Specificity Determinants for the Pyruvate Dehydrogenase Component Reaction Mapped with Mutated and Prosthetic Group Modified Lipoyl Domains*

Received for publication, December 20, 1999, and in revised form, January 27, 2000

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Efficient catalysis in the second step of the pyruvate dehydrogenase (E1) component reaction requires a lipoyl group to be attached to a lipoyl domain that displays appropriately positioned specificity residues. As substrates, the human dihydrolipoyl acetyltransferase provides an N-terminal (L1) and an inner (L2) lipoyl domain. We evaluated the specificity requirements for the E1 reaction with 27 mutant L2 (including four substitutions for the lipoylated lysine, Lys173), with three analogs substituted for the lipoyl group on Lys173, and with selected L1 mutants. Besides Lys173 mutants, only E170Q mutation prevented lipoylation. Based on analysis of the structural stability of mutants by differential scanning calorimetry, alanine substitutions of residues with aromatic side chains in terminal regions outside the folded portion of the L2 domain significantly decreased the stability of mutant L2, suggesting specific interactions of these terminal regions with the folded domain. E1 reaction rates were markedly reduced by the following substitutions in the L2 domain (equivalent site-L1): L140A, S141A (S14A-L1), T143A, E162A, D172N, and E179A (E52A-L1). These mutants gave diverse changes in kinetic parameters. These residues are spread over >24 Å on one side of the L2 structure, supporting extensive contact between E1 and L2 domain. Alignment of over 40 lipoyl domain sequences supports Ser141, Thr143, and Glu179 serving as specificity residues for use by E1 from eukaryotic sources. Extensive interactions of the lipoyl-lysine prosthetic group with the active site are supported by the limited inhibition of E1 catalysis by Lys172 or enzymatic addition of lipote analogs to Lys173. Thus, efficient use by mammalian E1 of cognate lipoyl domains derives from unique surface residues with critical interactions contributed by the universal lipoyl-lysine prosthetic group, key specificity residues, and some conserved residues, particularly Asp172 adjacent to Lys173.

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1 The abbreviations used are: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase component; TPP, thiamin pyrophosphate; E2, dihydrolipoyl acetyltransferase component; L1, NH₂-lipoyl domain of E2; E3, dihydrolipoyl dehydrogenase; E3BP, E3-binding protein; GST, glutathione S-transferase.

2 S. Liu, X. Gong, X. Yan, S. Ravindran, P. M. Robben, J. C. Baker, L. A. Andersson, A. B. Cole, and T. E. Roche, submitted for publication.
lipoyl domain structure is required to allow an E1 source to use its cognate lipoyl domains and not use lipoyl domains from other complexes. In the case of mammalian PDC, this specific molecular recognition by the E1 component must exclude using the lipoyl domains provided by other mitochondrial systems: the α-ketoglutarate dehydrogenase complex, the branched chain α-keto acid dehydrogenase complex, and the glycine cleavage enzyme. In marked contrast, the lipoyl domains of all of these multienzyme systems are substrates for a common E3 component which catalyzes the reversible conversion of each of their reduced lipoyl groups to the disulfide form coupled to the reduction of NAD⁺ (1, 5, 6).

Studies using bacterial PDC E1 and various lipoyl domain sources have delineated structural features of the lipoyl domains that contribute to specific recognition (7, 8). The first loop in the lipoyl domain structures, which is located adjacent to the lipoyl prosthetic group, was shown to contribute to reaction specificity (8–10). These studies have focused on residues fairly close to the lipoyl group and have provided limited insights into the relative importance of interactions of the prosthetic group and the lipoyl domain. Despite the high specificity of an E1 for its cognate lipoyl domain, only weak interactions between E1 and its lipoyl domain substrate have been detected (11). Indeed, dissociation constants for E1-lipoyl domain interactions appear to be much higher than the K_m of E1 for lipoyl domain substrates. A weak interaction is probably a desirable feature for sustaining high efficiency since the flexibly held lipoyl domains are concentrated in mammalian PDC at >1.0 mM (12, 13) next to E1 and should, therefore, efficiently ferry lipoyl groups into the active site of E1.

The recent crystal structure of the Pseudomonas putida branched chain α-keto acid dehydrogenase, also an α/β tetramer, show that the active site is a long funnel requiring the full reach of the lipoyl lysine prosthetic group (14). Consistent with specificity determining interactions being provided by specific interactions between E1 and the cognate lipoyl domains of a particular complex, it was predicted that the lipoyl domain would need to be positioned close to the surface of the E1 for the reaction to occur. Similar requirements for the lipoyl-lysine cofactor to use its length in reaching into deep active sites also formed at subunit interfaces in the E2 (15, 16) and E3 (23) reaction, as described previously (23). Compact mobility in native gel electrophoresis, which readily detects the degree of lipoylation, Lys173, was also mutated to Met or Leu with L2(120–233) and to Glu using the L2(128–229) structure. Neighboring the lipoylation site, the L2(129–229) mutations included: E170Q, D172N, and A174S. The peptides of mutated amino acids in the L2 structure are shown in Fig. 2. L1(1–128) was mutated by alanine substitution at Ser14, Lys46 (site of lipoylation), Glu179, and Asp197 in the L2 domain. Lipoyl domain sequences, obtained from GenBank were aligned by the Genetics Corporation Group Pileup program, Wisconsin Package version 9.0, University of Wisconsin.

Native and mutated lipoyl domains, purified as described previously (23), were evaluated by native gel electrophoresis, which readily detects the degree of lipoylation (23, 24, 26). The effective use of the lipoyl group is further tested by the use of lipoyl domains as substrates in the E3 reaction, as described previously (23). Compact mobility in native gel electrophoresis requires well folded domains. As reported here, changes to forms that tend to aggregate are also detected by native gel electrophoresis. In some cases, masses were also evaluated by matrix-assisted laser desorption ionization mass spectrometry using a Laserbot 2000.

