Flexible Regions of the E1 Component of the Pyruvate Dehydrogenase Complex from Gram-negative Bacteria

Most bacterial pyruvate dehydrogenase complexes from either Gram-positive or Gram-negative bacteria have E1 components with an α2 homodimeric quaternary structure. In a sequel to our previous publications, we present the first NMR study on the flexible regions of the E1 component from Escherichia coli and its biological relevance. We report sequence-specific NMR assignments for 6 residues in the N-terminal 1–55 region and for a glycine in each of the two mobile active center loops of the E1 component, a 200-kDa homodimer. This was accomplished by using site-specific substitutions and appropriate labeling patterns along with a peptide with the sequence corresponding to the N-terminal region and for a glycine in each of the two mobile active center loops of the E1 component. To study the functions of these mobile regions, we also examined the spectra in the presence of a reaction intermediate analog known to affect the mobility of the active center loops, (b) an E2 component construct consisting of a lipoyl domain and peripheral subunit binding domain, and (c) a peptide corresponding to the amino acid sequence of the E2 peripheral subunit binding domain. Deductions from the NMR studies are in excellent agreement with our functional finding, providing a clear indication that the N-terminal region of the E1 interacts with the E2 peripheral subunit binding domain and that this interaction precedes reductive acetylation. The results provide the first structural support to the notion that the N-terminal region of the E1 component of this entire class of bacterial pyruvate dehydrogenase complexes is responsible for binding the E2 component.

The pyruvate dehydrogenase complex (PDHc) is a molecular machine consisting of multiple copies of three distinct proteins in bacteria (5 MDa) (1, 2). In most bacteria (both Gram-negative and Gram-positive) the complex has octahedral symmetry. The PDHc catalyzes the oxidative decarboxylation of pyruvate according to Equation 1.

\[
\text{Pyruvate} + \text{coenzymeA} + \text{NAD}^{+} \rightarrow \text{acetylcoenzymeA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad \text{(Eq. 1)}
\]

The components of the Escherichia coli PDHc have the following roles (Equations 2–6) (Ref. 3–5). The thiamin diphosphate (ThDP)-dependent E1 component (E1ec, pyruvate dehydrogenase) is an α2 homodimer and carries out consecutively pyruvate decarboxylation and reductive acetylation of the E2 component (Equation 3). E2 (E2ec, dihydrolipoamide acyltransferase) has a dual function. Its covalently attached lipoamide is reductively acetylated by the E1 component and pyruvate, and subsequently, the acetyl group is transferred to coenzyme A (CoA), and the principal metabolic product acetyl-CoA is released (Equation 4). E3 (E3ec, dihydrolipoamide dehydrogenase) has tightly bound FAD and NAD⁺; it regenerates the lipoamide from the reduced dihydrolipoamide form (Equation 5) (Scheme 1).

\[
\text{Pyruvate} + \text{E1-ThDP-Mg(II)} \rightarrow \\
\text{E1-hydroxyethylidene-ThDP-Mg(II)} + \text{CO}_2 \quad \text{(Eq. 2)}
\]

\[
\text{E1-hydroxyethylidene-ThDP-Mg(II)} + \text{E2-lipoamide} \rightarrow \\
\text{E1-ThDP-Mg(II)} + \text{E2-acytyldihydrolipoamide} \quad \text{(Eq. 3)}
\]

\[
\text{E2-acytyldihydrolipoamide} + \text{CoA} \rightarrow \\
\text{E2-dihydrolipoamide} + \text{acetyl-CoA} \quad \text{(Eq. 4)}
\]

\[
\text{E2-dihydrolipoamide} + \text{E3-FAD} \rightarrow \text{E2-lipoamide} + \text{E3-FADH}_2 \quad \text{(Eq. 5)}
\]

\[
\text{E3-FADH}_2 + \text{NAD}^{+} \rightarrow \text{E3-FAD} + \text{NADH} \quad \text{(Eq. 6)}
\]

Our laboratory has been studying the PDHc from E. coli (PDHc-ec) as a representative of this large class of enzymes (6–10). In the x-ray structure of E1ec with ThDP, there were identified three disordered regions corresponding to the N-terminal 1–55 residues, to amino acids spanning residues 401–413 (inner active center loop) and 541–557 (outer active center loop) (7). We have now shown that all three of these regions have important function. To study the function of the N-terminal region of E1ec, we followed up on earlier work of de Kok and de Kok and...
co-workers (11, 12) on the related enzyme from *Azotobacter vinelandii* to elucidate the loci of binary interactions in the E1ec-E2ec complex. In our earlier study (13) we used the E1ec deletion variants (Δ16–25, Δ26–35, Δ36–45, and Δ46–55), single-site substituted variants in the region 7–15, and mass spectrometric analysis to confirm that the N-terminal region, although not seen in any x-ray structures of E1ec (7, 14, 15), is important for both overall activity of the complex and for interaction with the E2ec. We have extended the study to the entire N-terminal region of E1ec and here report the importance of this region in the interaction with E2ec. In a series of reports (9, 14, 16), we also demonstrated (a) the functional importance of the two active center loops in the organization of the active center during the reaction sequence and (b) that loop movement is correlated with catalysis. In this paper we explored the possibility that regions too mobile to be seen in the x-ray structure may give rise to resolvable resonances in the NMR spectrum notwithstanding the size of E1ec (2886 residues for an Mr of 200,000) (3). Our expectations were met; we have for the first time made sequence-specific NMR assignments for six amino acids in the N-terminal region of E1ec, which was made possible by the presence of some remarkably sharp resonances in the two-dimensional HSQC spectra. In addition, we also assigned one glycine resonance in each of the two active center loops. In fact, the total number of observable resonances corresponds well to the number expected on the basis of sequence, and the NMR observations pertain virtually exclusively to the three flexible regions. These “reporters” from the three regions were then examined in the presence of (a) a reaction intermediate analog known to affect the mobility of the active center loops, (b) an E2 component construct consisting of a lipoic domain and peripheral subunit binding domain (PSBD), (c) a peptide corresponding to the amino acid sequence of the PSBD, and (d) the independently expressed lipoic domain. The results show that the PSBD is the most important domain for recognition of E1 rather than the lipoic domain. The results also correlate the functional and structural data from our laboratories.

