Requirements for the Adaptor Protein Role of Dihydrolipoyl Acetyltransferase in the Up-regulated Function of the Pyruvate Dehydrogenase Kinase and Pyruvate Dehydrogenase Phosphatase*

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The dihydrolipoyl acetyltransferase (E2 component) is a 60-mer assembled via its COOH-terminal domain with exterior E1-binding domain and two lipoyl domains (L2 then L1) sequentially connected by mobile linker regions. E2 facilitates markedly enhanced function of the pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP). Human E2 structures were prepared with only one lipoyl domain (L1 or L2) or with alanines substituted at the sites of lipoylation (Lys-46 in L1 or Lys-173 in L2). The L2 domain and its lipoyl group were shown to be essential for markedly enhanced PDP function and were required for greatly up-regulated PDK function. The complete absence of the L1 domain reduced the enhancements of both of these activities but not the maximal effector-stimulated PDK activity through acetylation of L2. With nonlipoylated L2 present, lipoylated L1 supported a lesser enhancement in PDK function with significant stimulation upon acetylation of L1. Prevention of L1 lipoylation in K46AE2 removed this competitive L1 role and enhanced L2-facilitated PDK activity beyond that of native E2 when PDK activity was measured in the absence or in the presence of stimulatory effectors. Thus, the E2-L2 domain has a paramount role in facilitating enhanced PDK and PDP function but inclusion of E2-L1 domain, even in a noninteracting (nonlipoylated) form, contributes to the marked elevation of these activities.

The mammalian pyruvate dehydrogenase complex (PDC) has a strategic role in controlling the oxidative consumption of glucose (1). To limit consumption of body carbohydrate reserve, PDC activity is controlled by an intricately regulated cycle carried out by dedicated kinase and phosphatase components. PDC activity is reduced due to phosphorylation of the pyruvate dehydrogenase (E1) tetramers and increased by production of nonphosphorylated tetramers. Phosphorylation proceeds in a kinetically preferred order at three sites on the α subunit of E1 (2, 3), an αβ2 structure; however, incorporation of a phosphate into each site is capable of causing inactivation (4).

The dihydrolipoyl acetyltransferase (E2) component forms the structural core of the complex. It consists of four independently folded domains set apart from each other by interdomain linker (or hinge) regions, each having substantial reach (>40 Å) and high mobility (Fig. 1, E2). The largest domain, located at the COOH terminus, forms a catalytically active trimer which assembles at the 20 vertices of a pentagonal dodecahedron to form a 60-mer inner core structure with icosahedral symmetry. Then a flexible segment (H3) connects to an E1-binding domain followed by two lipoyl-bearing domains (L2 and then L1 at the NH2 terminus) sequentially connected by two more flexible hinge regions (H2 and H1). In the outer surface of the E260 structure, these mobile, multisegment NH2-terminal structures intercede in dynamic processes associated with catalytic transfers and regulatory interconversions (5, 6). Here we further define the latter adaptor protein roles in the regulation of PDC.

Bovine kidney pyruvate dehydrogenase kinase (PDK) activity and pyruvate dehydrogenase phosphatase (PDP) activity are greatly enhanced in the presence of E2. Furthermore, E2 mediates acetyl-CoA and NADH stimulation of PDK activity and facilitates Ca2+ stimulation of PDP activity (6). These regulatory inputs constitute important and sensitive response mechanisms in the control of cellular energy metabolism. The marked reduction in PDC activity due to elevated NADH:NAD+ and acetyl-CoA:CoA ratios stimulating PDK activity is a strategic response resulting from increased fatty acid oxidation and serves to preserve body carbohydrate stores (1, 6). To meet transitional energy needs, PDC activity is increased due to elevation of intramitochondrial Ca2+ ions in association with a wide variety of signal transduction cascades (7). Increasing Ca2+ from <0.1 μM to >1.5 μM can enhance PDP activity more than 10-fold (8).

In dissecting the intercessions of E2 in these regulatory mechanisms, we have used recombinant lipoyl domain constructs to establish that the L2 domain of E2 has a crucial role in these processes (9–11). L2 preferentially binds PDK through an interaction that requires the lipoyl cofactor of L2 (9); we have suggested that this is critical to E2 activation of PDK activity. Effector stimulation of bovine kidney PDK by NADH and acetyl-CoA ensues from using these products in reducing and acetylating, respectively, the L2 lipoyl prosthetic group (10). Reduction and acetylation are sequentially catalyzed by the reverse of the dihydrolipoyl dehydrogenase (E3) and E2 components. Additionally, the L2 domain exclusively binds PDP.

