Expression, Purification, and Characterization of the Dihydrolipoamide Dehydrogenase-Binding Protein of the Pyruvate Dehydrogenase Complex from *Saccharomyces cerevisiae*†

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ABSTRACT: Genes encoding dihydrolipoamide dehydrogenase (E_3) and the E_3-binding protein (E_3BP, protein X), components of the *Saccharomyces cerevisiae* pyruvate dehydrogenase (PDH) complex, were coexpressed in *Escherichia coli* to produce an E_3BP-E_3 complex, thereby minimizing proteolysis of E_3BP and facilitating its purification. The 2 genes were linked into a single transcriptional unit separated by a 31-nucleotide segment containing a ribosome-binding sequence. The E_3BP-E_3 complex was highly purified and then separated into E_3 and E_3BP by chromatography on hydroxylapatite in the presence of 5 M urea. The E_3BP-E_3 complex combined rapidly with a pyruvate dehydrogenase (E_1)−dihydrolipoamide acetyltransferase (E_2) subcomplex (E_1-E_2 subcomplex) to reconstitute a functional PDH complex, with pyruvate oxidation activity similar to that of PDH complex from bakers' yeast. The stoichiometry of binding of E_3BP and E_3BP-E_3 complex to the 60-subunit pentagonal dodecahedron-like E_2 was determined with a truncated form of E_2 (tE_2, residues 206−454) lacking the lipoyl domain and the E_1-binding domain, and with E_1-E_2 subcomplex, which contains intact E_2. Mixtures containing tE_2 or E_1-E_2 subcomplex and excess E_3BP or E_3BP-E_3 complex were subjected to ultracentrifugation to separate the large complexes from unbound E_3BP or E_3BP-E_3, and the complexes were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis. After staining with Coomassie brilliant blue and destaining, the gels were analyzed with a video area densitometer. The results showed that the E_1-E_2 subcomplex binds about 12 E_3BP monomers attached to 12 E_3 homodimers. Similar results were obtained by analysis of highly purified PDH complex from bakers' yeast. Somewhat more E_3BP (~15 molecules) and E_3BP-E_3 complex (~14 molecules) bound to tE_2. Structural considerations suggest that 1 E_3BP molecule, bearing an E_3 homodimer, is bound in each of the 12 faces of the pentagonal dodecahedron-like E_2.

Mammalian and *Saccharomyces cerevisiae* pyruvate dehydrogenase (PDH)[1] complexes are organized about a 60-subunit pentagonal dodecahedron-like E_2 core, to which multiple copies of E_1 ($\alpha_2\beta_2$), E_3BP (protein X), and E_3 are bound by noncovalent bonds (Reed & Hackert, 1990; Patel & Roche, 1990; Perham, 1991). E_3BP plays a structural role as an E_3-binding protein. It binds and apparently positions E_3 to the E_2 core in a specific manner that is essential for a functional PDH complex (Powers-Greenwood et al., 1989; Gopalakrishnan et al., 1989; Lawson et al., 1991a; Neagle & Lindsay, 1991). The functional unit of E_3 is a homodimer (Williams, 1992; Mattevi et al., 1991). The number of E_3-BP molecules bound per molecule of the bovine PDH complex has been estimated to be 6−12 (DeMarcucci & Lindsay, 1985; Jilka et al., 1986). The genes encoding E_2 and E_3BP from *S. cerevisiae* have been cloned, sequenced, and disrupted (Niu et al., 1988; Behal et al., 1989; Lawson et al., 1991a,b). Comparison of the deduced amino acid sequences of the two proteins indicates that they evolved from a common ancestor (Behal et al., 1989). The amino-terminal half of E_3BP resembles E_2, but the remainder is quite different. E_3BP possesses an amino-terminal lipoyl domain, an E_3-binding domain, and a carboxyl-terminal domain (Figure 1) which is involved in binding E_3BP to the inner core (assemblage of catalytic domains) of E_2 (Rahmatullah et al., 1989; Lawson et al., 1991a,b). The domains are connected by flexible segments.

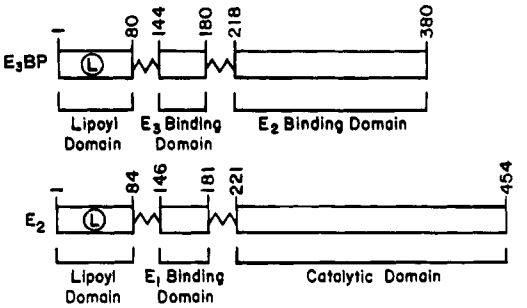

FIGURE 1: Diagrammatic representation of the structural domains of *S. cerevisiae* E_3BP and E_2. The domains are connected by hinge regions. The limits of these domains are approximate.