Melting transitions for the cooperative unfolding of domains were determined by DSC using MicroCal VP-DSC (27) and a scanning rate of 1 °C/min unless otherwise indicated. Lipoyl domains (0.7–2 mg/ml) were dialyzed into 20 mM potassium phosphate buffer (pH 7.0); scans of buffer alone were subtracted from those for the lipoyl domains. After the first scan of a sample, the solution was cooled and re-scanned to test the reversibility of unfolding. T_m values reported are the transition mid-point temperatures. For estimation of ΔH values, base line corrections were made using a smooth transition between the pre- and post-melting transition baselines. Protein concentrations were determined by measurements of absorbance at 280 nm standardized based on amino acid composition. For wild type L2e, this gives a predicted e_L2e = 2.37

EXPERIMENTAL PROCEDURES

Materials—Bovine kidney E1 was prepared as described previously from the purified kidney PDC (20, 21). As will be described in detail elsewhere, human E1 was prepared by restructuring the preparation of Korotchkin et al. (22) so that His-tag could be removed by treatment with Precision Protease and changing the purification procedure to eliminate a contaminant. The sodium salt of β-(2-14C)pyruvate purchased from NEN Life Science Products Inc. was quantified by measuring the incorporation of NADH formed (isospecific limiting) using the over-expressed PDC reaction and liquid scintillation counting and further analyzed by separation on AMINEX ion exclusion HPX87H column (Bio-Rad) with isocratic elution using 5 mM H₂SO₄ with continuous monitoring radioactivity using a PACKARD TRACE 7140. [2-14C]Pyruvate preparations gave a single peak; different preparations had specific radioactivities of 20.7 to 34 counts/min/mg. Oligonucleotides were purchased from Oligos Etc. or synthesized on an Applied Biosystems 381A DNA synthesizer. Pfu polymerase was from Stratagene.

Preparation and Evaluation of Lipoyl Domain Constructs—Expression conditions (23, 24) were compared and modified as described under "Results." Optimal expression of GST-lipoyl domain constructs was obtained with the host Escherichia coli BL21 grown at 37 °C to 0.8 to 0.9 absorbance at 600 nm followed by induction with 1 mM isopropyl-1-thio-β-d-galactopyranoside and expression at 22–24 °C for 6 h. Prior to preparation of mutated L1(1–128) and L2(120–233) structures, silent mutations were introduced flanking the lipoyl domains to produce restriction sites and the corresponding changes were introduced into the coding sequence for native E2 so mutated lipoyl domains could be produced. Oligonucleotide modified E2 oligomers (which had been introduced into the pGEX-2T plasmids to produce single amino acid substitutions in GST-L1(1–128) or GST-L2(120–233) were introduced using offset oligonucleotide primers containing the appropriate base change(s) and a design which allowed replication only of the parental strands. Replication was performed using Pfu polymerase through 10 (one base change) or 12 (two-base change) replication cycles. This was followed by digestion of the methylated parent strands with DpnI endonuclease (target sequence 5’-GmATC-3’). Mutants in L2(128–229) were introduced using the Altered Sites mutagenesis kit (Promega Corp.); some properties were previously described (26). All mutations were confirmed by dideoxy sequencing. In the L2(120–233) alanine was substituted for Tyr148, Leu151, Ser153, Thr157, Thr158, Val159, Lys163 (site of lipoylation), Glu179, Leu180, Asp187, Phe188, and Thr192. The basic residues Lys184 and Arg195 were substituted with glutamine, and selected acidic residues in the extended C-terminal tail were converted to their corresponding amides (e.g. D213N, D219N, and E224Q). The site of lipoylation, Lys173, was also mutated to Met or Leu with L2(120–233) and to Glu using the L2(128–229) structure. Neighboring the lipoylation site, the L2(129–229) mutations included: E170Q, D172N, and A174S. The peptides of mutated amino acids in the L2 structure are shown in Fig. 2. L1(1–128) was mutated by alanine substitution at Ser14, Lys46 (site of lipoylation), Glu47, and Asp50 which correspond to Ser141, Lys173, Glu179, and Asp197 in the L2 domain. Lipoyl domain sequences, obtained from GenBank were aligned by the Genetics Corporation Group Pileup program, Wisconsin Package version 9.0, University of Wisconsin.

Native and mutated lipoyl domains, purified as described previously (23), were evaluated by native gel electrophoresis, which readily detects the degree of lipoylation (23, 24, 26). The effective use of the lipoyl group is further tested by the use of lipoyl domains as substrates in the E3 reaction, as described previously (23). Compact mobility in native gel electrophoresis requires well folded domains. As reported here, changes to forms that tend to aggregate are also detected by native gel electrophoresis. In some cases, masses were also evaluated by matrix-assisted laser desorption ionization mass spectrometry using a Laserbot 2000.

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The numbers in parentheses after lipoyl domain designations correspond to the amino acid sequence of human E2 included in the construct.

Lipoyl Domain Determinants

9650 m⁻¹ cm⁻¹; both constructs of wild type L2 share all aromatic residues and therefore have the same molar extinction coefficient. On a per mg basis, this ε₉₅₀ translates for L2(120–233) to 0.74 A₉₅₀/mg/ml/cm and for L2(128–229) to 0.815 A₉₅₀/mg/ml/cm. Protein concentrations obtained with the BCA method using bovine serum albumin as a standard (23) generally gave protein values 55–65% as high as the values determined by 280 nm absorbance for domains exhaustively dialyzed into 20 mM phosphate buffer.