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1 The abbreviations used are: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase component; E2, dihydrolipoyl acetyltransferase component; L1, NH2-lipoyl domain of E2; L2, interior lipoyl domain of E2; B, E1 binding domain of E2; I, oligomer-forming, transacetylation-catalyzing COOH-terminal inner domain of E2; H1, H2, and H3, connecting hinge (or linker regions) sequentially located between the globular domains of E2; L1E2, E2 oligomer lacking L2 domain; L2E2, E2 oligomer lacking L1 domain; PDK, pyruvate dehydrogenase kinase; PDK1, PDK2, PDK3, and PDK4, PDK isozymes; E3, dihydrolipoyl dehydrogenase; E3BP, E3-binding protein (formerly protein X); PCR, polymerase chain reaction; MOPS, 4-morpholinepropane-sulfonic acid; PAGE, polyacrylamide gel electrophoresis.
and acetyl-CoA stimulated PDK activity to the maximal extent with the E2 assemblage lacking the L1 domain suggesting the selective interaction of acetylated L2 with bovine PDK markedly alters its function.

EXPERIMENTAL PROCEDURES

Materials—Bovine kidney PDC, E1 component, E2-E3BP/PDK subcomplex, the recombinant bilipoyl domain region of human E2, and one form of full sized human E2–60-mer were prepared as described previously (16–18). Porcine heart E3 was from Boehringer Mannheim or Sigma. Pfu DNA polymerase was from Stratagene Inc. DNA oligonucleotides used for plasmid construction were made by Eppendorf. Primers used for PCR reaction or sequencing were from Oligos Etc. or Biotechnology Core Facility at Kansas State University. T4 DNA ligase and BamHI were from Promega Corp.; other restriction enzymes were from New England Biolabs. Other materials used are the same as those described previously (9–11, 16, 19).

Polymerase Chain Reaction—Polymerase chain reaction (PCR) was performed according to Innis et al. (20) with a GeneAmp PCR System 2400 thermocycler from Perkin-Elmer. Primers (200 pmol) having about 50–60% G-C content (Tm > 45 °C) were reacted with 1.5 ng of purified template DNA, 200 μM dNTPs, 2.5 IU Pfu DNA polymerase in Pfu buffer (Stratagene). The reaction mixtures, overlaid with 50 μl of mineral oil, were denatured initially and incubated each cycle for 1 min at 95 °C, reacted for 20 to 30 cycles with 0.5 min annealing at 55 °C, and 0.5 min extension at 72 °C with the final extension reaction proceeding for 2 min.

Construction of the Expression Vectors for L1E2 and L2E2—As diagramed on the right side of Fig. 2, a cDNA fragment coding for L1 was amplified by PCR with pShE2 plasmid (codes for mature human E2 with the E2 leader sequence removed and a start Met inserted (16)) as a template using 5'-CATCCATGGTAGTTCTTCGGCGATC-3' (sense) and 5'-GATCGCCGAGAATCCGTTGA-3' (antisense) primers. This introduced flanking NcoI and EagI sites (compatible with EoeI site) at the 5'- and 3'- ends (sense direction), respectively. The DNA amplified from PCR was digested by NcoI and EagI, purified, and ligated to 1-kilobase pair DNA fragment purified after digestion of pShE2 plasmid with EoeI and BamHI. The resulting DNA fragment coding for L1E2 (Fig. 1) was ligated to pSE420 vector previously digested by NcoI and BamHI to produce pShL1E2. DNA sequencing was performed for the region produced by PCR and linking ligation sites.

As shown on the left side of Fig. 2, a segment including the 5'-region coding for L1 portion of E2 was removed from pShE2 vector by digestion with NdeI and BamHI and reinserted in combination with BamHI digestion to produce a 1.3-kilobase pair cDNA fragment. This fragment was ligated to a hybrid of two oligonucleotides that include 16 base pairs encoding the NH2 terminus of L2 plus two other amino acids (Gly and Ser) prior to start codon. This spliced fragment was cloned into the pSE420 vector previously digested by NcoI and BamHI to produce pShL1E2. DNA sequencing was performed for the region produced by PCR and linking ligation sites.

As shown on the left side of Fig. 2, a segment including the 5'-region coding for L1 portion of E2 was removed from pShE2 vector by digestion with Ndel and BamHI and reinserted in combination with BamHI digestion to produce a 1.3-kilobase pair cDNA fragment. This fragment was ligated to a hybrid of two oligonucleotides that include 16 base pairs encoding the NH2 terminus of L2 plus two other amino acids (Gly and Ser) prior to start codon. This spliced fragment was cloned into the pSE420 vector previously digested by NcoI and BamHI. The resultant expression vector, pShL2E2 (Fig. 2, left side), encoded L2E2 (Fig. 1). The DNA derived from the synthetic oligonucleotides along with their adjoining ligation sites were sequenced in pShL1E2 and pShL2E2.