**EXPERIMENTAL PROCEDURES**

**Materials**

The Wizard® Plus Miniprep 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). DNA sequencing was done at the Molecular Resource Facility of the New Jersey Medical School (Newark, NJ). The *E. coli* AT2457 strain (glycine auxotroph) was from *E. coli* Genetic Stock Center (Yale University). *E. coli* BL21(DE3) pLys cells were from Novagen (Novagen, EMD Chemicals, Gibbstown, NJ). The synthetic peptide corresponding to the N-terminal 1–35 amino acids of the E1ec was from SynPep (Dublin, CA). The synthetic peptide with sequence H2N-ATPLIRRLAREFGVNLAKVKGRGRGLREDVQAYKVEAI-OH corresponding to the E2ec PSBD was from CHI Scientific (Maynard, MA). RbCa TXN SALTS used to make super-competent cells was from BIO101, Inc. (Vista, CA). The 18 unlabeled L-amino acids used in the minimal medium were from Sigma, with the exception of L-Lys from EMD Chemicals, Inc. (La Jolla, CA). [15N]Glycine, [1-13C]glycine, and [15NH4]Cl were from Cambridge Isotope Laboratories, Inc. (Andover, MA).

**Construction of Single-substituted N-terminal Variants and Expression and Purification of E1ec and Its Variants**

See Ref. 17 and supplemental Experimental Procedures.
NMR Evidence for the Flexible Regions of PDHc E1 Component

Expression and Purification of 1-lip E2ec

See Ref. 18 and supplemental Experimental Procedures.

Activity and Related Measurements

The activity of E1ec and its variants was measured after reconstitution to PDHc complex with E2ec and E3ec components using a E1ec:E2ec:E3ec mass ratio of 1:1:0.5 (19). The E1-specific activity of E1ec and its variants was measured in the model reaction with 2,6-dichlorophenolindophenol (DCPIP) as the external oxidizing agent by monitoring the reduction of DCPIP at 600 nm (20).

Size-exclusion Chromatographic Test of the Dimerization of E1ec and Its Variants

Fast protein liquid chromatography Superose 6 10/300 GL column (Amersham Biosciences) was equilibrated with 50 mM KH$_2$PO$_4$ (pH 7.0) containing 0.15 mM NaCl. The column was calibrated using the following standards: thyroglobulin (669,000), ferritin (440,000), catalase (232,000), aldolase (158,000), bovine serum albumin (67,000), and ovalbumin (43,000). The proteins (1–2 mg) were eluted at a solvent flow rate of 0.4 ml/min and monitored by UV at 280 nm.

Interaction of E1ec and Its Variants with E2ec Monitored by Size-exclusion Chromatography

E1ec and its variants were incubated with 1-lip E2ec in 50 mM KH$_2$PO$_4$ (pH 7.0) containing 0.15 mM NaCl at 25 °C (molar ratio of E1ec:1-lip E2ec = 1:1). After 45 min, samples were centrifuged for 10 min at 17,530 × g, and the subcomplex was applied to the column. Circular dichroism (CD) experiments were carried out on an Applied Photophysics Chirascan spectrometer (Leatherhead, UK) at 30 °C as reported earlier from our laboratories (8, 10, 20).

Reductive Acetylation of the Lipoyl Domain and of 1-lip E2ec Monitored by MALDI-TOF/TOF Mass Spectrometry

See Refs. 18 and 21 and supplemental material.

Construction of Plasmid, Expression, and Purification of C-terminal Hist$_6$-tag E1ec

The pGS878 plasmid encoding E1ec was used as a template. The XbaI and Xhol restriction sites were created at the beginning and at the end of the E1ec gene by site-directed mutagenesis (22). The DNA fragment of 2307 bp encoding E1ec was subcloned into the pET-22b(-) vector pretreated with the same restriction enzymes. The resulting plasmid pET-22b(-)/H11001-1-lip E2ec (6946 bp) plasmid encoding 1-lip E2ec was used as the template (18), and the amplification primer 5'-CCATGCGAGTTGAGCGTGCTCAATGATCGACGCTGCTGTAAGCCC-3' and its complement were used for site-directed mutagenesis to introduce the TAA stop codon in place of Lys-191, which is located at the N-terminal region of the catalytic domain of the 1-lip E2ec. The 1-lip E2ec(1-190) was well expressed in BL21(DE3) cells at 37 °C with no parent 1-lip E2ec detected by SDS-PAGE. The culture was grown in LB medium with 50 µg/ml ampicillin supplemented with 0.30 mM lipoic acid, and protein expression was induced by 0.50 mM isopropyl 1-thio-β-D-galactopyranoside for 5–6 h at 37 °C. The expressed protein was purified using consecutively a DEAE anion exchange column and a G3000SW TSK size-exclusion column with a high performance liquid chromatography system. About 7 mg of protein was obtained from 6–7 g of E. coli cells (2.4 liters of LB medium). The identity of 1-lip E2ec(1-190) was confirmed by N-terminal sequencing for nine amino acids: AIIEIKVPD.