Circular dichroism (CD) spectra were obtained for all lipoyl domain structures using a quartz cuvette with a 1-mm light path in 20 mM potassium phosphate (pH 7.0) maintained by water jacketed holder at 20 °C using a Cary 5200 spectrometer by averaging at least 16 scans each accumulated at 50 nm/min. Spectra from 300 to 180 nm were collected at 0.13–0.15 mg/ml lipoyl domain. Protein concentrations were based on 280 nm absorbance. The instrument was calibrated with using Dl-10-camphorsulfonic acid as a standard.

E1 Activity Assays—The rate of acetylation of lipoyl domain constructs was measured by minor modification of procedure of Liu et al. (23). E1 was diluted in 50 mM potassium phosphate (pH 7.6) containing 7.5 mg/ml bovine serum albumin and 0.25 mM EDTA. In duplicate or triplicate, the lipoyl domain construct was equilibrated at 30 °C in 100 mM potassium phosphate (pH 7.0) and developed versus 20 mM potassium phosphate buffer (pH 7.0). To the rear of the folded domain, substitutions of well exposed hydrophilic groups, substitutions were made at hydrophobic side chains that are well exposed to solvent at the surface of the lipoylated domain next to and just interior to Lys173. Cys and Trp do not have significant exposure at the surface of the lipoylated domain. The positions of all mutated amino acids and the location of the specificity loop in the lipoyl domain. The positions of all mutated amino acids and the location of the specificity loop in the lipoyl domain construct to Lys173 (T145A, E170Q, D172Q, A174S, R196Q, and D197A).

RESULTS AND DISCUSSION

Mutation Sites in Lipoyl Domains—Fig. 1 shows a ribbon diagram and space filled presentations of the human L2 domain. These models are based on the structure of Howard et al. (19), but the extra amino acids on the N-terminal, which are not part of the L2 structure are removed. The ribbon diagram (panel A) shows L2 is a the flattened β-barrel structure made up of two antiparallel β sheet regions. The position of Lys173 is marked with an arrow and the specificity loop (loop 1) with a star. Panel B shows a space filled model positioned in a similar orientation to the ribbon diagram in Panel A and panel C shows the opposing side of the lipoyl domain. The positions of all mutated amino acids and the location of the specificity loop in the L2 structure are indicated. Residues are numbered according to their sequence in mature E2 which is 123 higher than the numbering used in Ref. 19. Each asterisk in panels B and C indicates a residue that is a different amino acid in the aligned sequence of the L1 domain. Colors used: Aap is red; Gua is orange; Lys and Arg are blue except Lys, which is magenta; Gin and Asn are burgundy; Ser and Thr are green; Pro, Met, and Gly are white; Leu, Ile, and Val are yellow; and Phe and Tyr are gray. Ala residues are white in panel A but are yellow in panel B. Cys and Trp do not have significant exposure at the surface of L2 and were left white.

The folded domain includes a high proportion of acidic residues with those on the upper side located away from basic residues and those on the lower side generally located near basic residues. The opposing side of the folded domain has a hydrophobic face (Y129 residues, Fig. 1, panel C) centered around Leu190, has only one acidic residue (Asp197), and prominent basic residues. Besides prominent hydrophilic groups, substitutions were made at hydrophobic side chains that are well exposed to solvent at the surface of the folded portion of the L2 domain (Leu186, Val186, and Leu190) and in terminal regions (e.g. Phe177). At the lipoylated end of the molecule, alamine substitutions were made for solvent exposed residues in the specificity loop (L140A, S141A, and T143A) and around the lipoyl domain next to and just interior to Lys273 (T145A, E170Q, D172Q, A174S, R196Q, and D197A).

The rear of the folded domain, substitutions of well exposed hydrophilic residues included Q134A, E162A, E179A, Q181A, and K188Q. Substitutions were also made for residues with a potential for functional importance in the N-terminal (Y129A)
and C-terminal (D213N, F217A, D219N, Y220A, R221Q, and E224Q) regions.

Corresponding to Ser\textsuperscript{141}, Glu\textsuperscript{170}, Asp\textsuperscript{197} in L2, Ser\textsuperscript{14}, Glu\textsuperscript{52}, and Asp\textsuperscript{70} were substituted in L1 based on these residues being seldom found in the aligned sequences of bacterial PDC lipoyl domains or domains of other \(\alpha\)-keto acid dehydrogenase complexes. Thus, there seemed to be a high likelihood of these being specificity residues. Beyond the pursuit of the full range of E1 interactions, these L2 mutants are also being used to determine the basis for the specific interactions of certain kinase and phosphatase isoforms of pyruvate dehydrogenase complex with the L2 domain.\textsuperscript{5} Indeed, Tyr\textsuperscript{129}, Glu\textsuperscript{134}, Thr\textsuperscript{145}, Gln\textsuperscript{181}, and Val\textsuperscript{156} would not be expected to be major specificity residues for E1 function since these residues differ in L1, a good E1 substrate;\textsuperscript{2} however, these residues may contribute to specificities of different regulatory enzymes. The testing of all mutants for E1 use is important, since effective use in the E1 reaction constitutes strong evidence that a mutant domain retains near native folding to allow correct presentation of the residues that interact with E1.

\textbf{Lipoyl Domain Properties—Besides substitutions for the Lys that undergoes lipoylation (Lys\textsuperscript{48} and Lys\textsuperscript{173} in L1 and L2, respectively), only the substitution E170Q prevented lipoylation of L2 by the bacterial lipoyl-protein ligase. Besides not being used in the E1 and E3 reactions, the lack of lipoylation was confirmed by the mass determined by matrix-assisted laser desorption ionization-MS. The E170Q-L2 is well folded (below). Fujiwara \textit{et al.} (29) demonstrated the importance of Glu residues in the corresponding position (3 residues to the N-terminal side of the lipoylation site) for the lipoylation of various lipoyl domain sources by the mammalian lipoyl-AMP: \(N^\alpha\)-lysine lipoyl transferase. Thus, the \textit{E. coli} lipoyl-protein ligase and mammalian enzyme apparently share a need for this residue.