Production of L1E2 and L2E2—pShL1E2 and pShL2E2 plasmids were introduced into BL21(DE3) strains by electroporation using the Transector 300 BTX. Expression of pShL1E2 and pShL2E2 was carried out as described previously for pShE2 to produce E2 (16). Expression of each of the modified E2 subunits, bearing only one E2 lipoyl domain, was analyzed by dot blotting and Western blotting techniques using lipoyl domain-specific monoclonal antibodies with 150.2 for detecting L1E2, 157.2 plus 315.2 for detecting L2E2 protein, and horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) as the second antibody under conditions previously described (16, 19).

Purification of L1E2 and L2E2—All steps were performed at 4 °C. Cells were resuspended in 50 mM potassium phosphate buffer, pH 7.3, containing 0.5 mM EDTA and 1 μg/ml aprotinin, and 1 μg/ml leupeptin (buffer A) and then disrupted by sonication. Cell debris was removed by centrifugation (15,000 × g for 20 min). PEG-8000 was added dropwise to 8% (v/v), and the precipitated protein was recovered by centrifugation (20,000 × g for 20 min). The pellet was in a cloudy state after being resuspended in buffer A. Upon addition of (NH4)2SO4 to 9% saturation, substantial clearing occurred; after ~20 min, the material still suspended was precipitated and discarded. Addition of (NH4)2SO4 was continued until the concentration of (NH4)2SO4 reached 25% saturation. The precipitated protein was pelleted at 20,000 × g for 20 min and redissolved in 50 mM potassium phosphate buffer, pH 7.2, containing 0.2 mM EDTA, 1 μg/ml aprotinin, and 1 μg/ml leupeptin.

Aliquots of purified samples were stored frozen at −80 °C. The pro-

<sup>#</sup> J. C. Baker, J. Dong, and T. E. Roche, unpublished observations.

<sup>4</sup> PDK3 is markedly activated (>10-fold) by E2, and this is a stable response; PDK1 and PDK2 are activated up to 4-fold by E2 when first prepared, but these responses fade with time.
tein concentration was measured by the BCA method. The E2 activity at each fraction step was followed by measuring the PTA activity (21), and the protein pattern was analyzed by SDS-PAGE (22). NH$_2$-terminal sequencing of SDS-PAGE-separated protein bands was conducted as described previously (16, 19) with thioglycolate increased in the sample loading buffer to 15 mM. The ratio of the different protein bands was analyzed by scanning the band patterns for Coomassie Blue R-250 and silver-stained gels following SDS-PAGE separation on samples from the preparations of L1E2 or L2E2. An Ittis Dos Program was used to do the image density analysis and analyze the intensity ratios.

Production of K46AE2 and K173AE2—As will be described in detail elsewhere, a variety of lipoyl domain mutants has been prepared and tested alone and incorporated into E2 oligomers. Expression vectors for mature E2 and for glutathione S-transferase fused L1 or L2 and have been designed with silent restriction sites to permit transfer of cDNA fragments encoding L1 or L2 mutants from vectors expressing glutathione S-transferase-lipoyl domains to vectors expressing these mutant domains in whole E2 structures. DNA fragments, encoding K46A-modified L1 or K173A-modified L2, were introduced by this approach into E2 structures.

The modified cDNA inserts expressing whole E2 have also been modified to include a removable His tag at the NH$_2$ terminus. The E2 assemblages are purified to >98% purity by a two-step procedure which involves fractionation with polyethylene glycol and gel filtration chromatography which is immediately preceded by removal of the His tag. The introduction of the His tag greatly increased the recovery of E2 by improving solubility of E2 assemblages and reduced the presence of truncated E2 subunits, probably by reducing the tendency of a codon for Met in the L1 domain operating as an internal start site (16). E2, K43AE2, and K173AE2 prepared by this approach were used for studies shown in panel B of Figs. 5–7. Other properties of these and several other constructs developed will be described elsewhere.

Binding of Bovine E1 to L1E2 and L2E2—To evaluate E1 binding to the truncated or full-sized human E2 constructs, ~20 µg of L1E2 or L2E2 subunit with or without E1 (20 µg) was incubated at 4 °C for 120 min. After incubation, the above mixtures and the control E1 sample were each loaded onto the top of a three-step sucrose gradient, and gradient separation was carried out as described (16, 23). SDS-PAGE analysis with silver staining (24) was conducted as described in Fig. 3.


