Assembly and Full Functionality of Recombinantly Expressed Dihydrolipoyl Acetyltransferase Component of the Human Pyruvate Dehydrogenase Complex*

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The dihydrolipoyl acetyltransferase (E2) component of mammalian pyruvate dehydrogenase complex (PDC) consists of 60 COOH-terminal domains as an inner assemblage and sequentially via linker regions an exterior pyruvate dehydrogenase (E1) binding domain and two lipoyl domains. Mature human E2, expressed in a protease-deficient Escherichia coli strain at 27°, was prepared in a highly purified form. Purified E2 had a high acetyltransferase activity, was well lipoylated based on its acetylation, and bound a large complement of bovine E1. Electron micrographs demonstrated that the inner core was assembled in the expected pentagonal dodecahedron shape with E1 binding around the inner core periphery.

With saturating E1 and excess dihydrolipoyl dehydrogenase (E3) but no E3-binding protein (E3BP), the recombinant E2 supported the overall PDC reaction at 4% of the rate of bovine E2-E3BP subcomplex. The lipoylas of assembled human E2 or its free bilipoyl domain region were reduced by E3 at rates proportional to the lipoyl domain concentration, but those of the E2-E3BP were rapidly used in a concentration-independent manner consistent with bound E3 rapidly using a set of lipoyl domains localized nearby. Given this restriction and the need for E3BP for high PDC activity, directed channeling of reducing equivalents to bound E3 must be very efficient in the complex.

The recombinant E2 oligomer increased E1 kinase activity by up to 4-fold and, in a Ca²⁺-dependent process, increased phospho-E1 phosphatase activity more than 15-fold. Thus the E2 assemblage fully provides the molecular intervention whereby a single E2-bound kinase or phosphatase molecule rapidly phosphorylate or dephosphorylate, respectively, many E2-bound E1. This, we prepared properly assembled, fully functional human E2 that mediated enhanced regulatory enzyme activities but, lacking E3BP, supported low PDC activity.

Pyruvate dehydrogenase complexes from various sources are among the largest enzyme systems that serve strategic roles in metabolism (1, 2). The mammalian complex has a highly organized structure in which the dihydrolipoyl transacetylase (E2) component has a central role in the organization and integrated chemical reactions of the complex, and supports enhanced functioning of dedicated kinase and phosphatase components (3–8). The other components of the mammalian complex required for the overall reaction are: the pyruvate dehydrogenase (E1) component, an αβγ tetramer present at 20–30 copies (1); the dihydrolipoyl dehydrogenase (E3), a homodimer present in about 6 copies (9); and the E3-binding protein (E3BP) estimated at 6–12 copies (10, 11). Dedicated and highly regulated kinase and phosphatase components control the conversion of E1 between an active (nonphosphorylated) form, E1a, and an inactive (phosphorylated) form, E1b.

Mammalian PDC-E2 subunits have four flexibly connected domains and form the core of the complex in which 60 of its COOH-terminal inner (I) domains assemble into a dodecahedral shape with its other three domains extending out around this porous surface. Theicosahedral I₆₀ inner core anchors the E3BP (12–14) and carries out the transacetylase reaction (15), probably via functional trimer units as established for the octahedral inner core of Azotobacter vinelandii PDC-E2 (16, 17). Trimer units were also shown to be important in the assembly of bovine heart E2 subunits dissociated to nonfunctional monomers in 4 M guanidinium chloride (18).

The outer globular domains of E2 consist of two lipoylate-bearing domains (L1 and L2), followed by a small E1 binding (B) domain. The connecting hinge regions are 20–30 residues in length, are enriched in Ala and Pro residues (19, 20), and are highly mobile but stiffer than random coil structures (5, 7). Three-dimensional structures have been derived for examples of each of E2’s domain classes from bacterial PDC-E2s (16, 21–23). However, the domains in mammalian E2 have significant differences both in structure and function (3–6). For instance, the mammalian PDC-E2 lipoyl domains appear to be larger and at least L2 has several specialized roles (see below). The small B domains of eukaryotic PDC-E2s only bind E1, whereas the structurally related domains in other PDC E2s bind only E3 (e.g. Escherichia coli PDC; Refs. 5 and 24) or bind both E3 and E1 (e.g. Bacillus stearothermophilus PDC; Refs. 5, 25, and 26). The I domains of eukaryotic PDC-E2s are apparently unique in binding the E3BP.

1 The abbreviations used are: E2, dihydrolipoyl acetyltransferase component; PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase component; L1, NH₂-lipoyl domain of E2; L2, interior lipoyl domain of E2; B, E1-binding domain of E2; I, oligomer-forming, transacetylase-catalyzing COOH-terminal inner domain of E2; E3, dihydrolipoyl dehydrogenase; E3BP, E3-binding protein (formerly protein XI); PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5’-dithiobis(2-nitrobenzoate); PEG, polyethylene glycol; Mops, 3-(N-morpholino)propanesulfonic acid.

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more, the L2 domain has a direct role in mediating regulatory of the lipoyl domain with E1 is essential for efficient E1 catalyzed to the E1, E2, and E3 components where the prosthetic connecting hinge regions allows the lipoyl domains to be delivered to performing their unique roles. High mobility of the added structure at their COOH-terminal ends that may contribute to their active site channels (8). Specific interaction of the lipoyl domain with E1 is essential for efficient catalysis and probably aids the E2 and E3 reactions (5, 28, 29). 4 The L2 domain binds the E1a kinase (30) and the E1b phosphatase (31). Binding of a kinase dimer probably engages two L2 domains plus their lipoyl prosthetic groups (30), while the binding of the phosphatase to L2 requires Ca2+ (31). Furthermore, the L2 domain has a direct role in mediating regulatory stimulations of the kinase by the PDC products, NADH and acetyl-CoA (32), and the marked enhancement of phosphatase activity by the Ca2+-facilitated binding to L2 constitutes an important means of second messenger activation (33, 34).