With many mutated L1 or L2, native gel electrophoresis detected small portions of nonlipoylated domain (upper band) with the level being <1 to 5\% for expression at 23–25 °C and 3 to 30\% when expression is at 37 °C for wild type or mutant L1 or L2. Like the wild type construct (lane 1, upper panel, Fig. 2), mutant L2(120–233) structures expressed at 37 °C (lanes 2 and 6, upper panel) generally contained low levels of nonlipoylated domain although the level of this upper band rose to as high as 30\% with the T145A mutant and with all L2(128–229) constructs (lane 8, wild type). Expression at 23–25 °C greatly reduced or eliminated the nonlipoylated constructs (lanes 3 and 10 in upper panel and lanes 1–4 in the lower panel) even in the case of the T145A construct (lane 5) or A174S (lane 4) and D172N (not shown) which are L2(128–229) structures mutated at the sites adjacent to the lipoylated Lys\textsuperscript{173}. The latter finding is consistent with mutations of the \textit{Bacillus stearothermophilus} PDC-E2 lipoyl domain in which Met and other residues were introduced on either side of the lipoylated lysine without affecting the relative extent of lipoylation (30). However, the wild type form of this bacterial domain was only partially lipoylated (~30\%) in \textit{E. coli}. Lipoylation is probably limited by the high expression of this small domain and hindered because it contains a glutamate at the position aligned with Glu\textsuperscript{170} for which glutamine substitution prevented lipoylation of L2. The lipoyl domains of \textit{E. coli} pyruvate and \(\alpha\)-ketoglutarate dehydrogenase complexes have a glutamate at this position (31, 32). The successful removal of nonlipoylated domain by chromatography on a small HIC column was established by native gel electrophoresis, e.g. Fig. 2 for L2(128–229), upper panel, lane 9, prepared from the mixture in lane 8 of lipoylated and nonlipoylated domain. For introduction of lipoate analogs (lanes 6–8, lower panel), nonlipoylated L2(120–233) was prepared by HIC, lower panel, lane 5, from an L2 preparation containing ~80\% nonlipoylated L2 that was made by expressing cells in media not supplemented with lipoate at 37 °C. L190A and F217A were well lipoylated based on the masses determined by matrix-assisted laser desorption ionization-MS, but native gel electrophoresis (Fig. 2) revealed a tendency to aggregate. Primarily dimer was observed with L190A (lane 11) and a combination of dimer and monomer with F217A (lane 12). Leu\textsuperscript{190} is exposed in the center of hydrophobic face in L2, Phe\textsuperscript{217} is located in the unfolded C-terminal region (Fig. 1). It would appear that Leu\textsuperscript{190} and Phe\textsuperscript{217} must participate in interactions that prevent aggregation elicited by the individual alanine substitutions. Consistent with this, deletion of the C-terminal regions of L1 or L2 also led to aggregation. An interaction of the C-terminal lobe with the surface of L2 was not detected in NMR studies (19) suggesting that any interaction is either weak or condition-dependent. An interesting prospect is that the hydrophobic face centered around Leu\textsuperscript{190} interacts with the hydrophobic residues in the C terminus. With the large number of charged residues in the C terminus and next to the hydrophobic face, such a reversible positioning of the C-terminal lobe against the folded domain might also be aided by

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favorable electrostatic interactions.

The above finding of high lipoylation of mutated domains constitutes strong evidence that lipoylated domains were folded into the appropriate β-barrel structure since the E. coli lipoyl-protein ligase requires a folded lipoyl domain with the lipoylated Lys residue located at the tip of a sharp β-turn onto which lipoate is added (28, 30). Additionally domains were tested for their effectiveness as substrates in the E3 reaction (cyclic activity measured) (23). All mutant lipoyl domains that were lipoylated functioned as effective substrates in the E3 reaction. Using 10 μM levels of lipoyl domains, rates within ±15% of 26.7 μmol/min/mg E3 were observed with each of these L2 mutants. This rate is about 5 times faster than the rate with 10 μM lipoamide, but a lipoylated peptide substrate (L2 construct 163–177) was also used at a higher rate.

Mutant lipoyl domains had CD patterns similar to those found with native L1 and L2 structures with a positive peak at 227 nm and a deep trough at 198–200 nm (Fig. 3). The trough depth is decreased with L2(128–229) structures in comparison to L2(120–233) structures, supporting a major contribution of the terminal regions to this feature of the CD spectra. Some variation is observed in the spectra of mutant lipoyl domains. However, CD spectra do not give good insight into the presence of the expected β-barrel fold because the spectra are not typical for a domain containing primarily antiparallel β-structure but are dominated by the contributions of terminal regions, particularly the large C-terminal lobe. This is in marked contrast to B. stearothermophilus PDC-E2 lipoyl domain (30, 33) which gave a typical CD pattern for a structure dominated by antiparallel β-structure. Similar spectra are found with the K173A mutant and native L2 indicating that the absence of a lipoyl group does not greatly change the CD pattern; this was also found with a nonlipoylated Lys173.2

To further establish that the lipoyl domains form stably folded structures, differential scanning calorimetry studies were conducted. Table I (data columns 1 and 2) summarizes Tm and ΔH values determined from these scans; Fig. 4 shows representative Cp profiles for these highly cooperative melting transitions. All but two transitions were nearly symmetric and Tm values were unchanged when multiple cycles of heating-cooling were performed which is consistent with unfolding transitions representing a two-state, irreversible equilibrium process. Tm values were reproduced with considerable accuracy (repeat determinations were invariably within ±0.2 °C). Although Tm values were unchanged in repeated melting cycles, the area of second and third scans were typically reduced 4–10%. The most likely cause is lack of refolding due to aggregation of unfolded domain prior to or during the lowering of the temperature. The combination of base line corrections and the combination of baseline corrections and dilution corrections that were: 0.2 °C, 0.5% for activities between 8 and 17%, and ±1% for activities between 8 and 17%, ±1% for activities between 8 and 17%, and ±1% for activities between 8 and 17%, ±1% for activities between 8 and 17%, and ±1% for activities between 8 and 17%, ±1% for activities between 8 and 17%, and ±1% for activities between 8 and 17%, ±1% for activities between 8 and 17%, and ±1% for activities between 8 and 17%, ±1% for activities between 8 and 17%, and ±1% for activities between 8 and 17%, ±1