*5 Porcine E3 is used for comparison to our previous data with recombinant human E2 (16). Lindsay and co-workers (26, 27) have indicated that, in the absence of the E3BP component, very high levels of bovine E3 (e.g. 100-fold excess) are more effective than similarly high levels of porcine E3 in supporting reconstituted bovine PDC activity. They suggest weak binding of bovine E3 to E2 may occur.
Facilitated Kinase and Phosphatase Function

of lipoyl groups in a cyclic E3 reaction (16, 19) was measured using 150 pmol of L1E2, L2E2, or E2.

Kinase Activation and Regulation—PDK activity, in the absence of effectors, was determined as described previously (16, 28) after the E2 constructs were incubated at 4 °C for about 120 min (domain truncated E2) or 20 min (mutated E2) to maximize binding of E1 and increase the solubility, particularly of L1E2 and L2E2. 25–30 μg of E1 and the molar levels of E2 construct indicated were added to PDK reaction mixtures. To evaluate maximal PDK activities, assays were conducted in the presence of 20 mM potassium phosphate (condition 1); to evaluate regulatory effects or otherwise assay PDK activity at a near-physiological level of K+ (as, were conducted in the presence of 50 mM MOPS-K, pH 7.5, 20 mM potassium phosphate, pH 7.5, 60 mM KCl (condition 2).

Under both reaction conditions, assays, additionally contained 1 mM MgCl2, 0.05 mM EDTA, 0.2% Pluronic-F-68, and 0.2% Triton X-100. Reactions were started by addition of [γ-32P]ATP (~3 × 104 cpm/pmol) to give a final volume of 50 μl and then terminated and worked up as described previously (28, 29). For evaluating effects of NADH and acetyl-CoA on PDK activity (10), assays (condition 2) also included 1 μM of E3 and, when indicated, 0.6 mM NADH plus 0.2 mM acetyl-CoA.

Phosphatase Activity—PDP activity was determined as described previously (11, 16) except that 0.4 mg/ml bovine serum albumin was included along with 2 mg/ml Pluronic-F-68 in reaction mixtures, and 14–15 μg of E1b was included in each assay. In studies involving L1E2 and L2E2, E2 sources were preincubated with E1b at 4 °C for about 120 min before the activity assays, while only 20 min was used with mutated E2 structures.

RESULTS AND DISCUSSION

Expression and Purification of E2 Oligomers with One Lipoyl Domain—By using the domain codes in Fig. 1 and the approaches outlined under “Experimental Procedures” and Fig. 2, the region coding for the H1-L2 was removed along with connecting the coding region for the L1 domain to yield a vector, pShL1E2 (Fig. 2, right side) expressing L1E2 (Fig. 1). L1E2 has one extra amino acid, a glycine at the NH2 terminus following the start Met codon, and was designed with residues 233–240 of H2 hinge region removed so that L1 was connected after Ser-98 to a hinge region, H2’, which starts with an Ala-Ala sequence (residues 241 and 242 of E2). This produced a transition similar to that between L1 and the beginning of H1; H2’ is still a hinge region over 20 residues in length. Similarly, the coding region for L1-H1 was removed from pShE2 expression vector for E2 to produce a vector, pShL2E2 (Fig. 2, left side), coding for L2E2 (Fig. 1). The last two residues of H1 region (Gly-126 and Ser-127) were retained following the start Met in L2E2. The accuracy of the cDNA inserts was confirmed by restriction enzyme digestion and DNA sequencing.

Western blotting confirmed that L1E2 or L2E2 expressed in Escherichia coli strain BL-21 reacted selectively with L1- or L2-specific monoclonal antibodies (19), respectively, that the single lipoyl domain-containing E2 structures had about the expected mobilities, and that at least 70% of L2E2 and a lower portion (~50%) of L1E2 remained in the soluble fraction upon centrifugation of cell lysates. By using the purification steps described under “Experimental Procedures,” nearly homogeneous L1E2 (Mr = 48.4 ± 1.5) and L2E2 (Mr = 51.2 ± 1.5) were obtained as indicated by their silver-stained patterns following SDS-PAGE separation (Fig. 3). The observed molecular weights are somewhat larger than calculated molecular weight values of 44.4 and 46.2 for L1E2 and L2E2, respectively, as is expected for hinge regions causing E2 structures to run anomalously slow (30, 31). The recombinant L2E2 was recovered completely intact. However, as occurred in the recombinant production of whole E2 (Ref. 16 and Fig. 3, lane 5), two of the bands in the L1E2 preparation were determined by NH2-terminal sequencing to be truncated products (within L1 (E2)) in Fig. 3 and just before the I domain in H3 (E2) in Fig. 3. Since these partial structures retain the inner domain, they can participate in the L1E2 assemblage and contribute to acetyltransferase activity. NH2-terminal sequencing of L1E2 gave a mixed sequence that fit about 50% each of MGSLPPHQK and GSLPPHQK, indicating about half the start Met residue was retained. L2E2 only gave a GSTYPHQK sequence, indicating all the start Met was removed. From the use of densitometric area scanning, bands densities were estimated in Coomassie-stained SDS-PAGE patterns of L1E2. Full-sized L1E2 was about 80% of the band density and therefore about 76% ± 5% on a molar basis. L2E2 was about 94 ± 5% of the protein.