Although recombinant lipoyl domain structures of human E2 have proved useful for determining binding sites and characterizing component reactions, some catalytic and regulatory processes require a higher level of structural complexity. For instance, markedly activated functioning of the kinase and the phosphatase, when bound to the complex, is not supported by these isolated domains and apparently requires the full oligomeric E2 structure, which can bind multiple copies of the E1 substrate as well as a regulatory enzyme and can facilitate a sequence of steps needed to produce enhanced activity. The capacity of the mammalian E2 core to support the overall PDC reaction has been previously evaluated only when a major portion of E3BP has been removed from the E2 core under highly chaotropic conditions (35, 36), making uncertain the status of the residual E2. Recombinant expression of assembled E2 would overcome these problems and allow development of altered structures in subsequent work.

Successful expression and recovery of assembled recombinant E2 could not be expected on the basis of previous studies, in which bacterial (E. coli and A. vinelandii) or Saccharomyces cerevisiae PDC-E2s were recovered only upon co-expression in E. coli of the cognate E1 or E1 + E3 (13, 37, 38). The successful expression of assembled but lipoyl-deficient E2 core of the bovine branched-chain α-keto acid dehydrogenase complex was encouraging (39), as well as evidence for the independent folding and assembly of unfolded bovine E2 subunits (18). Here, we have developed conditions for expressing fully assembled mature human E2 in E. coli and for obtaining highly purified preparations that have the expected structure and known catalytic functions. We have evaluated how well this E3BP-deficient E2 supports the PDC reaction, E3 catalysis, and the enhanced catalytic reactions of the kinase and the phosphatase.

Materials—Bovine kidney PDC (40), E1 component, E2-E3BP-kinase subcomplex (41), and the recombinantly expressed lipoyl domain region of human E2 (42) were prepared as described previously. Porcine heart E3 was from Boehringer Mannheim. The original pBTA vector that coded for all of mature human E2 as well as a portion of its leader sequence was kindly provided by M. E. Gershwin (University of California, Davis). The expression vector pSE420 and its recommended E. coli host strain, Top10, were from Invitrogen; and the host E. coli strain BL21(DE3), lacking the lon protease and ompT outer membrane protease, was from Novagen. PEG-8000 was from J.T. Baker Co., and Pluronic F-68 was from BASF Corp. Other chemicals and reagents were as described by Liu et al. (42).

Vector Construction and Expression—The plasmid pBTA harboring the human E2 cDNA insert was cut with restriction enzymes SfiI and BamHI to produce a DNA fragment lacking the coding region for the leader sequence and first 7 amino acids of mature E2. Two phosphorylated synthetic oligonucleotides (sequences in Fig. 1) were hybridized to produce a small double-stranded DNA fragment that coded for this remaining portion of mature E2 preceded by the ATG start codon and containing compatible splicing sequences for a NcoI site at the 5'-end of the coding sequence and a SfiI site at its 3'-terminus. This synthetic structure, the pBTA restriction fragment, and the pSE420 vector digested with NcoI and BamHI were ligated to produce the expression vector coding for mature E2 preceded by a start codon (Fig. 1). The ligated plasmid was transformed into E. coli strains by electroporation using Transfector 300 BTX under the conditions recommended in the manufacturer’s manual. The expression of E2 was first confirmed by dot blots on Western blots using a mixture of monoclonal antibodies 150.2 and 157.2 (42), which react with the first and second lipoyl domain, respectively. Clones were designated pHsE2 (pS for pSE420 vector, h for human, E2 for coding for full-sized mature E2). The DNA sequencing was conducted using a series of synthetic primers and the dideoxynucleotide sequencing procedure using the Sequenase Version 2.0 kit from U. S. Biochemical Corp.

Experiments to optimize expression used Western blot analyses employing not only the lipoyl domain-specific monoclonal antibodies (above) but also a polyclonal antibody that reacts with the inner domain of E2. With different host strains, induction at different stages of growth, and expression for variable periods, Western blots were used to detect production and cleavage of E2 when intact bacterial cells were extracted with hot SDS-sample buffer, soluble and particulate fractions were prepared from different host strains, and clarified lysates were incubated for various times.