Preparation of Labeled E1ec Variants for NMR Studies

Uniform 15N Labeling of Hist$_6$-tag E1ec (23)–20 ml of overnight culture from a single colony on the plate was grown in LB medium containing 50 µg/ml of ampicillin. After 15 h of growth at 37 °C, cells were collected by centrifugation (2236 g, 10 min, 4 °C) and washed with M9 minimal medium (24) sup-
plemented with 1g/liter of $^{15}$N$\text{NH}_4\text{Cl}$ and sodium acetate (2 mM) to remove the traces of LB medium. The cells were collected by centrifugation, dissolved in 25–30 ml of the minimal medium, and inoculated into 700 ml of the minimal medium supplemented with $^{15}$N$\text{NH}_4\text{Cl}$. Cells were grown to $A_{600}=0.60–0.80$ at 37 °C, then the temperature was lowered to 20 °C, and protein expression was induced by 0.50 mM isopropyl 1-thio-$\beta$-D-galactopyranoside for 15 h. Cells were collected by centrifugation at 2 236 $\times$ $g$ for 7 min and washed with 20 mM KH$_2$PO$_4$ (pH 7.0) containing 0.25 mM EDTA and 0.10M NaCl and stored at $-20$ °C. The 15N-labeled E1ec was purified using Ni$^{2+}$-Sepharose 6 Fast flow column similarly to that presented for His$_6$-tag E1ec above. In addition, the 15N-labeled E1ec was purified using an anion exchange DEAE column and a 0–0.50M gradient of NaCl in 20 mM KH$_2$PO$_4$ (pH 7.5). The protein was dialyzed against 20 mM KH$_2$PO$_4$ (pH 7.0) containing 0.20 mM ThDP, 2.0 mM MgCl$_2$, 1.0 mM DTT, and 0.50 mM EDTA (dialysis buffer), concentrated using a Centriprep 30 (Millipore) concentrating unit, and stored at $-20$ °C. Approximately 100 mg of purified 15N-labeled protein was obtained from 3.5 liters of culture.

Selective 15N Labeling and $^{13}$C-$^{15}$N Double Labeling of His$_6$-tag E1ec —Selective 15N and $^{13}$C,$^{15}$N enrichment of glycine residues in E1ec was achieved by transformation of the pET-22(b)-E1ec plasmid into the E. coli strain AT2457 glycine auxotroph. The E. coli AT2457 super-competent cells were prepared using RbCa TXN SALTS. Cells were grown on the minimal salt medium supplemented with 18 unlabeled L-amino acids and 0.35 g/liter $^{15}$N glycine for 15N-selective labeling or 0.35 g/liter $^{15}$N glycine and 0.35 g/liter $^{1-13}$C glycerine for $^{13}$C,$^{15}$N double-labeling of E1ec, similarly to that presented above for uniform 15N labeling of E1ec (25, 26). Approximately 46 mg of protein was purified from 1.0 liters of culture.

**NMR Spectroscopy**

Sample Preparation for NMR Studies —The concentration of His$_6$-tag E1ec in the NMR sample was measured using Bio-Rad Protein Assay Dye Reagent Concentrate and adjusted to 20 mg/ml (subunit concentration $= 211 \text{ M}^{-1}$) in 0.4 ml of dialysis buffer (see above), then 0.15 M NaCl and 7% D$_2$O were added.

### Table 1

| N-terminal substitution in E1ec | Overall PDHc activity | E1-specific activity | $K_{e_{\text{MAP}}}$ | Ability to form E1-E2 complex |
|---------------------------------|-----------------------|---------------------|---------------------|-----------------------------|
| none                            | %                     | %                   | $\mu$M               | +                           |
| $D7A$                           | 0.14                  | 90                  | 0.159               | +                           |
| $D9A$                           | Not detected          | 88                  | 0.199               | +                           |
| $E12D$                          | 2.3                   | 10                  | 0.100               | +                           |
| $E12Q$                          | 3.3                   | 41                  | 0.100               | +                           |
| $I11A$                          | 94                    | 62                  | 0.100               | +                           |
| $R14A$                          | 1.25                  | 100                 | 0.100               | +                           |
| $D15A$                          | 0.19                  | 84                  | 0.199               | +                           |
| $E21R$                          | 69                    | 89                  | 0.199               | +                           |
| $E26R$                          | 87                    | 89                  | 0.199               | +                           |
| $E27R$                          | 2.7                   | 67                  | 0.068               | +                           |
| $E30R$                          | 2.9                   | 61                  | 0.296               | +                           |
| $D37R$                          | 74                    | 75                  | 0.296               | +                           |
| $E42R$                          | 100                   | 86                  | 0.296               | +                           |

*Italics indicate data from Ref. 13.

*The $K_{e_{\text{MAP}}}$ for E1ec in the overall PDHc reaction after reconstitution with E2ec and E3ec was 90 s$^{-1}$.

*The $K_{e_{\text{MAP}}}$ for E1ec in the model reaction with DCPIP was 1.20 s$^{-1}$.

*+, only E1ec-E2ec complex; ++, E1ec and E1ec-E2ec subcomplex. Strength of subunit interaction: $+$ > ++ > +.

### Figure 1

**Time course of reductive acetylation of 1-lip E2ec by pyruvate and D9R E1ec monitored by MALDI-TOF/TOF mass spectrometry.**

- **A**, 2 min. **B**, 5 min. **C**, 15 min. **D**, 30 min. The lower mass peak corresponds to the unacetylated lipoyl domain excised by trypsin, and the higher mass corresponds to the acetylated domain.
The pH of the NMR samples was adjusted to 7.0 by the addition of 0.15 M CH₃COOH or 0.15 M NaHCO₃.

**NMR Spectroscopy**—All NMR experiments were performed on a Varian INOVA 600 MHz spectrometer at 20 °C. Two-dimensional ¹H,¹⁵N TROSY-HSQC (27) spectra were recorded for ¹⁵N-labeled His₆-tag E1ec and all its singly substituted variants and for the [¹⁵N]glycine containing variant. A ¹³C,¹H,¹⁵N triple resonance three-dimensional HNCO (28) NMR experiment was performed for the ¹³C,¹⁵N double-labeled glycine residues in His₆-tag E1ec, and the NH plane from the three-dimensional data was analyzed to assign Gly-47. Two-dimensional ¹H,¹⁵N HSQC (29) spectra were recorded for the 1–35 ¹⁴N synthetic peptide. NMR Pipe (30) and NMR ViewJ (31) were used for data-processing of all spectra.

**Two-dimensional HSQC NMR Method to Identify the Region of E1ec Interacting with E2ᵢ–₁₉₀, Lipoyl Domain, and PSBD**—For the HSQC NMR experiment, the ¹⁵N-labeled His₆-tag E1ec (20 mg/ml, concentration of subunits = 211 μM) in 20 mM KH₂PO₄ (pH 7.0) containing 0.20 mM ThDP, 2.0 mM MgCl₂, 1.0 mM DTT, and 0.50 mM EDTA was mixed with 1-lip E2ec₁–₁₉₀ (422 μM) in the presence of 0.10 M NaCl and 10 mM DTT in a total volume of 0.50 ml, then incubated for 1 h at 25 °C. D₂O (7%) was added before the NMR spectrum was recorded. Similar conditions and concentrations were used for the experiments on His₆-tag E1ec in the presence of PSBD and lipoyl domain.