Properties of L1E2 and L2E2—Besides having high acetyltransferase-specific activities (L1E2, 15.76 ± 0.61 μmol/mg min, L2E2, 16.93 ± 0.93 μmol/mg min), the lipoyl domain of each engineered oligomer served as effective substrates in the cyclic E3 assay. At equivalent full-sized subunit levels, substrate-limited E3 rates were 52–60% of that observed for E2 oligomer with L2E2 serving as a somewhat better substrate than L1E2 on a per mg basis (Table I). Upon applying a correction for contaminants and lipoyl domain-deficient subunits in the L1E2 preparation, the observed E3 rate supported by L1E2 is somewhat greater (9% higher) than that for L2E2 in this substrate-limited reaction. E3 catalytic utilizations (estimated Km and Vmax values) for the free individual lipoyl domains (L1 and L2) were very similar.6 Thus, this measure supports at least high lipoylation of full-sized L1E2 as L2E2. The higher E3 reaction rate for the bilipoyl domain containing E2 substrate versus the rates using the monolipoyl domain oligomers indicates that the unbound E3 is responding to the levels of the coupled lipoyl domains almost as if they were in independent structures. This is consistent with the high mobility of the linker regions aiding independent encounters of the co-tethered lipoyl domains with E3.

Lacking the E3BP and therefore the capacity for tight binding of E3, each E2 preparation gave a low level of reconstituted PDC activity (Table I) when combined with excess bovine E1 and porcine heart E3. As in the E3 assay, higher activities were observed with the full-sized E2. Under the same conditions, the E2-E3BP subcomplex supported reconstituted PDC activity >50-fold higher than either truncated structure (data not shown).

L2E2 was acetylated to a significantly higher level than L1E2 (Table I). The results support full or nearly full lipoylation of L2E2 with a small portion having to undergo biacetylation (probably via noncatalytic transfer of acetyl groups (19)) to attain the observed level of acetylation. When the level of acetylation of L1E2 was evaluated after corrections for contaminating proteins and partial subunits lacking functional lipoyl domains, an estimate of 88 ± 7% of full acetylation of L1E2 is obtained assuming monocetylation. That suggests that at least 80% of the full-sized L1E2 subunits are lipoylated. Based on these results and L1E2 giving a higher corrected activity in the E3 reaction, we conclude that high lipoylation of the L1E2 and L2E2 assemblages was achieved.

Both L1E2 and L2E2 were very effective in binding bovine

<table>
<thead>
<tr>
<th>E2 source</th>
<th>E3-specific activitya (pmol/min/mg)</th>
<th>Reconstituted PDC activity (nmol/mg)</th>
</tr>
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<tbody>
<tr>
<td>L1E2</td>
<td>2.02 ± 0.30</td>
<td>0.43 ± 0.05</td>
</tr>
<tr>
<td>L2E2</td>
<td>2.30 ± 0.11</td>
<td>0.55 ± 0.09</td>
</tr>
<tr>
<td>E2</td>
<td>3.85 ± 0.32</td>
<td>0.87 ± 0.07</td>
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* J. Baker and T. E. Roche, manuscript in preparation.
PAGE analysis was conducted on 10-μg samples of bovine E1, alone, or combined with 20-μg samples of L1E2 and L2E2. SDS-PAGE analysis was conducted on 10-μl samples of S1, and 3-μl samples of S2, S3, and P fractions. Silver-stained patterns are shown for each series as well as for 2 μg of bovine PDC, left-most lane.