![Table I](http://www.jbc.org/)

| L2 construct concentration | Tm(a) °C | ΔH(b) kcal/mol | % E1 activity(c) |
|---------------------------|--------|----------------|-----------------|
| w128–233                  | 68.7   | 74             | 100             |
| w128–229                  | 67.7   | 68             | 100             |
| Y129A                     | 63.6   | 72             | 101             |
| Q134A                     | 67.6   | 66             | 81              |
| L140A                     | 56.0   | 50             | 43              |
| S141A                     | 64.3   | 47             | 4.0             |
| T145A                     | 65.3   | 51             | 4.9             |
| T145A                     | 64.8   | 73             | 94              |
| V156A                     | 61.2   | 78             | 94              |
| E162A                     | 73.9   | 69             | 15              |
| E170Q                     | 68.3   | 74             | 1.4             |
| D172N3                    | 65.0   | 79             | 2.1             |
| K173nonlip                | 67.4   | 68             |                 |
| K173A1                    | 68.0   | 65             |                 |
| K173L                     | 68.6   | 76             |                 |
| K173M                     | 69.3   | 64             |                 |
| K173Q1                    | 70.1   | 67             |                 |
| A174S                     | 67.2   | 78             | 60              |
| E179A                     | 70.2   | 74             | 9.7             |
| Q181A                     | 66.5   | 72             | 88              |
| K188Q                     | 64.2   | 72             | 92              |
| L190A                     | 49.0   | 49             | 62              |
| R196Q                     | 63.0   | 80             | 96              |
| D197A                     | 66.8   | 71             | 112             |
| D197N1                    | 60.6   | 79             | 84              |
| F217A                     | 52.3   | 67             | 60              |
| D218N                     | 66.7   | 52             | 103             |
| Y220A                     | 63.1   | 64             | 84              |
| R221Q                     | 67.8   | 64             | 103             |
| E224Q                     | 67.8   | 69             | 88              |

Values were determined by differential scanning calorimetry as determined under “Experimental Procedures.” E1 activity assays were conducted in 15-μl reaction mixtures using 8 ng of E1 and 40 s reaction time. Data from several experiments are combined and normalized with the rates with the wild type L2 (range for rates at 50 μM wild type L2 was 5.8 to 8.7 μmol/min/mg E1) assigned a value of 100% for each lipoyl domain concentration. The average ratio of rates using wild type L2 at 20 μM to rates at 50 μM L2 in different experiments was 0.58 ± 0.04. Other conditions were as described under “Experimental Procedures.”
appear to involve an entropy-based Boltzmann distribution. The entropy difference between the unfolded and the folded domain and \( T_m \) is in K values were therefore also reduced relative to native L2 with the largest \( \Delta S \) decrease (36%) occurring with S141A mutation. One possibility is that alanine substitution enhances the flexibility of the specificity loop leading to a smaller gain in entropy upon unfolding S141A-L2 while also reducing specific interactions that contribute to stability of the folded domain which also reduces the \( \Delta H \) difference. This suggests that significant organization is contributed by the specificity loop in the native structure even though this region is somewhat flexible (19).

Marked decreases in \( T_m \) were also observed with L190A and F217A L2 mutants. This is consistent with loss of a stabilizing interaction involving the C-terminal lobe which results in the tendency of these mutant L2 to aggregate, as described above. With L190A and F217A a second transition was observed after the main cooperative melting transition. The area of this second (but not the first) transition was greatly reduced by increasing the scan rate from 1 °C/min to 1.5 °C/min. That is consistent with the second phase arising from aggregation of the unfolded domain. Besides F217A substitution, the D213N (Fig. 5) and Y220A mutations in the C-terminal lobe caused significant reductions in \( T_m \). The combination of a reduced \( T_m \) with maintenance of high \( \Delta H \) indicates a \( \Delta S \) gain with the D213N-L2 mutation. This suggests that the C-terminal lobe has reduced flexibility upon converting Asp to Asn and a larger gain in entropy upon unfolding this structure. Given the large number of C-terminal residues separating Asp219 from the folded domain, it is surprising that this mutation reduces the \( \Delta H \) value estimated from melting transitions. Since the D219N mutation lowered the \( \Delta H \) by about 20 ± 6 kcal/mol without significantly affecting \( T_m \), this indicates \( \Delta S \) is significantly decreased, suggesting a decrease in the organization within the C-terminal lobe due to this mutation. Asp219 likely forms an electrostatic interaction that reduces the flexibility of the C-terminal lobe in the native domain. Smaller decreases in \( \Delta H \) were estimated for other substitutions in C-terminal residues (Y220A and R221A) suggesting Tyr220 and Arg221 contribute to structure within this lobe.

Similarly, mutation of the N-terminal Tyr129, mounted off the folded domain by two intervening Pro residues, reduced the \( T_m \) to 63.6 °C; this outcome suggests Tyr129 interacts to stabilize the folded domain, possibly through a H-bond. Conversion of Glu162 to alanine produced a domain with a significantly increased \( T_m \) value (Fig. 5, Table I). \( T_m \) values were also increased by the E179A and K173Q substitutions. Removal of a charge repulsion between Glu162 and Glu173 (with concomitant gain in flexibility of the remaining acidic residue) and favorable H-bonding by a Gln173 side chain are likely sources of increased stability of these mutant L2.