Purification of the Recombinant E2 Oligomer—All steps were performed at 4 °C. PEG and (NH4)2SO4 fractions involved continuous stirring with centrifugation conducted 20 min after addition of these precipitants were completed. E2 activity was measured in a rapid continuous assay (below) with corrections in the case of crude extracts for coli PDC-E2 activity: for coli assuming the ratio of E2 to PDC activity as measured in the nontransformed host strain. (The expression of human E2 had no effect on the E. coli PDC activity.) Cells (12–14 g) harvested from 1.5 liters were thawed and resuspended at 12.5% w/v in 50 mM potassium phosphate (pH 7.2), containing 0.5 mM EDTA, 1 µg/ml aprotinin and leupeptin (buffer A) and immediately ruptured using Vibra-cell high intensity ultrasonic processor (Sonics and Materials Inc.) equipped with a 0.5-inch probe at a setting of 4 using 10 1-min bursts. Particulate material was removed by centrifugation at 15,000 × g for 20 min. PEG-8000 (50% w/v) was added dropwise to the clarified lysate to 8% (v/v), and precipitated protein, removed by centrifugation, was discarded. An additional 8% (v/v) was added followed by centrifugation. This 8–16% PEG pellet was dissolved in buffer A. The protein-concentration was diluted to 2–3 mg/ml and solid (NH4)2SO4 was added to 25% saturation. The E2-containing precipitate was removed by centrifugation and dissolved in 4 ml of buffer A containing 0.2 mg/ml Pluronic F-68. After 12 h, the fraction was clarified by centrifugation and applied to 2.7 × 75-cm bed volume Sephacryl S-400 column equilibrated with 40 mM sodium phosphate, 0.5 mM EDTA, 1 µg/ml aprotinin, and leupeptin, and 0.2 mg/ml Pluronic F-68 (buffer B). Fracions (1 ml) were assayed and those containing E2 were clarified by centrifugation at 15,000 × g for 20 min and then simultaneously analyzed by SDS-PAGE (43) with silver staining (44) and concentrated 10–12 fold by centrifugation in Centricon 10 units. Concentrated fractions were then stored in aliquots at −80 °C.

The purified E2 preparations were analyzed to identify cleavage products, establish the NH2-terminal sequences of all E2 bands, and determine the proportion of intact E2 subunits. Putative intact E2 and

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2 J. K. Stoops, R. H. Cheng, M. A. Yazdi, C.-Y. Maeng, J. P. Schroeter, U. Kleeberg, S. J. Kolodziej, T. S. Baker, and L. J. Reed, submitted for publication.

3 The Kd of E3 reaction for L1 lipoyl domain of human PDC-E2 was found to be well below the Kd of E3 for dihydrolipoamide (J. C. Baker and T. E. Roche, manuscript in preparation).

4 Potential pockets for lipoyl domains appear to exist at the entrance to the active sites of the E2 and E3 components (W. H. G. Hol and J. Hendle, personal communication).
cleaved E2-polyepitopes, initially identified by immunoblottedting were blotted onto ProBlott, stained with 0.1% Coomassie Blue R250 in 1% acetic acid, 40% methanol for 1 min, and destained in 50% methanol, and then NH2-terminal sequencing was conducted as described previously (42). To estimate the proportion of E2-protein and relative levels of intact E2, quantitative analysis of silver-stained patterns was performed following SDS-PAGE separation of 0.4, 0.8, 1.6, and 2.4 μg of purified human E2 and 0.6, 1.2, 2.0, and 3.2 μg of bovine E2-E3BP loaded into lanes. Alternatively, 0.48, 0.96, 1.6, 2.4 and 4.0 μg of human E2 and 0.8, 1.2, 2.4, 5.0 μg of bovine E2-E3BP were analyzed in Coomassie Brilliant Blue R-250-stained patterns. Band densities were evaluated by scanning with an Epson E2200 flatbed scanner acquiring area density data with Adobe Photoshop program, and analyzing the data using NIH Image software. Each series gave a linear increase in area with the amount loaded (data not shown).

E2 Activity Assays—Routine E2 activity assays monitored acetyl-dihydrolipoamide production by recording the ΔA232 nm with time (45). Reaction mixtures contained 30 μM Tris-HCl (pH 7.4), 1 mM dihydrolipoamide, 1 mM acetyl-phosphate, 50 μM CoA (prepared as concentrate 1:1 with cysteine), and 1 unit of phosphotransacetylase in a 1-ml cuvette at 30 °C. After the absorbance became constant due to formation of acetyl-CoA (~10 s), the E2 source was added and increased absorbance at 232 nm measured. The activities are recorded simply as absorbance/min because the precise extinction coefficient of the immediate 8-acetyl derivative in acetic acid-containing disks; protein-bound [32P]phosphate was determined as described previously (54).

[3P]Phosphate release from PDCb or E1b was measured at 30 °C in 30-μl reaction mixtures containing 20 mM Mops-K buffer (pH 7.3), 0.4 mM dithiothreitol, 0.6 mM EDTA, 1.0 mM EGTA, 1.2 mM CaCl2, and the indicated level of E2 oligomer (56, 57). E1b phosphatase was added for 120 s and then activity initiated by addition of MgCl2 to 10 mM. After 120 s, reactions were terminated by addition of 50 μl of 20% trichloroacetic acid and precipitated protein was pelleted after at least 30 min of incubation at 4 °C by centrifugation at 10,000 × g at 8000 × g. Aliquots (28 μl) were withdrawn and radioactivity determined by counting in scintillation fluid. All kinase and phosphatase assays were conducted in duplicate or triplicate; absolute deviations are shown.

RESULTS

Construction and Sequencing of E2-coding Expression Vector and Development of Optimal Expression Conditions—The initial expression vector that was provided coded for all of mature E2, 16 amino acids of the presequence (location of a convenient EcoRI restriction site), and a small terminal portion of β-galactosidase (58). The expression product accumulated only in inclusion bodies and lacked E2 activity before and after a variety of solubilization procedures but was fully antigenic in Western blots using polyclonal and monoclonal antibodies. The construction of the expression vector, as outlined in Fig. 1, removed the leader sequence and adjointed a start codon at the front of the coding region for mature E2. The pSE420 vector was chosen for its bacteriophage T7 gene 10 element that enhances translation of eukaryotic genes. Cell lysates from pShE2 transformed colonies gave strong responses in dot blots using lipoyl domain-specific monoclonal antibodies and contained soluble E2 activity well beyond that of E. coli PDC (data not shown).