E1 based on E1 co-sedimenting with these assemblages (Fig. 4). Thus, the B domain of each of these structures is fully functional. The high sedimentation rate of the complexes supports the assemblage of L1E2 and L2E2 into large oligomers, presumably 60-mers as demonstrated by electron microscopy results with recombinant full-sized human E2 (16). In the absence of E1, a larger proportion of the truncated assemblages sedimented through the step gradient into the pellet fraction (data not shown). This indicates that binding of E1 reduced a tendency of these assemblages to form further aggregates. This is consistent with previous results that suggest reversible unfolding (partial or full) by a low portion of the very small (~45 amino acids) B domain has a tendency to cause inter-oligomer aggregation (32). Binding of E1 was allowed to proceed for 60 min prior to separation in the step gradient; E1 presumably captures B domains in the folded state, thereby reversing aggregation. In the following regulatory studies E1 (active, non-phosphorylated E1a for kinase assays and inactive, phosphorylated E1b for phosphatase assays) was incubated at 4 °C for at least 120 min with L1E2 and L2E2 prior to assays to minimize aggregation of E2 oligomers. In the case of the mutated, full-sized E2 structures, 20 min preincubation with E1a or E1b was fully adequate.

**L1E2 and L2E2 Enhancement of PDK Activity**—The capacity of L1E2 and L2E2 oligomers to support enhanced PDK activity was compared with mature E2 using reaction condition 1. The concentration of L1E2 was based on the mass proportion of L1E2 subunits estimated following corrections for other polypeptides based on area densitometric analysis of a Coomassie-stained SDS-polyacrylamide gel. As shown in Fig. 5, lane 5, the E2 oligomer with fewer partial E2 subunits was not significantly more effective than the previous E2 preparation (Fig. 3, lane 5) in activating PDK or PDP (below).

As shown in Fig. 5, panel B, A173KE2 (L2 not lipoylated) was not nearly as effective in supporting PDK function as unmodified E2. This further supports the lipoyl group of L2 being critical for fully activated PDK function. Nevertheless, A173KE2 was much more effective in activating PDK than L2-truncated L1E2 (condition 1, Fig. 5, panel A, or condition 2, Fig. 6, panel A, in the absence of effectors). Thus, the presence of a nonbinding L2 domain does enhance the functionality of L1 although it remains low.

Enhancement of PDK Activity by Full-sized E2 with One Lipoyl Group—Because the above results suggest a need for the L1 domain for full PDK activity and yet the lipoyl prosthetic group is required both for the strong binding of PDK to L2 and weak binding to L1 (9), we evaluated the effects of substitution in full-sized E2 constructs of alanines at the lipoylation site in either lipoyl domain. For direct comparison of these results to those showing regulatory effects (below), the relative PDK activations are shown for studies in the high salt buffer (condition 2). As shown in Fig. 5, panel B, 3.5–10.5 μg E2 increased PDK activity from nearly 3-fold to over 6-fold. Higher PDK activities (2.5-fold) but lower fold activations were obtained when assays were conducted under the nonphysiological low salt assays (condition 1) with this E2 preparation (data not shown). For the studies in Fig. 5, panel B, E2 constructs, produced with a His tag which was then removed, contained much lower levels of modified E2 subunit with a partial L1 domain than the L1-containing constructs shown in Fig. 3, lanes 3 and 5. However, the E2 oligomer with fewer partial E2 subunits was not significantly more effective than the previous E2 preparation (Fig. 3, lane 5) in activating PDK or PDP (below).

As shown in Fig. 5, panel B, A173KE2 (L2 not lipoylated) was not nearly as effective in supporting PDK function as unmodified E2 (Fig. 5, panel B). Thus, inclusion of the L1 domain along with removing its capacity to undergo lipoylation led to a substantial enhancement in PDK activity. It seems likely that the L1 domain has some capacity to bind PDK in a lipoyl-dependent manner and that when interacting with L1 PDK is less active than when solely bound to L2. This is consistent with earlier findings suggesting weak lipoyl-dependent binding by glutathione-S-transferase-L1 and PDK inhibition by L1 (9). We assume the unlipoylated L1 domain of A46KE2 lacks the capacity to bind PDK as was observed following delipoylation of glutathione-S-transferase-fused L1 (9). Apparently, exclusive binding to L2 markedly enhances PDK activity or at least the activity of the bovine kidney PDK isozyme(s) that fractionate with E1 upon resolution of the bovine kidney PDC. At the same time, the presence of L1 at the surface of the E2 oligomer must contribute to enhanced PDK function since, in the absence of L1, a much lower PDK activity was supported by L2E2. The nature of the contribution of a nonlipoylated L1 versus the absence of this domain for PDK (and PDP) activity is considered below.

