis complex could be detected. In the present investigation, for very low activity inner loop variants (E401K, K403E, N404A, and H407A) the Michaelis complex could be detected under both conditions, i.e. with or without

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### TABLE 3

| Variant | $\Delta G$ | $\Delta H$ | $\Delta S$ |
|---------|------------|------------|------------|
|         | kcal mol$^{-1}$ | kcal mol$^{-1}$ | kcal mol$^{-1}$K$^{-1}$ |
| E1ec    | -8.32 ± 0.95  | -0.88 ± 0.002 | 7.44 ± 0.87 |
| E401K   | -5.43 ± 0.22  | 0.51 ± 0.21   | 5.94 ± 0.02 |
| K403E   | -5.29 ± 0.03  | 0.53 ± 0.036  | 5.82 ± 0.003|
| H407A   | -5.03 ± 0.10  | 0.3 ± 0.09    | 5.53 ± 0.003|

The values for E1ec are reported for 30 °C.

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FIGURE 4. Stereo view of the active site of variant E401K-ThDP (red) and E401K-PLThDP (blue) complexes. The cofactor ThDP (cyan) or intermediate analog PLThDP (green) is at the interface between the N-terminal and middle domains from different subunits. Residues numbered <470 and >500 are from the N-terminal and middle domains, respectively.
of incubation, unacetylated lipoyl domain could still be detected (Fig. 7).

Residue His-407 in the Active Center Influences the Stereoenzyme Chemical Outcome of the Carboligase Reaction—According to CD and chiral GC analysis, the H407A and H407C (data not shown) variants produced excess (R)-acetoin (Fig. 5), whereas E1ec produced an excess of (S)-acetoin (~10% enantiomeric excess). This result suggests that the enzyme intermediate changes its facial preference vis-à-vis the acetaldehyde, with these variants, preferring attack on its re face. Apparently, the residue His-407 also has an influence on the facial selectivity of the carboligase reaction.

Positively Charged Residues on the Loop Lining the Active Site Tunnel Help Ligand Entry—The CD and chiral GC studies (data not shown) revealed that the major carboligation product of the K410A, K410E, K411A, and K411E variants is (S)-acetoin and a small amount of racemic acetolactate. More importantly, NMR analysis also revealed that the total amount acetoin formed was higher than the total amount of acetolactate as compared with E1ec (Table 4). This suggests that, unlike with E1ec and other loop variants with neutral and charge-reversed substitution at Lys-410 and Lys-411, the second pyruvate cannot readily access the active center to add to the enamine to generate acetolactate. Thus the reactive enamine reacts more frequently with acetaldehyde generated in the active center in the absence of oxidizing agent. Therefore, the positive charge at Lys-411 and Lys-410 is needed to assist entry of the second molecule of pyruvate and presumably of the first one as well. Also, binding curves of ThDP to apo-Lys-410 and Lys-411 variants were sigmoidal, a marked deviation from the hyperbolic curves generated by E1ec (data not shown). Curve fitting of these data yielded Hill coefficient values (nH) that indicated that substitutions at Lys-410 and Lys-411 induced cooperativity in ThDP binding (see above). However, the Kd(ThDP) values remained unchanged in the variants. Therefore, the positive charges lining the active site entrance appear to help entry of ThDP independently in both active sites of the dimer, but in the absence of these charges, structural changes in the active site (composed of residues from both subunits) that occur (25) upon binding of ThDP in one site are needed to bind the second molecule of ThDP in the other active site. These structural changes presumably propagate to the second active site and prepare it for binding the second ThDP. An analysis of the dependence of the activation lag phase on ThDP concentration (35) for these variants suggests that, whereas the concentration dependences were essentially identical for variants and E1ec within experimental error for ThDP < 1 μM and for ThDP > 20 μM, the activation lag phase diminishes faster with increasing ThDP concentration in the variants (supplemental Fig. S1). We speculate that this might be because of the stronger positive cooperativity evident even at higher concentrations of ThDP in

![Figure 6. Stabilization of transient Michaelis complex with pyruvate in low activity loop variants. Representative trace of each variant at a particular active site concentration is shown. In actual titration intensity of negative peak (centered at 327 nm) was proportional to active site concentration for all variants in the figure.](image)

**TABLE 4**

| Substitution | Acetolactate/acetoine |
|--------------|-----------------------|
| E1ec         | 1.25                  |
| E401A        | 1.73                  |
| E401K        | 2.47                  |
| K403A        | 1.81                  |
| K403E        | 2.13                  |
| N404A        | 2.39                  |
| H407A        | 0.24                  |
| K408A        | 1.77                  |
| K410A        | 0.83                  |
| K410E        | 0.69                  |
| K411A        | 0.95                  |
| K411E        | 0.70                  |