The pSE2 plasmid was sequenced not only at the ligated 5'-end but throughout most of the E2-encoding insert, including all regions in which the sequences of Coppel et al. (19) and of Thekkumkara et al. (20) differed. Sequencing confirmed that the desired construct was obtained and established a sequence for this placental-cDNA derived construct was identical to that reported for the human liver E2 (20), establishing that previously reported differences were due to sequencing errors.

Immunoblot analysis revealed substantial full-sized E2 (as expected with a mobility in SDS-PAGE somewhat faster than bovine E2; Ref. 59) along with degradation products, which were not reduced by inclusion of a variety of protease inhibitors in cell lysates. Furthermore, there was no time-dependent change in the pattern of intact versus partially cleaved E2 when cell lysates were incubated at 25 °C in buffer A and the same pattern of E2 bands was observed when cells were disrupted in 2% SDS-sample buffer by immediate insertion in boiling water (data not shown). Co-expression of groELS chaperonin proteins did not reduce E2 cleavage or increase the portion of E2 fractionating into the supernatant fraction. Thus,
if chaperonins are required to aid the folding or assembly of E2, the endogenous levels in E. coli are sufficient. Using pShE2 transformed into the protease-deficient BL21 strain, a greater proportion of full-sized E2 was found in the soluble fraction from cell lysates than with the Top10. Using the BL21 host, changing the IPTG induction from stationary to mid-log phase, and lowering the temperature of expression to 27 °C gave a higher level of soluble E2, significantly increased the proportion of intact E2, and reduced the number of E2-derived bands arising from the limited proteolysis. With a correction for the low level of E. coli PDC E2, the transacetylase activity indicated that recombinant E2 constituted about 3% of the soluble protein after 8 h of post-induction growth.

**Purification of Recombinant E2**—Using the steps detailed under “Experimental Procedures,” the recombinant E2 was purified to near homogeneity. The initial PEG fractionation step left >99% of the contaminating E. coli PDC in solution, but a significant portion of the bacterial α-ketoglutarate dehydrogenase complex co-precipitated with the E2 in the 8–16% cut. Human E2 (intact E2αβ has calculated Mr of 3.57 × 10^6) eluted prior to the similarly sized α-ketoglutarate dehydrogenase complex in gel-filtration chromatography, demonstrating that E2 subunits assembled into a large aggregate.6 The most prominent protein (Mr = 42,000 in SDS-PAGE) not separated from E2 by the gel filtration, as well as the E. coli α-ketoglutarate dehydrogenase complex, were removed by fractionation with ammonium sulfate prior to the size exclusion step; both contaminants stayed in solution when E2 was precipitated.

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**Fig. 2. Change in protein composition during the purification of human E2 and comparison with bovine PDC and bovine E2-E3BP subcomplex.** SDS-PAGE separation and silver staining were performed on: lane 1, 1.6 μg of PDC; lane 2, 1.0 μg of bovine E2-E3BP; lane 3, 4.1 μg of clarified lysate; lane 4, 3.3 μg of PEG fraction; lane 5, 0.7 μg of ammonium sulfate fraction; lane 6, 0.6 μg of the post-Septacryl-S400 fraction that was cleared of aggregated protein by centrifugation.

Gel-filtration chromatography then removed low molecular weight contaminants, followed by a clarifying centrifugation step, which reduced or removed additional contaminants. A variety of other procedures either failed to allow E2 to be recovered (ion exchange or hydroxylapatite chromatography) or failed to significantly improve the purification (additional PEG fractionations, sucrose gradient centrifugation).

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6 The E2 eluted in a broad peak from Sephacryl S 400 prior to a sharp peak for the α-ketoglutarate dehydrogenase complex. Since the latter complex has a similar mass (Mr = 2.8 × 10^6 versus 3.6 × 10^6 for unclipped E2 oligomer) and both structures have high frictional coefficients, this suggests that the E2 oligomer has a tendency to form aggregates. The inclusion of Pluronic F-68 (2 mg/ml) reduced but did not eliminate peak broadening due to decreased aggregation and minimized peak trailing due to interaction with the gel matrix.
products of E2 retaining the oligomer forming inner domain. The other minor contaminants all changed in their ratio to mature E2 during the purification process. NH$_2$-terminal sequencing gave SLPHPKIV, AKILVAEG, and VVPPTGP for bands identified as E2, E2', and E2", respectively, establishing that the upper band is mature E2 with start codon Met removed and that E2' and E2" resulted from cleavage following Met-59 (located in L1 domain) and Leu-318 (located in the third linker region just prior to the inner domain of E2), respectively.

Densitometric area scanning of silver-stained bands of SDS-PAGE patterns of E2 loaded over a 6-fold range (see “Experimental Procedures” for samples of bovine E1 (20 μg), purified human E2 (25 μg), or a combination thereof. SDS-PAGE analysis was conducted on a 10-μl sample of the S1, and 3 μl each of the S2, S3, and P fractions. Silver-stained patterns are shown for each series as well as for 3 μg of bovine PDC (leftmost lane).