**L1E2 and L2E2 Facilitation of Effector Stimulation of PDK**—By using physiological level of potassium salts (condition 2) required for significant effector stimulations of PDK activity, we evaluated the capacity of truncated E2 oligomers to support stimulations by NADH or acetyl-CoA, alone or in combination with a low level (2 μM) of oligomeric E2 structures. As shown in Fig. 6, panel A, very small enhancements of PDK activity were supported by L1E2 despite effective reductive acetylation of its lipoyl groups by the combination of E3-catalyzed reduction and E2-catalyzed acetylation of the lipoyl groups of L1 (Table I). However, in the presence of L2E2, NADH and acetyl-CoA gave a pronounced stimulation of PDK activity, and the combination of both effectors so markedly stimulated PDK activity that it rose to essentially the same level as supported by mature E2 (Fig. 6, panel A). In the case of
L2E2, NADH gave a fractional stimulation higher than that achieved with E2, but the absolute stimulated activity was still below that with E2.

Previous studies (10) have shown that acetyl-CoA stimulation requires transacetylase activity (catalyzed by trimers in the E2I assemblage) and that acetylation of a low proportion of sites in E2 can mediate kinase stimulation (33). Consistent with this trend of low levels of acetylated E2 being effective in enhancing kinase activity, marked stimulation with NADH and acetyl-CoA was observed with low, 2 μM L2E2 (Fig. 6, panel A). The combination of results contribute further support for the conclusion that acetylation leads to development of an altered, higher affinity interaction of PDK with one or more reductively acetylated L2 domains in the L2E2–60-mer.

Acetyl-CoA stimulation in the absence of NADH is a function of the portion of reduced lipoyl groups in the E2 preparations. That portion is normally low, but reductants that reduce lipoyl groups raise this stimulation to the level achieved in the presence of NADH (34, 35).

**Effector Stimulation of PDK Mediated by E2, K173AE2, and K46AE2—** With the full-sized E2 sources included at 7 μM, both modified forms facilitated NADH and acetyl-CoA stimulation of PDK, Fig. 6, panel A. As found in the absence of effectors (Fig. 5, panel B), the enhanced PDK activity mediated by K46AE2 remained greater than that supported by native E2 for all conditions. However, the fractional enhancement by effectors was higher when native E2 mediated the product stimulation of PDK activity (e.g. the combination of products gave 2.9-fold stimulation with E2 versus 2.5-fold stimulation with K46AE2).

Considering the very limited capacity of NADH plus acetyl-CoA to stimulate PDK in the absence of L1E2 (Fig. 6, panel A), it was surprising that substantial stimulations of PDK activity were facilitated by products with K173AE2 (Fig. 6, panel B). Indeed the 3.3-fold fractional stimulation by the combination of products was the highest observed with any E2 source although the total PDK activity remained well below that attained with either of the other E2 sources. Although the
large decrease in PDK activity with K173AE2 confirms that the lipoate of L2 is very important, the substantial difference between removing the L2 domain (L1E2) and just the lipoyl cofactor (K173AE2) indicates that a lipoylated L1 is much more effective when located in the surface environment with two lipoyl domains (considered below). Because all our preparations of bovine E2 contain very high levels of bound PDK, we were not able to compare the effects of human E2 to bovine E2 in supporting PDK activity. However, the magnitude of the effects look similar to those previously found using bovine E2 in which PDK was mostly released by a mercurial treatment (29).

Ca\(^{2+}\)-dependent Activation of PDP by the Different E2 Constructs—E2 greatly increases the rate of dephosphorylation of E1b by a process involving Ca\(^{2+}\)-dependent binding of PDP to the L2 domain of E2. Fig. 7, panel A, compares the effects of E2 and the E2 structures engineered to contain one lipoyl domain. L1E2 failed to activate PDP, whereas 1.7–5.0 \(\mu M\) L2E2 enhanced PDP activity 2–6-fold, further supporting a highly specific interaction with the L2 domain. However, equivalent levels of full-sized E2 supported even higher enhancements of PDP activity (4–7.5-fold). 7

Studies with the alanine-substituted E2s gave results consistent with previous studies and some surprises. As shown in Fig. 7, panel B, large (6.4–12-fold) Ca\(^{2+}\)-dependent enhancements of PDP activity were obtained with 1.5–4.5 \(\mu M\) E2 and K46AE2. In Fig. 7, panel B, E2 gave a somewhat higher stimulation than K46AE2, but the opposite was observed in other experiments. The only experimental difference associated with this variation was the age of the normal or modified E2 preparation; small decreases in enhanced PDP catalysis were observed for preparations stored for some time or frozen and thawed more than once. These results indicate that the lipoate of L1 is not contributing to the large stimulation of PDP activity. Equivalent levels of the E2 component of bovine E2-E3BP-PDK subcomplex were stimulated to essentially the same level (data not shown). Since a greater enhancement was found with K46AE2 than L2E2, above, this probably reflects the altered environment at the surface of E2–60-mer when the L1 domain is present (discussed further below).