**FIGURE 2.** ¹H,¹⁵N TROSY-HSQC spectra of His₆-tag E1ec. a, uniformly ¹⁵N-labeled type His₆-tag E1ec; b, glycine residues only ¹⁵N-labeled; c, spectra a and b superimposed showing perfect overlap.

**TABLE 2** ¹H and ¹⁵N chemical shifts of assigned residues of the flexible regions of His₆-tag E1ec compared with the reference chemical shift of random coil.

| Residue | Random coil chemical shifts at pH 5*, ¹⁵N | Residue | Random coil chemical shifts at pH 7, ¹⁵N |
|---------|------------------------------------------|---------|------------------------------------------|
| Glycine | 109.30                                   | Gly-28  | 8.454                                   |
|         |                                          | Gly-47  | 8.294                                   |
|         |                                          | Gly-402 | 8.389                                   |
|         |                                          | Gly-542 | 8.208                                   |
|         |                                          | Gly-28  | 8.454                                   |
| Gln-18  | 7.454                                   | Gln-33  | 7.596                                   |
| Gln-38  | 7.692                                   | Gln-38  | 7.692                                   |
| Trp-16  | 10.12                                   | Trp-16  | 10.12                                   |

*Random coil chemical shifts reported for glycine in Ref. 40 for Gln and Trp side chains from the Biological Magnetic Resonance Data Bank at the University of Wisconsin-Madison.
Two-dimensional HSQC-TROSY NMR to Identify the Region of E1ec Interacting with C2-
phosphonolactyl-ThDP—Two-dimensional HSQC-TROSY spectra were recorded for the His6-
 tagged E1ec and its G402A and G542A variants (20 mg/ml, concentration of subunits
in 20 mM KH₂PO₄ (pH 7.0) containing 0.20 mM ThDP, 2.0 mM MgCl₂, 1.0 mM DTT, and
0.50 mM EDTA. To identify the region of E1ec interacting with C2-
phosphonolactyl-ThDP (PLThDP), apoE1ec (20 mg/ml)
was prepared by dialysis against 20 mM KH₂PO₄ (pH 7.0) contain-
ing 2.0 mM MgCl₂, 1.0 mM DTT, and 0.50 mM EDTA, and apo-
enzyme was titrated by CD to saturation with 1 mM PLThDP, then
0.15 M NaCl and 7% D₂O were added for NMR experiment.

RESULTS AND DISCUSSION

Functional Evidence for the Importance of the Entire N-terminal E1ec Region in Its Interac-
tion with E2ec

The sequences of E1 components from Gram-negative bacteria reveal a large percentage of highly conserved acidic amino
acid residues in their N-terminal 1–55 region (13). The patch of
highly conserved positively charged residues in the PSBD of
E2ec led us to hypothesize that the negatively charged residues
in the N-terminal region of E1ec interact with the positively
charged residues on the PSBD (32). Earlier, we reported that the
negatively charged residues in the N-terminal region of E1ec
Asp-7, Asp-9, Glu-12, Asp-15, and a positively charged Arg-14
are important for the overall PDHc activity and for the interac-
tion with E2ec (13). To extend our study to the entire N-
terminal E1ec region, the E21R, E26R, E27R, E30R, D37R, and
E42R variants were created and analyzed. Because the nature
of the substitution of the negatively charged residues such as
Glu-12 to Arg, Asp, or Gln appeared to make a modest dif-
culty (Table 1), we created E1ec variants with Arg substitutions, assuming that substi-
tution of the negatively charged residue to a positive will be
informative.

Negatively Charged Residues in the Entire N-terminal Region of E1ec Have No Role in Either Formation of the First Covalent
Intermediate or in Decarboxylation—In addition to the nega-
tively charged residues studied earlier (Asp-7, Asp-9, Glu-12,
Asp-15), Glu-27 and Glu-30 displayed low overall activity (2.7
and 2.9%, respectively) on reconstitution with E2ec-E3ec sub-
complex (Table 1), whereas the others had activities of
69–100%. However, none of the substitutions in E1ec here
reported produced significant activity reduction according
to the E1-specific 2,6-dichlorophenolindopenol assay, sug-
gesting that the reactions were little affected through decar-
boxylation (Scheme 1, reactions k₁, k₂, and k₃). The addition
of substrate analogue methyl acetylphosphonate (MAP) to
ThDP to the E27R and E30R variants revealed formation of a
tetrahedral intermediate PLThDP by CD via its 1',4'-imi-
nopyrimidine tautomeric form, as demonstrated before (33, 34). The values of $K_{d,MAP}$ of 0.068 μM (E27R) and 0.296 μM (E30R) were similar to the $K_{d,MAP} = 0.159$ μM for E1ec, demonstrating that the formation of the first predecarboxylation intermediate was not affected by these substitutions either (Scheme 1, $k_1$, $k_2$).

Reductive Acetylation of the Lipoyl Domain and of Intact E2ec Are Affected Differentially by N-terminal E1ec Variants—As pointed out in our earlier publication on this topic, there are two loci of interaction between E1ec and E2ec; (a) between ThDP and the lipoyl domain, the site of reductive acetylation, and (b) between the N-terminal region of E1ec and E2ec (suggested to be the PSBD, perhaps with additional interaction with the N-terminal region of the catalytic domain). We had developed a MALDI-TOF mass spectroscopic method to monitor the reductive acetylation of either the independently expressed lipoyl domain or of the entire E2ec by E1ec and its variants in the presence of pyruvate (18, 21). Recently, we extended this method to MALDI-TOF-TOF and Fourier transform mass spectrometry. On incubation of E27R and E30R E1ec variants with lipoyl domain (0.30 or 0.60 mM) in the presence of pyruvate and ThDP, only the acetylated form of the lipoyl domain was detected within 1 min of incubation as with E1ec (a molecular mass of acetylated dihydrolipoyl domain of 9,019.2 Da was detected resulting from the mass of the lipoyl domain of 8,975 Da + 44).