![Table 4: Carboligation ratio in E1ec and its inner loop variants](image)

**Multiple Roles of Dynamic Active Center Loop in E1ec**

**FIGURE 5. Carboligation reactions of H407A.** I, proposed mechanism of formation of carboligation side reaction products on E1ec. Chiral GC analysis of carboligation reaction of reference standard of racemic acetoin (II), E1ec (III), and H407A (IV). Acetoin was extracted from overnight-incubated carboligation reaction mixture (10 mM KH2PO4, pH 7.0) containing 0.2 mM ThDP, 1.0 mM Mg2+, 8.0 mM pyruvate, and 30.0 μM E1ec or its variants) with chloroform, and 20 ml of this aliquot was analyzed for the enantiomeric excess on Chiralix B-DM chiral column. CD spectra of E1ec carboligation reaction of E1ec (I) and H407A (IV) protein in each case was removed before recording the spectra with the help of Centricon YM 30 (Millipore).

![Figure 5: Carboligation reactions of H407A.](image)
Multiple Roles of Dynamic Active Center Loop in E1ec

FIGURE 7. Reductive acetylation of lipoyl domain with E1ec loop variants. A, principle of active center communication assay using MALDI-TOF/TOF (10). Reaction was started by adding 2.0 mM pyruvate to the preincubated mixture of lipoyl domain and E1ec in 20 mM HEPES (pH 7.5) containing 2.0 mM MgCl$_2$, 0.2 mM ThDP. Reaction was stopped by adding equivalent amounts of matrix at various time intervals. B, time dependence of reductive acetylation in E1ec and some loop variants.

the variants and is consistent with fluorescence binding studies of ThDP reported in this study.

DISCUSSION

Dynamic loops are important in catalysis of many enzymes and have been studied extensively with respect to their dynamic and biochemical roles. The inner dynamic loop in E1ec, particularly residue His-407, is important for catalysis and communication between components. In this study we set out to characterize the roles of other conserved residues in this important active center dynamic loop and to further explore the effects of the H407A substitution.

Substitution of charged residues to alanine in the inner loop (other than of His-407) resulted in reduction of $k_{cat}$ for the overall reaction by as much as 10-fold. This is a modest reduction and rules out any major roles of these residues in catalysis. However, there are other possible sources of reduction in $k_{cat}$ such as reduced coenzyme and/or substrate affinity. The former could be ruled out because $K_{d(ThDP)}$ was unchanged in all charge-reversed variants as compared with E1ec. However, there was a 3-fold increase in $K_{m(pyruvate)}$ and a significant reduction in $k_{cat}/K_{m(pyruvate)}$ for E401A and K403A indicating that substitution at these positions affected substrate binding and predecarboxylation steps. There are two plausible explanations for this observation: 1) either the charges on these residues assist directly in substrate binding and specificity, or 2) this effect points to disordering of the loop perhaps caused by neutralization of charges on these residues thus eliminating the stabilizing interactions.

Charge-reversed variants were created to address this issue. However, the charge-reversed variants retained little activity (reduced the $k_{cat}$ as much as 177-fold) and could not be analyzed by steady-state kinetics. Instead, we assessed the effects by direct measurements of the apparent dissociation constant for the substrate analog MAP ($K_{d(MAP)}$). The $K_{d(MAP)}$ values for charge-reversed variants E401K and K403E were 129- and 136-fold higher, respectively, than with E1ec (Table 2), whereas the $K_{d(MAP)}$ values for E401A and K403A were only 9- and 12-fold higher, respectively. In K410E this value was reduced 7-fold but remained unchanged in K411E compared with E1ec. These observations indicate greatly reduced $K_{d(MAP)}$ hence impaired covalent addition of substrate to ThDP when charges are reversed, and strongly suggest that charges on loop residues have an important role in this step, via modulation of loop dynamics. Not surprisingly, in H407A, which is shown to be essential for disorder to order transition of the loop (11), $K_{d(MAP)}$ increased 221-fold. Presumably, the hydrogen bond from His-407 to PLThDP with the closed loop conformation is also present when substrate pyruvate binds to the coenzyme forming LThDP, and this interaction would thus stabilize this intermediate. A residue structurally analogous to His-407 in E1ec in yeast transketolase (His-263) has been shown to have an important role in donor substrate binding (26, 27).