Overall the DSC results support all mutant L2 being cooperatively folded. Differences in stability of a few mutants seem to reflect changes in the interaction at the surface of L2 with this being most pronounced in the case of F217A and L190A mutant L2 domains. Both these structures are well lipoylated and, as described below, both were used as substrates in the E1 reaction. As noted above, E1 will only reductively acetylate folded lipoyl domain structures (10).

### Mapping of Specificity Residues in the Lipoyl Domains in E1 Catalysis—Table I (data columns 3 and 4) compares use of wild type and all lipoylated-mutant L2 by bovine E1 using 20 and 50 \( \mu \)M L2 construct; these concentrations are below and just above the \( K_m \) of E1 for wild type L2. The L2 mutants L140A, S141A, T143A, E162A, D172N, and E179A were reductively acetylated by E1 at greatly reduced rates. Much smaller rate reductions were observed with the A174S, L190A, and F217A mutants. The reduced rates with L190A and F217A may, in part, be due to their tendency to aggregate. In comparison to wild type L2, very small decreases or no change in E1 activity were observed with the Y129A, T145A, V156A, K188Q, R196Q, D197A, D213N, D219N, Y220A, R221Q, and E224Q mutant L2 domains. This indicates that these residues do not directly contribute to the interaction of L2 with E1 and that these mutations do not greatly alter the L2 structure in regions required for E1 binding.

The D172N mutant gave the greatest reduction in E1 activity; whereas the A174S mutation on the other side of the lipoylated K173 was used at a rate that was only modestly reduced (Table I). The effects of substitutions for the corresponding Asp and Ala residues preceding and following, respectively, the lipoylated Lys of the B. stearothermophilus PDC-E2 lipoyl domain have been characterized (30). The D42A mutation had a smaller effect on the rate of the bacterial E1 reaction than our D172N substitution had on the mammalian E1. It would appear that Asp172 plays a more important role in the reaction catalyzed by mammalian E1. Introduction of a charged residue (Glu) for Ala144 greatly reduced the activity of B. stearothermophilus E1 while substitution of Met had even less effect
Lipoyl Domain Determinants

TABLE II

Variation in kinetic parameters of E1 with mutant lipoyl domains that had markedly supported markedly reduced activities in Table I

| L2 structure Mutations | \(K_m\) (micromolar) | \(V_{max}\) (micromolar/min/mg) | \(k_{cat}/K_m\) |
|------------------------|----------------------|-------------------------------|---------------|
| None                   | 35.0 ± 3             | 14.3 ± 0.4                    | 1.0           |
| L140A                  | 12 ± 1.7             | 3.3 ± 0.1                     | 0.7           |
| S141A                  | 80.0 ± 9             | 1.3 ± 0.3                     | 0.04          |
| T143A                  | 40 ± 7               | 1.6 ± 0.4                     | 0.1           |
| E162A                  | 61.4 ± 2.3           | 3.0 ± 0.25                    | 0.12          |
| D172N                  | 555 ± 50             | 3.0 ± 0.4                     | 0.014         |
| E179A                  | 168 ± 20             | 5.8 ± 0.5                     | 0.09          |

than the corresponding A174S substitution had in reducing the activity of bovine E1. The substantial effects of the Leu 140, Ser 141, and Thr 143 mutations add further support to studies on bacterial domains (8–10) which indicated that the loop formed following the first run of N-terminal \( beta \)-structure (Fig. 1) is a key specificity loop for the E1 reaction. The size and sequence of this loop region varies for different lipoyl domain sources.

Substitution of alanine in the L1 domain at the Ser 14 (aligns with Ser 141 in L2) and the Glu 162 position (aligns with Glu 179 in L2) gave similar decreases in E1 activity as the corresponding substitutions in L2 (Fig. 5). As in the case of D197A mutation in L2, the corresponding D70A mutation in L1 did not affect E1 activity.

Most of our kinetic studies were performed with bovine E1 prior to our developing human E1 preparations with nearly equivalent specific activities in reconstituted PDC assays. Human E1 use of lipoyl domain substrates and characterization of human E1, E2-E3BP, and the Glu 162 position (aligns with Glu 179 in L2) gave similar decreases in E1 activity as the corresponding substitutions in L2 (Fig. 5). As in the case of D197A mutation in L2, the corresponding D70A mutation in L1 did not affect E1 activity.

Changes in Kinetic Parameters with Key Residues—Mutations at the sites that greatly reduced E1 activity produced folded domains that underwent highly cooperative melting transitions. Thus, the substantial effects of these mutations favor the direct involvement of these residues in interactions with the E52 component. Apparent \( K_m \) and \( V_{max} \) values were obtained for all mutant L2 domains that greatly lowered E1 activity (Table II). The apparent \( K_m \) of bovine E1 (pyruvate fixed at 120 \( \mu \)M) is 35 \( \mu \)M L2; its true \( K_m \) determined from a full steady state study in which the pyruvate level was also varied, is 49 ± 6 \( \mu \)M L2. The changes in E1 kinetic parameters were very different with different mutants. The L140A mutation actually gave a reduced \( K_m \) in conjunction with decreasing the \( V_{max} \) so that the \( k_{cat}/K_m \) ratio in the E1 reaction was not significantly changed (Table II). In native L2, Leu 140 may aid E1 catalysis by favoring dissociation of the reductively acylated lipoyl domain. With S141A, the \( K_m \) of E1 for this L2 mutant was increased along with a decrease in the maximal velocity to produce overall a 26.8-fold change in the catalytic efficiency of the E1 reaction. Thus, it seems likely that Ser 141 contributes to specific binding of the lipoyl domain by E1. With the T143A mutant, the \( k_{cat} \) for the E1 reaction was markedly decreased while there was little change in the apparent \( K_m \) for this mutant (Table II) suggesting that Thr 143 contributes to E1 specificity by enhancing the rate of acetylation of the specific substrate.