![Fig. 3. Binding of bovine E1 to human E2. Microsucrose gradient separation was conducted and fractions derived as described under “Experimental Procedures” for samples of bovine E1 (20 μg), purified human E2 (25 μg), or a combination thereof. SDS-PAGE analysis was conducted on a 10-μl sample of the S1, and 3 μl each of the S2, S3, and P fractions. Silver-stained patterns are shown for each series as well as for 3 μg of bovine PDC (leftmost lane).](image)

![Fig. 4. Electron micrographs of human E2 and human E2-bovine E1 complexes. Upper panel is a field of purified human E2 oligomer with specific particles indicated that are observed along the 5-fold, 3-fold, and 2-fold axes; bottom panel shows examples with a high (left) and low (right) level of bound E1. Measurement of a large number of particles observed along the 5-fold axis gave diameters of 22.7 ± 1 nm (left center). The inner core structure revealed in the center of the E2-E1 complex in the lower panel on the right appears to be viewed along the 5-fold axis; this inner core structure had a diameter estimated at 24.0–24.4 nm.](image)

### Human Dihydrolipoyl Acetyltransferase

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**TABLE I**

| Fractions | Protein | Volume | Specific activity | Purification | Yield |
|-----------|---------|--------|------------------|--------------|-------|
|           | mg      | ml     | ΔA$_{232}$/min   | ΔA$_{232}$/min/mg | fold | %    |
| Clarified lyate | 689 | 110 | 457 | 0.86 | 1.0 | 100 |
| 8–16% PEG | 136 | 62.5 | 333 | 2.45 | 3.7 | 73  |
| 25% (NH$_4$)$_2$SO$_4$ | 35 | 8.0 | 263 | 7.6 | 11.5 | 58  |
| S-400 gel filtration | 11 | 48 | 110 | 10.0 | 15.2 | 24  |
| Centrifugation/concentration | 1.7 | 2.3 | 33 | 19.4 | 29.4 | 7   |

*Activities are at least averages of duplicates and have a deviation of ±±4%.*
unmodified, whereas with previous studies with bovine E2 (35) from which E3BP was removed, E3BP has not been able to prepare an active form of E3BP in the last column. The E2 oligomer supported a low but significant capacity to support the overall PDC reaction was compared for E2 oligomer relative to E2

Table III shows E3 reaction rates using 0.4–1.6 nmol/min/mg. 

Table II

Comparison of activities of human E2 (hE2) and bovine E2-E3BP subcomplex

| E2 source | Transacetylation activity | E3 activity in E2-E3BP subcomplex | Enzyme specific activity of E2 oligomer | Reconstructed PDC activity |
|-----------|---------------------------|------------------------------------|----------------------------------------|---------------------------|
| hE2       | A<sub>max</sub>/min/mg     | [1-<sup>14</sup>C]acetyl-dihydrolipoamide | µmol/min/mg | µmol/min/mg |
| hE2-E3BP  | 19.4 ± 0.5                | 4.79 ± 0.2                         | 24.7 ± 1.4                         | 0.93 ± 0.06 |
|           |                           |                                    | 33.0 ± 2.1                         | 24.6 ± 2.5 |

Table III

E3 activity as function of E2 lipoic domain level

| Lipoyl domain source | E3 specific activity for E2 lipoic domain concentrations<sup>a</sup> | µmol/min/mg |
|----------------------|---------------------------------------------------------------|-------------|
|                      | 0.40 µM                                                       | 0.80 µM     | 1.20 µM     | 1.60 µM     |
| E3 (0.1 µg) + hE2<sub>L1-L2</sub> | 4.53 ± 0.33                                                   | 7.88 ± 0.39 | 11.49 ± 0.24 | 14.11 ± 0.33 |
| E3 (0.1 µg) hE2      | 3.45 ± 0.27                                                   | 6.57 ± 0.13 | 8.89 ± 0.24 | 11.86 ± 0.49 |
| Endogenous E3 in E2-E3BP subcomplex<sup>c</sup> | 9.34 ± 0.39                                                   | 8.13 ± 0.99 | 8.58 ± 0.54 | 8.92 ± 0.08 |
| E3 (0.1 µg) + E2-E3BP minus endogenous | 9.26 ± 1.38                                                   | 8.77 ± 2.25 | 8.40 ± 2.49 | 8.60 ± 0.72 |
| E3 activity in E2-E3BP<sup>d</sup> | 6.366                                                       |             |             |             |

<sup>a</sup> Each structure contains both L1 and L2 lipoic domain of E2, and concentration is that of individual E2 subunits or of the free bilipoyl domain structure.

<sup>b</sup> This recombinant bilipoyl domain construct contains amino acids 1–233 of sequence of human E2 and was prepared as described previously (31).

<sup>c</sup> The level of bound E3 was estimated to be 0.015 µmol/min/mg subcomplex by matching staining intensities of SDS-PAGE analysis on several concentrations of subcomplex and several levels of E3. E3BP also contributes a lipoic domain, and its concentration is 10–20% of the concentration of E2 subunits (so E3BP contributes 5–10% of lipoic domains in subcomplex).

<sup>d</sup> Deviations are the sum of the deviation range in assays with and without added E3 to E2-E3BP.

Subcomplex (Table II). The other transacetylase assay measuring [14C]acetyl-dihydrolipoamide, which uses a 20-fold higher acetyl-CoA concentration, also gave a higher activity for the recombinant E2 than for E2-E3BP subcomplex (Table II, second column). We estimate the E2 specific activity of the E2 oligomer (following corrections due to non-E2 protein (decrease) and due to the presence of active, truncated forms of cleaved E2 (increase)) to be about 5.0 µmol/min/mg. 

**Participation in the Overall PDC and E3 Reactions**—The capacity to support the overall PDC reaction was compared for E2 oligomer relative to E2-E3BP subcomplex when both were reconstituted with excess E1 and the E3 components (Table II, last column). The E2 oligomer supported a low but significant rate of the overall reaction in the absence of E3BP. Even when a 4-fold higher level of E2 was added to the E2 oligomer, there was no increase in reconstituted PDC activity (data not shown). We have not been able to prepare an active form of E3BP in the absence of E2. Retention of about 4% activity is consistent with previous studies with bovine E2 (35) from which E3BP was removed, whereas with S. cerevisiae E2 no activity in the overall reaction was observed in the absence of E3BP. Even not as extreme as in the case of the yeast PDC, the retention of such a low activity indicates that the overall PDC reaction is facilitated by molecular mechanisms that efficiently channel reducing equivalents to E3BP-bound E3 (cf. “Discussion”).