Incubation of the intact 1-lip E2ec component with E1ec in the presence of pyruvate for 2 min at 25 °C followed by trypptic digestion led to the appearance of the lipoyl domain with a mass of 10,160.9 Da, clearly signaling an acetylated lipoyl domain (the molecular mass of the unacetylated lipoyl domain liberated by trypsin is 10,119 Da). The time course for reductive acetylation as monitored by MALDI-TOF/TOF was significantly different between E1ec and its D9R, E12R, and E30R variants. Within 2 min, only the acetylated lipoyl domain was detected with E1ec, but with the D9R, E12R, and E30R variants, we could monitor the time course of interconversion of the unacetylated and acetylated forms of the lipoyl domain of 1-lip E2ec. With increasing incubation time, the peak for the acetylated form of the lipoyl domain increased, whereas the peak corresponding to the unacetylated form diminished in size. Even after 30 min of incubation, the unacetylated forms were still observed. A typical time-course of the reductive acetylation of 1-lip E2ec by D9R E1ec and pyruvate is shown in Fig. 1. The E12R and E30R E1ec variants displayed the same behavior, indicating that substitutions of the negatively charged residues in the N-terminal region of E1ec affect its assembly with the E2ec and, as a result, the overall PDHc

FIGURE 4. Resonance assignment for Gly-402 (inner loop) and Gly-542 (outer loop) of His$_6$-tag E1ec in $^1$H,15N TROSY-HSQC spectra. a and b, superimposed spectra of His$_6$-tag E1ec and G402A variant. c and d, superimposed spectra of His$_6$-tag E1ec and G542A variant.
reaction rate. According to these experiments, binding of the lipoyl domain of E2ec to E1ec does not limit the rate of reductive acetylation of intact E2ec or of the overall rate of the complex, as is also supported by the structural studies presented below.

The E1ec-E2ec Interaction Is Affected by N-terminal E1ec Substitutions According to Size-exclusion Chromatography—Using an analytical size-exclusion chromatographic column calibrated with standards in the mass range of 67,000–669,000 Da, we demonstrated that all N-terminal-substituted variants are

FIGURE 5. Resonance assignment of three sets of Gln side chains in $^1$H,$^15$N TROSY-HSQC spectra. a and b, shown are superimposed spectra of His$_6$-tag E1ec (black) and Q18H variant (red). Also shown are superimposed spectra of His$_6$-tag E1ec (black) and Q33H variant (red) (c and d) and superimposed spectra of His$_6$-tag E1ec (black) and Q38H variant (red) (e and f).
eluted from the column as a dimer, similar to E1ec (retention time of 38.96 min, which corresponds to a molecular weight of dimer; theory, 198,948 Da) (see supplemental Fig. S1).

On assembly of E1ec with E2ec, only one peak corresponding to their subcomplex was detected by analytical size-exclusion chromatography and SDS-PAGE (not shown). The E21R, E26R, D37R, and E42R E1ec variants were apparently converted quantitatively to the complex similarly to E1ec, perfectly accounting for their high overall PDHc activity (69–87% in Table 1). Incubation of E2ec with the D7A, D9A, D9R, E12R, E12D, E12Q, R14A, D15A, E27R, and E30R E1ec variants gave two peaks; according to SDS-PAGE, the first peak to elute contained the E1ec-E2ec subcomplex, and the second peak contained only E1ec. Hence, whereas these variants displayed impaired interaction with E2ec (explaining their low overall PDHc activity), their normal E1-specific activity could be attributed to the fact that the latter is measured in the absence of E2 and E3 components (Scheme 1, $k_1$, $k_2$, $k_3$).

**Structural Evidence for the Interaction of the Flexible Regions of E1ec with E2ec**

To undertake the NMR studies reported below (the ultimate goal of this section), we created a His$_6$-tag E1ec to enable thorough purification needed for the NMR studies and demonstrated that this construct could function analogously to the parental E1ec (see supplemental Experimental Procedures).

**Sequence-specific Resonance Assignments in the Flexible Regions of E1ec**

The lack of interpretable electron density for residues 1–55, 401–413, and 541–557 in the high resolution x-ray structure of E1ec (7) implied that these regions are highly disordered, at the same time suggesting that perhaps they could be sufficiently mobile to give rise to narrow NMR signals. Guided by this hypothesis, the $^1$H,$^{15}$N TROSY-HSQC spectrum of a uniformly mobile to give rise to narrow NMR signals. Guided by this hypothesis, the $^1$H,$^{15}$N TROSY-HSQC spectrum of a uniformly mobile regions.

**A Glycine Auxotroph Assisted in the Assignment of Resonances to Glycines**—There were well resolved resonances in the non-TROSY spectrum of the same protein (Fig. 3a). This resonance was superimposed on the $^1$H,$^{15}$N TROSY-HSQC spectrum of the uniformly $^{15}$N-labeled His$_6$-tag E1ec was compared with the non-TROSY spectrum of the same protein (Fig. 3a). Because there is only a single pair of adjacent glycines in the mobile regions, there should be only one $^1$H,$^{15}$N (of Gly-47) directly bonded to a $^{13}$CO (of Gly-46), giving rise to a unique resonance in the three-dimensional HNCO NMR experiment, as is indeed the case in Fig. 3b. This resonance was superimposed on the $^1$H,$^{15}$N TROSY-HSQC spectrum of the uniformly $^{15}$N-labeled His$_6$-tag E1ec, and the NH corresponding to residue Gly-47 was assigned (Fig. 3c expanded in Fig. 3d, Table 2).