The E179A mutation caused a marked reduction in E1 catalysis (Table I) as a result of an ~5-fold increase in \( K_m \), and ~2.5-fold decrease in \( k_{cat} \) (Table II). The change in apparent \( K_m \) with the E52A mutant was not nearly as large as with Glu 179 (data not shown). This distinction suggests that E1 interacts somewhat differently with the L1 domain than it interacts with the L2 domain. Glu 162 is located behind Glu 179 (Fig. 1B). Alanine substitution at this position primarily reduced \( V_{max} \) while also modestly elevating the \( K_m \) of E1 for this substrate (Table II). The strong effects of mutations at Glu 179 and Glu 162 indicate that the E1 interaction extends well beyond the specificity loop (below). However, E1 activity was not significantly affected by substitutions for Thr 143, Arg 196, or Asp 197 which are located almost directly opposite from Thr 143 and Ser 141, respectively, at the lipoylated end of L2. Asp 197 is the only acidic residue on this side of the folded domain (panel C, Fig. 1) and was selected as a potential specificity residue based on sequence alignments. Substitutions of the corresponding amides for the three acidic residues in the C-terminal tail (Asp 213, Asp 219, and Glu 214) also failed to significantly affect the E1 reaction.

The marked decrease in activity due to the D172N mutation was a consequence of the largest increase among the L2 mutants in the apparent \( K_m \) of E1 and some reduction in \( V_{max} \) (Table II). The catalytic efficiency (\( k_{cat}/K_m \)) was decreased about 75-fold. Replacing the acid side chain with the corresponding amide did not affect E3 catalysis, alter lipoylation, change parameters in scanning calorimetry from those for native L2 (Table II), or greatly change the CD spectra (Fig. 3). It would therefore seem likely that the presentation of the adjacent lipoyl group is probably not substantially modified by the D172N mutation. Thus, this residue, which is found in virtually all lipoyl domains, makes an important contribution to catalysis by mammalian E1.

Key Specificity Residues and Lipoyl Domain Alignment—Residues that have a particularly important role in providing the high specificity of mammalian PDC-E1s for their cognate lipoyl domain substrates would not be expected to be found in the aligned positions in bacterial PDC lipoyl domain sequences or in lipoyl domains of the E2 components of various sources (including mammalian) of the \( \alpha \)-ketoglutarate and branched chain \( \alpha \)-keto acid dehydrogenase complexes. Leu 140 or a similar amino acid (particularly Ile) is commonly found in the aligned position in a wide range of \( \alpha \)-keto acid dehydrogenase complexes. However, Ser 141, Thr 143, or Glu 179 are not found even once in the aligned positions in 19 known sequences of bacterial PDC lipoyl domains and only one instance of a Ser residue is found aligned with Ser 141 in the sequences of non-PDC lipoyl domains. These residues are present not only in the aligned position in the L1 domain of mammalian E2 but also in most of the known lipoyl domain sequences of other eukaryotic PDC-E2. In the lipoyl domain of the human E3BP component, Glu 179 is conservatively substituted by Asp 50. This residue in the
E3BP lipoyl domain is preceded by a leucine which is a substantial change from the phenylalanine located in the aligned position of L1 and L2. Further studies will be needed to evaluate the importance of Glu occupying this position in L1 and L2 and an Asp residue occupying this position in E3BP.

Competitive Inhibition by Lipoyl Domains Modified at the Site of Lipoylation—Graham and Perham (11) observed a weak interaction of E. coli PDC-E1 with its cognate lipoyl domain despite high specificity for this substrate. Using GST-H1-L2, this laboratory has only been able to detect weak binding to L2 by E1 containing transition state analogs of HE-TPP and even under turnover conditions in which the inner domain of E2 and E3 supported slow catalysis in the overall reaction. A potential explanation for the capacity of E1 to be highly selective for a lipoyl domain substrate while a weak affinity is that binding energy from the required protein-protein interactions between E1 and its lipoyl domain is used to move the lipoyl group into the active site. This would predict that the L2 mutant K173A would be a good competitive inhibitor. Alternatively, the long lysyl-octanoyl chain or specific interactions of the dithiolane ring of the lipoyl group may make an important contribution to specific binding or catalysis. To evaluate this, L2 was modified at the site of lipoylation either by substitution of the amino acids alanine, methionine, leucine, or glutamine for Lys173 or by introduction of the lipoyl substitutes: octanoyl, 8-thioloctanoate, or heptanoyl into nonlipoylated L2. Although 8-thioloctanoyl-L2 was an efficient substrate for the transacylation reaction catalyzed by the E2 component, it was not used by E1 at a significant rate (<2 nmol/min/mg E1).

Using a driven NADH regenerating system (α-ketoglutarate dehydrogenase complex reaction) to convert and maintain lipoyl groups via the E3 reaction in the reduced form, rates of acetylation by E1 of reduced lipoyl groups using pyruvate at least 100-fold faster than the above rate obtained using the 8-thioloctanoyl-L2. In both cases, O$_2$ presumably serves as the electron acceptor for the required oxidation of the HE-TPP to acetyl-TPP (4). Assuming that very low levels of oxidized lipoyl groups were not contributing to the higher rates with the dihydrolipoyl-containing substrate, this finding suggests that the presence of a thiol in the 6-position is important for the reductive acetylation reaction to proceed even though acetylation is at the 8-position (4).