Liu et al. (42) found that the individual lipoic domains of E2, prepared by recombinant techniques, were effectively utilized by the E3 component in a cyclic form of the reverse reaction involving NADH-dependent reduction by E3, followed by rapid chemical reoxidation to the disulfide by reaction with DTNB. Table III shows E3 reaction rates using 0.4–1.6 µM intact E2 subunits of the E2-E3BP subcomplex or recombinant oligomer and of the bilipoyl domain portion of E2 (L1-H1-L2). With the recombinant sources (lines 1 and 2, Table III), concentration-dependent increases in rates fit E3 using these lipoic domain sources as a substrate with a K<sub>m</sub> above this concentration range. Indeed, increasing the level of a free lipoic domain yields a maximal specific activity that is much higher. The somewhat higher rates with the free bilipoyl domain structure than with the E2 oligomer may reflect more efficient delivery of lipoic domains to the E3 active site with the freely diffusing structure than when lipoic domains are grouped in close proximity within an E2 structure that E3 cannot bind.

Results from varying the E2-E3BP subcomplex were very different (Table III). The subcomplex contained a low level of endogenous E3 even after additional resolution steps to reduce the E3 level. Both this endogenous E3 (line 3) and added E3 (line 4) gave a fixed and nearly identical specific activity as the subcomplex level was increased. This implies that E3 bound to E3BP is not responding to variation in the E2 concentration but only to the fixed stoichiometry of lipoic domains surrounding it within the E2-E3BP subcomplex. Addition of E3 to the E2-E3BP to levels exceeding 30% of its E3 binding capacity lowered E3 specific activities (data not shown). This outcome suggests an increased competition for localized lipoyl of more E3 is bound in the subcomplex. The results are consistent with E3, localized by binding to E3BP, encountering a fixed availability of lipoic domains to introduce reducing equivalents.

**Capacity of the Recombinant E2 Oligomer to Support Kinase and Phosphatase Function**—Bovine kidney E1α kinase binds to the inner (L2) domain of E2 (30); binding increases the rate of phosphorylation of E1α several-fold by lowering the K<sub>m</sub> for E1α 20-fold and increasing the V<sub>m</sub> of the kinase 2-fold (51). In the resolution of bovine kidney PDC that produces the E2-E3BP subcomplex and E1, a major portion of the kinase fractionates with the subcomplex and a small portion with E1. The activation state of the dilute kinase that fractionates with E1 is enhanced with the level of E2. With 2.9 µM E1, 6 µg of the recombinant E2 oligomer gave somewhat more than a 2-fold increase and 19 µg provided >4-fold increase in this kinase activity (Fig. 5). In contrast, no increase in kinase specific activity was found when E2-E3BP-kinase subcomplex was varied to provide a similar range of E2 levels. The activation of

<sup>7</sup> Using a constant level of kinase-free recombinant human E1 (prepared as described by Korotchkin et al. (62)) as the kinase substrate, at least as high kinase activity was obtained with 7 µg (specific activity 12.4 nmol/min/mg of subcomplex) as with 18 µg of subcomplex, indicating no change in kinase binding to E2 in this range (J. C. Baker and T. E. Roche, unpublished result).
the kinase associated with E1 but not that associated with E2-E3BP as the concentration of recombinant or subcomplex E2 was varied in the same range may reflect enrichment of different kinase isozymes (63–65) with E1 or with E2-E3BP during the resolution of bovine PDC.

The Ca$^{2+}$-dependent E2 enhancement of the Mg$^{2+}$-requiring E1b phosphatase activity is pronounced, with activation in the presence of saturating Mg$^{2+}$ primarily resulting from a decrease in the $K_m$ of the phosphatase for E1b (66). Fig. 6 shows that, in 25-μl reaction mixtures, the recombinant E2 oligomer markedly increased phosphatase activity with as little as 2 μg of E2 giving a 10-fold increase in activity and 8 μg of E2 facilitating up to a 16-fold increase in phosphatase. Relatively low E1b (1.52 μM) was included, which maximizes E2 activation in these assays. From a reciprocal plot of activity to E2 subunit concentration, half-maximal activation of the phosphatase is estimated to occur at 0.74 ± 0.1 μM E2 subunit concentration, consistent with tight binding of the phosphatase to E2.

When 8 μg of phosphorylated PDCb and 4 μg of E1b (3.8 μg of bovine E2, 1.88 μM E1b in total) were present, phosphatase activity was increased by further addition of 8 μg of recombinant E2 by 2-fold over the enhancement by bovine E2 (right series, Fig. 6). Comparing the absolute activities for a change from 3.8 μg of bovine E2 to a total of 7.8 μg of E2 (3.8 μg of bovine plus 4 μg of human E2) to that for a change from 4 μg to 8 μg of human E2, the initial activity is 2-fold higher with 4 μg of human E2 than with the bovine E2 and the final activity is still 41% higher with 8 μg of human E2 than with the combination of human and bovine. These data suggest human E2 is more effective than bovine E2 in activating the phosphatase. It remains to be determined whether that is due to interference of other components (E3BP or E3), damage to bovine E2 during preparation of the complex, or an innately greater capacity of the human E2 to activate the bovine phosphatase in dephosphorylating bovine E1b. Regardless, these data further support high functionality of the recombinant human E2.