Direct involvement of charged loop residues in substrate binding can also be ruled out because charged residues are on opposite sides and spatially separated from the bound intermediate. Also, the value of $K_{d(MAP)}$ increased with a reduction in activity and responded dramatically to charge variations. The fractional increase in $K_{d(MAP)}$ in loop variants (as compared with H407A) most probably is because of impaired juxtaposition of His-407 with respect to intermediate due to loop disorder. In light of the above observations, it is probable that His-407, which clamps down on the substrate, is also directly responsible for stabilizing the LThDP form. This substrate-induced “clamping” or “capping” action is reported in many enzymes whose catalysis is controlled by loop gating. This is further supported by the observed stabilization of the otherwise transient Michaelis complex in those loop variants in which the degree of loop disorder can be said to be at a maximum. The substitutions at Lys-410 and Lys-411 did not result in significant reduction in activities; however, results of carboligation side reactions and ThDP binding studies, and the position of these residues along the active site channel indicated that these residues help with ligand entry into the active site. The N404A substitution resulted in the greatest reduction in overall activity among the alanine-substituted variants and also greatly affected the $K_{d(MAP)}$ (33 mM) (Table 2). This is expected because Asn-404 is within hydrogen bonding distance and most probably interacts with the outer loop (11), and this interaction is essential for proper closure of this loop (36).

Thermodynamics of MAP binding to E1ec and its loop variants supported the above hypothesis. The van’t Hoff plot for E1ec was nonlinear in the accessible temperature range resulting in $\Delta C_p = -245.73$ cal mol$^{-1}$ K$^{-1}$ (Fig. 3). $\Delta C_p$ has been related to the change in polar ($\Delta A_p$) and nonpolar ($\Delta A_{np}$) surface area ($\AA^2$), which occurs during binding (28).
Binding of MAP to E1ec yielded ΔAnp value of −646.28 Å² and ΔAp value of −173.45 Å² indicating that binding is predominantly accompanied by burial of the nonpolar surface area. These are typical values reported for ligand binding processes coupled to folding events (28). This observation is also consistent with the crystal structure, which shows the intermediate analog PLThDP in the active site in the ordered conformation being surrounded by hydrophobic residues; these residues may not be juxtaposed in the disordered conformation, exposing these residues to solvent. However, because we do not have a crystal structure of the disordered conformation, we were not able to get a theoretical estimate of ΔAnp and ΔAp during the disorder to order transition. The finding that with the inner loop variants ΔC_p is zero suggests the absence of binding-coupled conformational changes upon MAP addition, and hence impaired ordering of the loop(s).

The activity data of all the loop variants also revealed that the fractional reduction in E1 subunit-specific activity is always smaller than the fractional reduction in overall complex activity. This is an indication of disruption in communication between the E1ec and E2ec components. Disordering of the inner loop structure therefore affects the transfer of the acetyl group from E1ec to the lipoamide on E2ec. It has also been shown that the lipoyl domain of pyruvate dehydrogenase multienzyme complex E2 from E. coli and Azotobacter vinelandii works only with pyruvate dehydrogenase multienzyme complex E1 and not with E1o from 2-oxoglutarate dehydrogenase complex from both sources and vice versa (29, 30). From these observations it was suggested that the E1 component confers specificity on an otherwise unremarkable folded lipoyl domain and also provides a molecular mechanism for substrate channeling in the complex (31). Protein sequence alignment of E1ec with E1o did not result in any sequence homology in the 401−413 region of E1ec (data not shown). Based on the above observations and those from the crystal structure of E1ec with PLThDP in which a new surface is formed in the active site channel as a result of loop ordering (Fig. 1B), it was proposed that this new surface facilitates E2ec-lipoyl domain binding and receiving lipoamide in the active center (11). In H407A this newly ordered surface was not seen because of disorder of the loops and could explain the disruption of active site coupling in H407A. An analogous mechanism could also explain impaired active site coupling in some variants studied in this report. This is also additional evidence in support of our above-mentioned argument that charged loop residues help stabilize the ordered loop conformation.