As shown in Fig. 6 (left panel), the substitution of Leu or Glu for Lys$^{173}$ yielded L2 that were inhibitory at a 1:1 ratio versus native L2, whereas Ala- and Met-substituted L2 gave much less inhibition. Introduction of heptanoyl gave weak inhibition and 8-thioloctanoate gave stronger inhibition (Fig. 6, right panel). However, octanoyl-L2 was a very weak inhibitor. It is possible that substitutions like Leu or Glu for Lys$^{173}$ or heptanoyl for lipoylate produce L2 that can interact with E1 in artificial ways that do not mimic the prosthetic group on the native domain. However, the substantial inhibition by 8-thioloctanoyl-L2 probably involves specific interactions of the thiol group since octanoyl-L2 is a much weaker inhibitor. The poor inhibition due to substitutions like K173A and octanoyl-L2 supports the lipoyl lysine group interacting via its specific structure. Specifically the dithiolane ring of lipoylate may contribute to binding by forming favorable interactions and increasing the probability of cofactor conformation that accommodates the active site. In combination, the results with Lys$^{173}$ substitutions and lipoylate substitutions support significant interactions with E1 occur throughout the lipoyl-lysine prosthetic group and do not support lipoylate being forced into the active site. This is consistent with the active site channel of the P. putida branched chain E1 being lined with hydrophobic groups which could interact favorably with the mostly aliphatic lipoyl-lysine prosthetic group (14).

L2 Structure and Relation to the E1 Reaction—We have prepared a versatile set of mutant lipoyl domains that are well folded. All of the mutant domains, that had major effects on E1 activity, were well behaved in native gel electrophoresis and, with the exception of L140A-L2 mutant, unfolded with $T_m$ values in excess of 64 °C. We would emphasize that D170Q-L2, which was not lipoylated gave near wild type values in DSC and CD studies indicating it had a near native structure. Thus, our studies further support the concept that lipoylation requires both proper folding and the presentation of essential residues. Since the lipoyl domains used to test E1 function were well lipoylated, that further supports their retaining a well folded structure that properly presented the tight turn bearing Lys$^{173}$ for the lipoylation reaction.

The specificity loop 1 (Leu$^{140}$, Ser$^{141}$, and Thr$^{143}$), the conserved Asp$^{172}$ and, anterior to those residues, Glu$^{179}$ and Glu$^{162}$ are located on one side of the L2 domain (panel B, Fig. 1) in a region abundant in acidic residues (10 on the folded part of the domain). Glu$^{179}$ is located in a long β-strand leading away from Lys$^{173}$; its α-C is 16 Å from Ser$^{141}$ and the α-C of

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6 Using α-ketoglutarate dehydrogenase prepared free of PDC activity, this was measured by M. Rahmatullah and T. E. Roche (unpublished data) using the bilipoyl domain fragment derived from bovine E2 as a substrate. Reaction of a re-neutralized, acid quenched sample with 5,5'-dithiobis(2-nitrobenzoate) supported full reduction of the lipoyl domain substrate; however, the complete absence of oxidized lipoyl groups is uncertain because the presence of NADH with no NAD$^+$ tends to cause E3 to be converted to the four electron reduced state which allows oxygen to react at the E3 active site to form H$_2$O$_2$ which, in turn, may serve to oxidize small portion of the dihydrolipoyl groups.
these amino acids are separated by 19 Å and 11 Å, respectively, from the α-C of Lys173. Glu162 is located an additional 6 Å behind Glu179. These acidic residues are well separated from basic residues; this is in contrast to the acidic residues that line the bottom edge of the panel B structure. Electrostatic interactions of Glu162 and Glu179 with basic residues at the surface of E1 are predicted to contribute to the E1-L2 complex. Thus, E1 interacts over a substantial region with the surface of the lipoyl domain. The opposite side of the lipoyl domain has a hydrophobic face (Fig. 1, panel C) with Leu190 in the center and prominent residues, Arg196, Asp197, and Thr145, which were not important for E1 catalysis. Apparently, there is very limited interaction of E1 with this side of the lipoyl domain structure although mutation of Val156 and Leu190 reduced activity.

The mutants developed here have been used to establish specificity residues for specific kinase and phosphatase isoforms.5 These studies have revealed an overlapping pattern in supporting of the function of the regulatory enzymes and E1 but also established the involvement of many distinct residues and large differences in the relative importance of overlapping residues. This further indicates that these established mutations do not propagate extended changes in the structure of the lipoyl domain to thereby interfere with normal lipoyl domain roles. Now that this initial mapping has been achieved, further substitutions at the key specificity sites should greatly add to our understanding of the roles and relative importance of these key specificity residues in the L2 domain for E1 function.

Based on the capacity of lipoylated L2 to compete very effectively versus analogs lacking structural features of the lipoyllysine prosthetic group, structural requirements for the interaction of the lipoyl prosthetic group include distinct structural features, probably including active site complementarity favoring binding of the dithiolane ring of the lipoylproteostatic group. The stronger inhibition in substituting a Gln rather than Ala or Met for Lys173, probably results from Gln participating in H-bonding with E1 which cannot be formed by the Met and Ala side chains. However, the alternative explanation that the Gln participates in a relatively stable internal H-bond within the lipoyl domain that enhances binding to E1 cannot be eliminated. Since extensive interactions between E1 and L2 are supported and yet, to date, the binding of the lipoyl domains to E1 appear to be weak (k on values above k off values (11)), this suggests that intrinsic interaction energy is used to facilitate catalysis. That is consistent with the high specificity for cognate lipoyl domains of an E1 source of an F1

**Acknowledgments**—We thank Xiaohua Yan and Jason C. Baker for help in preparing PDC and E1 and Shane Kasten for help in preparing wild type and mutant lipoyl domains.

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Specificity Determinants for the Pyruvate Dehydrogenase Component Reaction Mapped with Mutated and Prosthetic Group Modified Lipoyl Domains
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J. Biol. Chem. 2000, 275:13645-13653.
doi: 10.1074/jbc.275.18.13645

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