**DISCUSSION**

E2 components have many roles in the organization, operation, and regulation of α-keto acid dehydrogenase complexes. Because E2 subunits invariably have modular structures with independently folded domains set off from each other by mobile linker regions, the domain-specific roles can be fruitfully diagnosed by recombinant DNA approaches as exemplified by using individual domains or groups of domains in native or mutated forms in studies of *E. coli* (e.g. Refs. 37 and 67–69), *B. stearothermophilus* (e.g. Refs. 21, 22, and 24–26), *A. vinelandii* (16, 17, 70), and *S. cerevisiae* PDC-E2s (e.g. Refs. 13 and 61). However, as noted in the Introduction, some roles require assembly of E2 structures. Guest and colleagues have characterized a variety of assembled *E. coli* PDC-E2 structures for lipoyl domain roles and found the three lipoyl domains have equivalent capacities in supporting the overall PDC reaction (67–69). As in the case of *S. cerevisiae* PDC (27), production of mammalian E2 free of E3BP is important for distinguishing the contribution of these two lipoyl-bearing components in catalytic and regulatory processes. Mammalian PDC is regulated by phosphorylation and dephosphorylation, but that is not the case in the well characterized bacterial PDCs, and yeast PDC has only recently been shown to produce a kinase and phosphatase under specialized growth conditions (71).

We have successfully expressed and prepared in a highly purified state the assembled E2 core of human PDC without coexpression of other components of the complex. Beyond deleting the upstream coding region (including a partial leader sequence), key conditions were expression of E2 in a protease-deficient host (*E. coli* BL21), lipoate supplementation, and induction of expression in mid-log growth phase at a reduced temperature of 27 °C. The latter two conditions minimized but did not completely eliminate proteolytic cleavage and allowed recovery of an assembled structure with about 75% of the subunits intact. Sequencing of NH$_2$ termini identified a truncated structure, labeled E2', whose sequence began after Met-59 located in the first domain; E2' may have been produced by the Met-59 codon serving as an internal start site. There is a good Shine-Dalgarno sequence (GGAGGAG) located 6 nucleotides upstream of the Met codon. This Met residue aligns with Leu, Val, and Ile in other PDC-E2 lipoyl domains and corresponds to a buried amino acid in the established three-dimensional structures of bacterial domains (22, 23), suggesting that production of E2' by proteolysis might require cleavage to occur prior to folding of the lipoyl domain. A second site of cleavage is located near the end of the third linker region. By whatever means, the E2' and E2 structures were created prior to rupture of *E. coli* cells since they were observed in immunoblots when cells were extracted with immediate heating in SDS-sample buffer. Residual contaminants in E2
preparations are large structures that do fractionate somewhat differently in sucrose gradients (e.g. Fig. 3), and there is no indication that these E. coli proteins interact with human E2.

Although not fully intact, the recombinant E2 exhibited high activity in the acetyltransferase reaction, bound the E1 component, and supported the activated function of the kinase and phosphatase. The observable inner core has the expected decahedron structure. Supporting the independent folding of lipoyl domains, internal disruption of the terminal lipoyl domain in E2’ did not prevent folding and lipoylation of the inner lipoyl domain since it could be effectively acetylated by the E1 (data not shown) or E2 reactions.

Cryoelectron microscopy and quasielastic light scattering measurements indicate that hydrated mammalian PDC has a diameter of ~500 Å, that the E1 and E3 components are tethered with gaps between them and the inner core, that there is considerable solvent entrapped at the surface of the complex (53, 72). This solvent-rich environment can support domain and component movement, a capacity eminently important for many explanations are possible (cf. “Results”), testing the interesting prospect that E3BP has an inhibitory role will require preparation of human E2-E3BP.

In agreement with an earlier preparation (35) in which bovine E2 was freed of E3BP (then designated as protein X), the recombinant E2 oligomer was able to support about 4% of the PDC activity obtained with the E2-E3BP subcomplex. These results differ from those obtained with yeast PDC-E2, prepared in the absence of the E3BP, which failed to support any PDC activity even in the presence of high levels of E3 (61). Nevertheless, both outcomes demonstrate that the binding of E3 by the E3BP component must greatly enhance the capacity to feed reducing equivalents through E3 in the PDC reaction. This suggests unique organizational features allow limited E3 dimers, tethered at the surface of the complex, to function efficiently. The results in Table III indicate E3BP-bound E3 has access to a fixed number of lipoyl domains based on the unchanging specific activity of low levels of E3 (endogenous or recombinant E2 oligomer was able to support about 4% of the PDC activity). These conditions may not only facilitate reducing equivalents being directly channeled to E3 but also aid a rapid thiol-disulfide interchange between lipoyl. The latter may be coupled to electrons being fed to E3 with some degree of preference via the lipoyte on E3BP’s lipoyl domain (8, 73). The E3 component must operate at >280 μmol/min/mg of E3 to give the observed specific activity of 20 μmol/min/mg of bovine heart PDC in the overall reaction of the complex (bound E3 is maximally 7% of the protein; Ref. 9). Given this high rate, it likely seemed that reaction of 0.2 μM DTNB with reduced lipoylates is probably limiting when E3 was confined by binding E3BP. In support of this possibility, halving the DTNB concentration to 0.1 mM reduced the E3 reaction rates of E3 associated with E2-E3BP structures by ~40% (data not shown). Thus, a rapid E3 reaction followed by a rate-limiting chemical reoxidation of lipoylates is indicated.