**Assignment of Sole Glycines in the Mobile Active Center Loops**—Fortuitously, the inner loop (401–413) and the outer loop (541–557) each has only a single glycine, and these were converted to alanine to help assign the resonance to residues Gly-402 (inner loop) and Gly-542 (outer loop) by the difference of the spectra of E1ec and the G402A (activity in complex = 54.2%) or G542A (activity in complex = 20.1%) variants (Fig. 4, a and b, Table 2). Such side chains are characterized by a pair of resonances with the same $^1$N but different $^1$H chemical shifts, due to slow rotation around the amide bond on the NMR time scale. The chemical shift regions are 6.6–7.7 ppm for $^1$H and 115–118 ppm for $^1$N in the spectrum of His$_6$-tag E1ec. Site-directed mutagenesis was carried out, substituting glutamine with histidine at positions 18, 33, and 38, and uniformly $^{15}$N-labeled enzymes were prepared for each E1ec variant. The activities of the PDHc-ec complex with these substitutions were (in parentheses, % compared with His$_6$-tag E1ec) Q18H (100%), Q33H (55%), and Q38H (66%), indicating no significant effect on the activity (ThDP enzymes accelerate the rates by 10$^{12}$–13 or more (35); such reductions in activity as reported here for all of the variants make only minor or insignificant changes in the structure and will not complicate interpretation of the glycines in His$_6$-tag E1ec were labeled by having an equimolar concentration of $^{15}$N-labeled and $^{13}$C-labeled glycine in the medium (see “Experimental Procedures”), and the NH plane was processed from the three-dimensional data. The $^1$H,$^{15}$N TROSY-HSQC spectrum of His$_6$-tag E1ec was compared with the non-TROSY spectrum of the same protein (Fig. 3a).

**Assignment of Sole Glycines in the Mobile Active Center Loops**—Fortuitously, the inner loop (401–413) and the outer loop (541–557) each has only a single glycine, and these were converted to alanine to help assign the resonance to residues Gly-402 (inner loop) and Gly-542 (outer loop) by the difference of the spectra of E1ec and the G402A (activity in complex = 54.2%) or G542A (activity in complex = 20.1%) variants (Fig. 4, a and b, Table 2).

**Sequence-specific Assignment of Glutamine Side-chain Resonances in the N-terminal Region of His$_6$-tag E1ec**—Such side chains are characterized by a pair of resonances with the same $^1$N but different $^1$H chemical shifts, due to slow rotation around the amide bond on the NMR time scale. The chemical shift regions are 6.6–7.7 ppm for $^1$H and 115–118 ppm for $^1$N in the spectrum of His$_6$-tag E1ec. Site-directed mutagenesis was carried out, substituting glutamine with histidine at positions 18, 33, and 38, and uniformly $^{15}$N-labeled enzymes were prepared for each E1ec variant. The activities of the PDHc-ec complex with these substitutions were (in parentheses, % compared with His$_6$-tag E1ec) Q18H (100%), Q33H (55%), and Q38H (66%), indicating no significant effect on the activity (ThDP enzymes accelerate the rates by 10$^{12}$–13 or more (35); such reductions in activity as reported here for all of the variants make only minor or insignificant changes in the structure and will not complicate interpretation of the glycines in His$_6$-tag E1ec were labeled by having an equimolar concentration of $^{15}$N-labeled and $^{13}$C-labeled glycine in the medium (see “Experimental Procedures”), and the NH plane was processed from the three-dimensional data. The $^1$H,$^{15}$N TROSY-HSQC spectrum of His$_6$-tag E1ec was compared with the non-TROSY spectrum of the same protein (Fig. 3a).

**Assignment of Sole Glycines in the Mobile Active Center Loops**—Fortuitously, the inner loop (401–413) and the outer loop (541–557) each has only a single glycine, and these were converted to alanine to help assign the resonance to residues Gly-402 (inner loop) and Gly-542 (outer loop) by the difference of the spectra of E1ec and the G402A (activity in complex = 54.2%) or G542A (activity in complex = 20.1%) variants (Fig. 4, a and b, Table 2).

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**Assignment of Sole Glycines in the Mobile Active Center Loops**—Fortuitously, the inner loop (401–413) and the outer loop (541–557) each has only a single glycine, and these were converted to alanine to help assign the resonance to residues Gly-402 (inner loop) and Gly-542 (outer loop) by the difference of the spectra of E1ec and the G402A (activity in complex = 54.2%) or G542A (activity in complex = 20.1%) variants (Fig. 4, a and b, Table 2).
The dispersion of the resonances was essentially superimposable on those for E1ec, confirming no change in secondary or tertiary structure with the substitutions. Next, a $^1$H,$^15$N TROSY-HSQC spectrum was acquired for each variant and compared with the E1ec spectrum (Fig. 5, a–f). We could assign each resonance to a specific glutamine in the sequence from such comparisons (Table 2); with each substitution a set of two peaks with the resonance assignments).
same $^{15}$N chemical shift but different $^1$H chemical shift was missing.

**A Synthetic Peptide with Sequence Corresponding to the N-terminal 1–35 Residues Enabled Assignment of Gly-28 and Confirmed That of Trp-16**—A peptide corresponding to the N-terminal 1–35 amino acids of E1ec was synthesized (SERFPNDVDPITRDWLQAIIESVIREEGVERAQYL) (N-term peptide; mass, 4161.4 Da; theory, 4161.6 Da). The CD spectrum of N-term peptide exhibits the negative band at 200 nm at pH 7.0, characteristic of unfolded peptides. Two negative bands were observed at 222 and 206 nm at pH 5.0, characteristic of $^1$H9251/H9251-$^1$H helix conformation (Fig. 6). To determine whether the synthesized peptide forms a functional binding domain, the E2ec-E3ec subcomplex was incubated with 0.050–1 mM concentrations of N-term peptide. On reconstitution of E2ec-E3ec subcomplex with E1ec, ~40% of activity was lost, indicating that the N-term peptide competes with E1ec for binding to E2ec. The limited inhibition suggests that N-term peptide is insufficient in length to fully mimic the N-terminal region of E1ec.

We next carried out a two-dimensional $^1$H,$^{15}$N HSQC experiment (non-TROSY this time to provide better sensitivity) of the unlabeled N-term peptide (7.6 mM) (supplemental Fig. S2). The peptide gave an excellent two-dimensional HSQC spectrum notwithstanding the low $^{15}$N natural abundance (0.36%). The spectrum was aligned to the TROSY spectrum of His$_6$-tag E1ec for comparison, leading to several conclusions: 1) the narrow dispersion of the resonances suggests that both the synthetic peptide and the N-terminal region of E1ec (and dynamic regions in general) are in the random conformation; 2, two residues are immediately assignable, Trp-16 and Gly-28 NH (Table 2), also providing $^1$H and $^{15}$N chemical shifts for the random coil form of the Trp-16 side chain.