To test the hypothesis that covalent substrate addition and reductive acetylation (active site coupling) are greatly impaired as a result of disorder in the loop caused by substitutions, we crystallized E401K in the presence of PLThDP. Because, in the wild type enzyme, the presence of this intermediate analog orders both inner and outer loops, the crystal structure may provide direct proof for the above hypothesis. Indeed, we found that both loops are disordered in both the E401K-ThDP and in the E401K-PLThDP complexes; in the variants the presence of PLThDP in the active site is insufficient to form a strong hydrogen bond with His-407 (present in the disordered loop 401−413) for ordering this loop in the active site channel. Compared with the E1ec-PLThDP structure (11), there is no conformational rearrangement of active site residues in the E401K-PLThDP complex. Analysis of the E1ec-PLThDP structure revealed that residues present in the inner loop are involved in strong hydrogen bonding interactions with the residues present in loop region 392−400 (Fig. 1). This 392−400 loop region is ordered in all E1ec structures in the complexes with ThDP or PLThDP present in the active site, and in the structures of different 401−413 loop variants. As seen in Fig. 1, the Glu-401 side chain is hydrogen-bonded to the main chain nitrogen atoms of Gly-395 and Gly-397. The main chain nitrogen atom of Gly-402 is hydrogen-bonded to the main chain carbonyl oxygen atom of Lys-392. Lys-403 also forms hydrogen bonds with residues Asp-398 and Gln-262. The residue Gln-262 is proximal to the stretch 258−260, known to create part of the ThDP fold, specifically binding Mg²⁺ (32). Therefore these interactions also play a significant role for ordering the loop 401−413 in the active site channel.

Disruption of loop closure over the active center in loop-substituted variants (as supported by the above data) would also mean impaired sequestering of active site chemistry from the solvent. Therefore, reactive intermediates such as the enamine would readily add to excess pyruvate in solution and one would expect that the unusual carboligation product acetolactate should form in excess. NMR studies of carboligation products formed by loop variants showed that this is indeed the case (Table 4), reconfirming our conclusion that charged loop residues do not directly participate in catalysis but rather stabilize the closed conformation. The carboligation reaction catalyzed by H407A showed a marked deviation from that of E1ec (Fig. 5).

In the carboligation reaction of H407A, acetolactate, which should form if a pyruvate adds to the intermediate enamine, was detected in a very low amount; instead a small amount of (R)-acetoin is formed in >90% enantiomeric excess (Fig. 5). Therefore, not only does His-407 appear to play a central role in chemical steps, its presence in the active center also determines stereoselectivity of carboligation reaction, presumably via active site packing.

In general, and as can be expected from the above arguments, the magnitude of the acetolactate signal (relative to the acetoin signal) is directly proportional to the magnitude of subunit-specific activity reduction as a result of substitution. This was also true for alanine and charge-reversed variants of the same residue; charge-reversed (except in Lys-410 and Lys-411) resulted in a higher amount of acetolactate relative to acetoin (Table 4). Therefore, we conclude that substitutions at different positions along the loop affected ordering of the loop to varying degrees, and the magnitude of disorder induced is qualitatively paralleled by their carboligation product profile.

We expected that any Gln-408 substitution would have a deleterious effect on overall catalysis of E1ec, because structurally it corresponds to Ser-292α (11), the phosphorylation site in the α2β2 heterotetrameric E1b component involved in activation-deactivation of enzyme. However, the Q408A substitution had little impact on activities, yet it yielded a higher concentration of acetolactate relative to acetoin. Because Gln-408 is not
involved in any major interactions other than the one with the intermediate analog through water, it is unlikely that the unusual carboligation results with this variant are because of loop disorder. This is particularly interesting because other important amino acids such as Glu-636 and Tyr-177 also interact with the intermediate analog through water, and their substitution also caused remarkable changes in the biochemical behavior of enzyme (15, 18). The E636A substitution converted the enzyme to an acetolactate synthase with very high stereoselectivity, which, based on Fourier transform ion cyclotron resonance mass spectrometry of tryptic digest of variant and E1ec, could be attributed again to changes in outer loop dynamics. From structural comparisons and biochemical data, the residue Tyr-177 was suggested to have a role in stabilizing the enamine-like transition state. Therefore, from the structural (11) and kinetic data here reported, we could speculate that the water-mediated interaction of Gln-408 with the intermediate could stabilize the transition state rather than loop dynamics, and its presence in the active center might help modulate premature pyruvate entry.

In conclusion, we have demonstrated that disorder to order transformation of active center dynamic loops (particularly the inner loop) modulates steps through LThDP formation. Ordering of loops also facilitates E1ec to E2ec active center communication reconfirming our previous hypothesis, presumably by acting as a recognition site for E2ec lipoyl domain, and acts as a regulatory switch for the next committed step in E1ec catalysis. Among the conserved amino acid residues on the inner loop, the charged ones assist in loop dynamics and also in sequestering the active site from undesirable carboligation reactions. This study for the first time gives biochemical evidence for the active site sequestering role of the inner loop, and also provides important information regarding determinants of substrate utilization. Data in this study also reveal that the Asn-404 residue is important for catalysis and subunit communication and provides biochemical evidence that this residue interacts with the outer loop and thus might control outer loop dynamics (possibly independently from the inner loop). These features of E1ec are analogous to many enzymes that use conformational changes to fine tune catalysis and may be a common feature of ThDP-dependent homodimeric E1 components.

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