The production of assembled recombinant E2 that functions in all the roles of mammalian PDC-E2 creates abundant opportunities for further studies in which recombinant techniques are used to dissect catalytic, binding, and regulatory roles of this central component of mammalian PDC. Furthermore, new insights and questions are raised by the differences that we have observed between bovine E2-E3BP and human E2 in supporting catalytic processes and in their relative capacities to facilitate high E1b phosphatase activity.

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REFERENCES

1. Reed, L. J. (1974) Acc. Chem. Res. 7, 46–48
2. Randle, P. J. (1986) Biochem. Soc. Trans. 14, 799–806
3. Patel, M. S., and Roche, T. E. (1990) FASEB J. 4, 3224–3233
4. Reed, L. J., and Hackert, M. L. (1990) J. Biol. Chem. 265, 8971–8974
5. Perham, R. N. (1981) Biochemistry 20, 6501–6512
6. Roche, T. E., Liu, J., Ravindran, S., Baker, J., and Wang, D. (1996) in α-Keto Acid Dehydrogenase Complexes (Patel, M. S., Roche, T. E., and Harris, R. A., eds) pp. 33–52, Birkhauser Verlag Press, Basel, Switzerland
7. Perham, R. N. (1996) in α-Keto Acid Dehydrogenase Complexes (Patel, M. S., Roche, T. E., and Harris, R. A., eds) pp. 1–16, Birkhauser Verlag Press, Basel, Switzerland
8. Roche, T. E., and Cox, D. J. (1996) in Channeling in Intermediary Metabolism (Agius, L., and Sherar, H. S. A., eds) pp. 115–132, Portland Press, London
9. Wu, T.-L., and Reed, L. J. (1984) Biochemistry 23, 221–226
10. Jilka, J. M., Rahmatullah, M., Kazemi, M., and Roche, T. E. (1986) J. Biol. Chem. 261, 1885–1891
11. Sanderson, S. J., Miller, C., and Lindsay, J. G. (1996) Eur. J. Biochem. 236, 56–77
12. Rahmatullah, M., Gopalakrishnan, S., Radke, G. A., and Roche, T. E. (1989) J. Biol. Chem. 264, 1245–1251
13. Lawson, J. E., Niu, X.-D., and Reed, L. J. (1991) Biochemistry 30, 11249–11254
14. Maeng, C.-Y., Yazdi, M. A., Niu, X.-D., Lee, H. Y., and Reed, L. J. (1994) Biochemistry 33, 13851–13860
15. Bleile, D. M., Hackert, M. L., Pettit, F. H., and Reed, L. J. (1981) J. Biol. Chem. 256, 514–519
16. Matteri, A., Obmolova, G., Schulz, E., Kalk, K. H., Westphal, A., de Kok, A., and Hol, W. G. J. (1992) Science 255, 1544–1550
17. Matteri, A., Obmolova, G., Kalk, K. H., Westphal, A. H., de Kok, A., and Hol, W. G. J. (1993) J. Mol. Biol. 230, 1185–1199
18. Behal, R., DeBuysere, M. S., Demeler, B., Hansen, J. C., and Olson, M. S. (1994) J. Biol. Chem. 269, 31372–31377
19. Coppel, R. L., McNeilage, L. J., Surh, C. D., Vande Water, J., Shippith, T. W., Whittingham, S., and Gershwin, M. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7317–7321
20. Thekkumkara, T. J., Ho, L., Westler, I. D., Pena, G., Lui, T.-C., and Patel, M. S. (1988) FEBS Lett. 244, 45–50
21. Kalia, Y. N., Brocklehurst, S. M., Hips, D. S., Appella, E., Sakaguchi, K., and Perham, R. N. (1993) J. Mol. Biol. 230, 323–341
22. Darde, F., Davis, A. L., Laure, E. D., and Perham, R. N. (1993) J. Mol. Biol. 239, 1047–1048
23. Green, J. D. F., Laure, E. D., Perham, R. N., Ali, S. T., and Guest, J. R. (1995) J. Mol. Biol. 248, 328–343
24. Packman, L. C., and Perham, R. N. (1986) FEBS Lett. 206, 193–196
25. Hips, D. S., Packman, L. C., Allen, M. D., Fuller, C., Sakaguchi, K., Appella, E., and Perham, R. N. (1994) Biochem. J. 297, 137–143
26. Lessard, I. A. D., and Perham, R. N. (1995) Biochem. J. 306, 727–733
27. Rechal, R. H., Browning, A. S., Hall, T. R., and Reed, L. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8732–8736
28. Graham, L. D., Packman, L. C., and Perham, R. N. (1989) Biochemistry 28, 1574–1581
29. Wallis, N. G., and Perham, R. N. (1994) J. Mol. Biol. 236, 209–216
30. Liu, S., Baker, J. C., and Roche, T. E. (1995) J. Biol. Chem. 270, 783–800
31. Chen, G., Wang, L., Liu, S., Chaux, C., and Roche, T. E. (1996) J. Biol. Chem. 271, 28064–28070
32. Ravindran, S., Radke, G. A., Guest, J. R., and Roche, T. E. (1996) J. Biol. Chem. 271, 653–662
33. Denton, R. M., and McCormack, J. G. (1990) Annu. Rev. Physiol. 52, 451–466
34. Hansford, R. G. (1991) J. Bioenerg. Biomembr. 23, 823–834
35. Powers-Greenwood, S. L., Rahmatullah, M., Radke, G. A., and Roche, T. E. (1989) J. Biol. Chem. 264, 3655–3657