**Identification of Residues/Regions of E1ec Interacting with E2ec**

N-Terminal E1ec Residues That Interact with an N-terminal E2ec Construct According to NMR—The E2ec component is composed of five domains. Starting from the N-terminal end, there are three lipoyl domains (LD) followed by the PSBD and the catalytic or core domain (Scheme 2). Although the lipoyl domain undergoes reductive acetylation by the E1ec and pyruvate, the PSBD has been long believed to be important as an anchor for both the E1ec and E3ec components. We used here a single lipoyl domain construct of E2ec (1-lip E2ec, Ref. 36) that can indeed be reconstituted to active PDHc and is only modestly less active than the wild-type three-lipoyl domain enzyme. One of the continuing challenges to those working with these important and ever-fascinating complexes is the high degree of oligomerization of the E2 components (24-mer for E2ec). This oligomerization renders the full-length E2ec difficult to handle. To circumvent the oligomerization issue, starting with the 1-lip E2ec, we created a construct consisting of the lipoyl domain and the PSBD for a total length of 190 amino acids from the N-terminal amino acid (E2ec$^{1–190}$, see the supplemental Experimental Procedures). Such a construct is usually denoted as a “didomain.” To
gain insight to the residues of E1ec reacting with the didomain, we inspected the two-dimensional HSQC spectrum of E1ec on the addition of E2ec1–190. The premise of the experiment is that, should the N-terminal region become organized (less mobile or dynamic) on complexation with the E2ec1–190, the resonances pertinent to the N-terminal region will lose much (or all) of their intensity. This expectation is indeed met in Fig. 7: 1) the side-chain NH resonance for Trp-16, 2) the NH resonance for Gly-47, and 3) the pairs of resonances for the side chains of glutamines 18, 33, and 38 are all reduced/absent in the complex (red) compared with the E1ec spectrum per se. We believe that these residues of E1ec indeed interact with the E2ec construct and their positions (16, 18, 33, 38, and 47) give us confidence that the entire N-terminal region does participate in the complex in a dramatic fashion. It is also important to point out that there are other mobile E1ec resonances unperturbed by the complexation with E2ec1–190, presumably located at other flexible regions of the E1ec (see below), but also providing excellent control for the interpretation that the observations do pertain selectively to the N-terminal region.

**N-Terminal E1ec Residues That Interact with PSBD**—A two-dimensional HSQC spectrum was recorded for His6-tag E1ec in the presence of a 2-fold molar excess of a synthetic peptide with a sequence corresponding to the E2ec PSBD: H2N-ATPLIRLAREFGVNLAKV-KGTGRGKRLREDVQAYVKEL-OH (CHI Scientific, Maynard, MA). The spectrum (Fig. 8) appeared very similar to that obtained with E2ec1–190, indicating that the PSBD of the didomain is mostly responsible for E1ec-E2ec recognition.

**N-Terminal E1ec Residues That Interact with Independently Expressed Lipoyl Domain**—When the experiment outlined in the previous paragraph was repeated in the presence of a 2-fold molar excess of independently expressed lipoyl domain over His6-tag E1ec, 106 resonances were detected of 109 total detected for His6-tag E1ec (see supplemental Fig. S3, a and b). None of the resonances assigned in the N-terminal region and found to interact with either didomain or PSBD was affected by the lipoyl domain.

**Interaction of the Flexible Active Center Loops of E1ec with the Stable Predecarboxylation Intermediate PLThDP**—Having assigned resonances that could act as reporters of each of the two active center loops (Gly-402 and Gly-542), we are in an excellent position to determine any changes in mobility on the addition of PLThDP, a stable predecarboxylation intermediate that resembles LThDP in Scheme 1 ($k_1, k_2$). We have very strong evidence from both x-ray and dynamic measurements (9, 14, 16, 43) that in the presence of PLThDP these two loops become organized and are now observed in the x-ray structure. In fact, the addition of PLThDP to His6-tag E1ec, selectively broadens the NH resonances corresponding to Gly-402 and Gly-542 but not those resonances assigned to the N-terminal residues (Fig. 9). This experiment is not only important to support all of the previous data on this important dynamic property but also serves as an outstanding control.
experiment, indicating that the observations are indeed selective to the particular interaction loci; the PLThDP interacts with the active center loops but not with the N-terminal region.

Quantification of Residues Identified in the Flexible E1ec Regions—Given that we have only made sequence specific resonance assignment to 8 of the 886 amino acids present in E1ec, it is important to address the issue of specificity of the observations. First of all, we have several reporters spanning residues 1–47 and 1 in each of the two active center loops. A summary of the resonances observed under different conditions is given in Table 3. In the region of chemical shifts presented in the two-dimensional $^1$H,$^1$N HSQC spectra, we expect to see resonances pertinent to (a) main-chain NHs, one for each peptide bond with the exception of those to proline, (b) two side-chain NHs for each Asn and Gln and one for each Trp. The number of resonances accounting for these is 64 for the N-terminal 1–55 residues, 19 for the inner (401–413), and 26 for the outer (541–557) active center loops for a total of 109. Using the program NMRviewJ peak counting with the same noise filtration for all spectra, we estimate the presence of 103 resonances for E1ec in the presence of saturating ThDP and 101 resonances in the absence of ThDP (i.e. apoenzyme).

Apparently, in terms of number of resonances observed, the numbers anticipated in the three flexible regions account for all of the observations within experimental error; coupled with the sequence specific assignments, we have strong evidence that these indeed are the regions observed in the spectra (Table 3). For the complexes, we conclude the following. (i) The addition of the E2$^{1–190}$ didomain results in the elimination of 44 resonances (of the 103 total observed and of the 64 calculated for the N-terminal region); apparently, not all residues in the N-terminal region are highly ordered in the complex. (ii) The addition of the synthetic peptide corresponding to PSBD results in the elimination of 38 residues, the same ones as missing on addition of E2$^{1–190}$ in (i). (iii) The addition of independently expressed lipoyl domain results in no assigned resonance being eliminated within experimental error. These numbers strongly support the conclusion that the PSBD is the important moiety of the didomain that binds to the E1ec, specifically to its N-terminal regions, whereas the lipoyl domain binds very weakly at best. (iv) The addition of PLThDP results in the elimination of 33 resonances (of the 101 total observed and of the 45 resonances observed in the absence of ThDP.