Since, in marked contrast to L2 domain, the L1 domain alone exhibits no capacity to competitively bind PDP (11) and since L1E2 failed to enhance PDP activity (Fig. 7, panel A), it was surprising that K173AE2 enhanced PDP activity. Considering the exclusive requirement for an L2 domain in our other studies, the prospect that somehow the L1 with its lipoate works in combination with the nonlipoylated L2 domain to elicit this effect would be very surprising considering that a maximal enhancement was achieved at a very low K173AE2 level (1.5 \(\mu M\)). A more likely prospect is that E1b, which is bound to an E2 containing two lipoyl domains, makes E1b a better substrate than free E1b for an unbound PDP, particularly when located next to a nonlipoylated L2. A modest (40%) increase in PDP activity was observed using an E1-binding preparation of bovine E2 in which the bilipoyl domain region was selectively removed by collagenase treatment (11). In support of this prospect of improved presentation of E1b for being dephosphorylated, K173AE2 gave 2.1-fold increase in PDP activity in the absence of Ca\(^{2+}\). However, we are unable to explain why L1E2 did not facilitate some increase in PDP activity via this mechanism.

Basis of Greater Effectiveness of K46AE2 Than L2E2 in Supporting PDK and PDP Function—The activation of PDK and PDP requires the lipoyl prosthetic groups on the lipoyl domains of E2 (11, 36), and PDK and PDP do not bind to delipoylated L1 (9, 11). Removal of L1 reduced PDP and PDK function, whereas prevention of lipoylation of L1 allowed fully activated PDP function and supported enhanced PDP function exceeding native E2. The latter gain in PDK activation and maximal stimulation can be explained by a lack of competitive binding of PDK to L1. However, to explain the diminished PDP and PDP activations upon removal of the L1 domain requires either that the bilipoyl domain E2 is more effective in enhancing PDK and PDP activity due to an active role of L1 or that these activities are diminished by the marked change in the environment at the surface of E2 following removal of the L1 domain in L2E2. Although many explanations are possible, for simplicity we would emphasize the latter. Each lipoyl domain has a high

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7 As indicated under “Experimental Procedures” E1b is prepared using E2-E3BP-PDK subcomplex as a source of kinase, and this is then removed by centrifugation. Trace levels of E2 in this E1b preparation (<1% of the protein based on SDS-PAGE pattern from a heavily loaded sample) reduced the activation observed with various E2 sources. The presence of contaminating bovine E2 was also indicated by EGTA reducing PDP activity by 40% in the absence of an added E2 source.
proportion of charged residues (>25%) with an excess of acidic over basic amino acids. Thus, 60 L1 concentrated at the surface of the E2 oligomer can change electrostatic interactions and additionally introduce molecular crowding effects that could alter component interactions. Considering components must move for one PDK or PDP molecule to efficiently modify many bound E1, it seems likely that the absence of L1 in L2E2 alters the environment at the surface of the complex to such an extent that it would alter these dynamic protein-protein interactions. PDK activation may be complex since PDK isomers are oligomers and can probably bind at least temporarily to more than one lipoyl domain (9). Whatever role L1 has in aiding PDK function, it is by-passed upon stimulation of PDK by acetylation, supporting a preferential interaction of the PDK used in these studies with acetylated-L2 (cf. below).

Final Considerations—Our results emphatically support an essential mediatory role of the L2 domain and its lipoyl group in facilitating PDK and PDP operation at accelerated rates and extent that it would alter these dynamic protein-protein interactions. PDK activation may be complex since PDK isomers are oligomers and can probably bind at least temporarily to more than one lipoyl domain (9). Whatever role L1 has in aiding PDK function, it is by-passed upon stimulation of PDK by acetylation, supporting a preferential interaction of the PDK used in these studies with acetylated-L2 (cf. below).

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* In the L1 structure, >25% of its residues are acidic (16 Asp + Gla or basic (10 Arg + Lys, excluding Lys-46); similarly L2 has 17 acidic and 9 basic residues. For these values, we include residues 1–96 in L1 domain and residues 128–229 in the L2 domain. Residues 82–96 in L1 and residues 209–229 in L2 do not align with domain residues in bacterial lipoyl domains of known structure (5). However, these regions cannot be removed without significant loss of functional properties of L1 and L2 (A. Yakhnin, X. Yong, and T. E. Roche, unpublished data).

* Recombinant PDK preparations tend to form aggregates. A dimer appears to be the lowest aggregation state.
Requirements for the Adaptor Protein Role of Dihydrolipoyl Acetyltransferase in the Up-regulated Function of the Pyruvate Dehydrogenase Kinase and Pyruvate Dehydrogenase Phosphatase

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