**TABLE 3**

| Experimentally estimated and theoretical number of resonances on E1ec in the three flexible regions in the $^{15}$N-1H HSQC spectra |
|---|
| N-terminal region | Inner loop | Outer loop |
| No. of residues$^a$ | 55 (1–55) | 13 (401–413) | 17 (541–557) |
| No. of Gln | 3 | 1 | 4 |
| No. of Asn | 2 | 2 | 1 |
| No. of Pro | 2 | 0 | 1 |
| No. of Trp | 1 | 0 | 0 |
| Theoretical no. of resonances | 64 | 19 | 26 |
| No. of resonances estimated (experimental/theoretical) | 103/109$^b$ |
| No. of resonances undetected on complexation | 101/109$^c$ |

$^a$ Each backbone NH contributes one resonance. The side chains of glutamine and asparagine give rise to two resonances on two-dimensional HSQC spectra. For each proline present, the total number of resonances was decreased by one. The Trp gives rise to the indole NH resonance.

$^b$ Spectrum of His$_6$-tag E1ec.

$^c$ Spectrum of His$_6$-tag E1ec without ThDP.

$^d$ Spectrum of His$_6$-tag E1ec in the presence of E2ec$^{1–190}$.

$^e$ Spectrum of His$_6$-tag E1ec in the presence of synthetic peptide corresponding to PSBD.

$^f$ Spectrum of His$_6$-tag E1ec in the presence of PLThDP.

**FIGURE 10.** A, the predicted secondary structure of the N-terminal 1–55 region (program from Protein Homology/analogY Recognition Engine) is shown. B, the Cu atoms were generated from ExPASy Proteomics server. Main-chain and side-chain atoms were generated with the program COOT (41); the final figure was with RIBBONS (42).
estimated for the inner and outer dynamic active center loops), and the specificity is confirmed by the elimination of the resonances assigned to Gly-402 and Gly-542, reporting on the inner and outer loops, respectively, but not any of those assigned to specific residues in the N-terminal region. These results confirm our series of studies on these loops and, equally importantly, point to the specificity of the NMR observations.

Summary and Conclusions—These NMR results provide the first structural support to the notion that the entire N-terminal region (residues 1–55) of the E1ec is responsible for binding the E2ec component. The N-terminal region of E1ec, although not seen in the x-ray structures, gives well resolved resonances and permitted sequence specific assignment for six resonances (Trp-16, Gln-18, Gly-28, Gln-33, Gln-38, and Gly-47). These assignments complement the biochemical findings on this region; substitution of several residues in this region dramatically reduced the activity of the reconstituted complex and affected intercomponent assembly (interaction of E1ec with E2ec). It is also clear, however, that not all residues in this N-terminal region have a role in assembly. For example, the chemical shift of the resonance for Gly-28 does not change under various conditions.

The narrow dispersion of the NMR resonances observed both in the N-terminal 1–35 synthetic peptide and in the N-terminal region of E1ec suggest that both are in the random conformation, i.e. they are disordered in the absence of E1-E2 assembly.

Interaction of E1ec with either E2ec1–190 or PSBD, but not with the lipoyl domain, leads to reduction/absence of the side-chain NH resonance for Trp-16, the NH resonance for Gly-47, and of three pairs of resonances for the side chains of glutamines 18, 33, and 38 and indicate that residues from the entire N-terminal region participate in the complex and that the PSBD, but not the lipoyl domain, is essential for E1-E2 interaction.

Concerning the effect of the N-terminal substitutions on individual reaction steps, we conclude the following. (a) None of the substitutions affected the rate of the E1-specific reaction, i.e. the reaction was unaffected through pyruvate decarboxylation. (b) Similarly, we showed that these substitutions do not affect formation of the first covalent intermediate LThDP. (c) Studies on the reductive acetylation of E2ec suggested that binding of the N-terminal region of E1ec to the PSBD of E2ec is essential before reductive acetylation could take place, complementing the NMR results.

The N-terminal region of E1ec is predicted to have a propensity for forming two α-helices (residues 11–26 and 29–45) and a small loop (residues 27 and 28) joining the two α-helices (Fig. 10, a helix-turn-helix motif), similar to the model suggested for the N-terminal region of the E1 component from the related bacterium A. vinelandii (12). According to the structural and functional analysis here presented, if this is the true secondary structure of the N-terminal region of the E1ec in the complex, both helices participate in the interaction with E2ec. Should assembly indeed require that the N-terminal region assume a secondary structure modeled in Fig. 10 and predicted, one could speculate that the residues “silent” to substitution do not participate in assembly.

The addition of PLThDP to His6-tag E1ec selectively broadens the NH resonances corresponding to Gly-402 and Gly-542, consistent with its effect on the active center loops, but does not affect those resonances assigned to the N-terminal residues. This indicates that the NMR observations are selective to the particular interaction locus.

The results here reported have broader significance/consequence to other members of this important class of multienzyme complexes. (i) The N-terminal region of the E1 component of the E. coli 2-oxoglutarate dehydrogenase complex was also suggested to interact with its E2 component (37). (ii) For this class of bacterial 2-oxoacid dehydrogenase complexes, the PSBD is the most important domain for recognition of E1, rather than the lipoyl domain, as was suggested by others (38, 39). To our knowledge our results here presented offer the first experimental evidence for this conclusion. (ii) The results also clearly show that with such large proteins, the NMR and x-ray results are indeed complementary; what is not seen in the x-ray may be visible in the NMR spectrum and vice versa. In particular, the N-terminal 1–55 residues and the two active center loops not seen in the x-ray structure have now been shown to provide resolvable resonances in the NMR spectrum of E1ec.

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