Attempts to separate intact E_3BP from E_2 have met with only limited success (Li et al., 1992). To obtain adequate amounts of E_3BP for further characterization and functional

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[1] Abbreviations: PDH complex or PDC, pyruvate dehydrogenase complex; E_1, pyruvate dehydrogenase; E_2, dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; E_3BP or BP, E_3-binding protein, also known as protein X; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-thiogalactoside; r, recombinant; t, truncated; FPLC, fast protein liquid chromatography; GST, glutathione *S*-transferase.

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studies, the genes encoding E_3 and E_3BP were linked in that order into a single transcriptional unit separated by a 31-nucleotide segment containing a ribosome-binding sequence. Coexpression of E_3 and E_3BP in *Escherichia coli* produced an E_3BP-E_3 complex, which was purified and then separated into E_3 and E_3BP. Binding studies demonstrated that the pentagonal dodecahedron-like E_2 binds approximately 12 molecules (or about 1 per face) of E_3BP-E_3 complex.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and Bethesda Research Laboratories. Plasmids pKK223-3 and pGEX-2T, glutathione−Sepharose 4B, and *E. coli* strain JM105 were obtained from Pharmacia. Plasmid pGroESL was provided by Dr. George Lorimer. [1-^{3}H]Acetyl-CoA was purchased from New England Nuclear. Rabbit antibodies to *S. cerevisiae* E_2 and E_3BP were prepared as described (Niu et al., 1988; Lawson et al., 1991a). Immobilon-P [poly(vinylidene difluoride)] membrane was purchased from Millipore, DEAE-cellulose (DE52) was from Whatman, heparin−agarose, HA-Ultrogel, and thrombin were from Sigma, Affi-Gel Blue gel was from Bio-Rad, and hydroxylapatite (fast flow) was from Calbiochem. Other reagents and materials were of the highest grade available commercially.

Preparation of Oligonucleotides. Primers for PCR and DNA sequencing were synthesized on an Applied Biosystems Model 381A DNA synthesizer.

PCR. PCR consisted of 30 cycles (3 min at 94 °C followed by 29 cycles of 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C; extended to 10 min at 72 °C in the last cycle).

Amino-Terminal Sequence Analysis. The purified E_3BP and tE_2 were subjected to SDS−PAGE (Laemmli, 1970) in a mini-slab gel apparatus, and the protein bands were transferred electrophoretically to an Immobilon-P membrane (Matsudaira, 1987). The membrane was stained with Coomassie brilliant blue in 45% methanol, destained with 45% methanol, washed with deionized H_2O, and air-dried. The major and minor bands were excised and subjected to automated sequence analysis with an Applied Biosystems Model 470A gas-phase sequencer equipped with an on-line Model 120A phenylthiohydantoin amino acid analyzer.

Immunoblotting. Proteins were separated by SDS−PAGE (12.5% acrylamide) and then transferred electrophoretically to an Immobilon-P membrane. Immunoblot analysis was performed with rabbit anti-E_2 or anti-E_3BP serum and goat anti-rabbit IgG conjugated to alkaline phosphatase as described by the supplier (Bio-Rad).

Enzyme Assays. Assay of E_3BP is based on reconstituting a functional PDH complex from fixed amounts of yeast E_1-E_2 subcomplex and varying amounts of E_3BP-E_3 complex, and then measuring the initial rate of the CoA- and NAD^+-dependent oxidation of pyruvate by the reconstituted PDH complex. The assay solution contained 50 mM potassium phosphate, pH 8.0, 0.2 mM thiamin diphosphate, 0.13 mM CoA, 2.5 mM NAD^+, 1 mM $MgCl_2$, 0.32 mM dithiothreitol, 2 mM sodium pyruvate, 10 μg of E_1-E_2 subcomplex (~160 units of E_2 activity per milligram), and E_3BP-E_3 sample in a final volume of 0.5 mL. The pH of the solution was 7.4. E_3BP-E_3 was added last, and the production of NADH was monitored at 340 nm and 30 °C with a Hewlett-Packard diode array spectrophotometer. When E_3BP was assayed, recombinant yeast E_3 (~20 μg) was added to the assay solution. One unit of E_3BP-E_3 complex corresponds to the production of 1 μmol of NADH per minute by the reconstituted PDH complex. Assay of E_2 activity is based on the initial rate of transfer of radioactive acetyl groups from [1-^{3}H]acetyl-CoA to dihydrolipoamide (Niu et al., 1990). Units are expressed as nanomoles of acetyl groups transferred per minute. Protein was determined as described by Bradford (1976).

Purification of E_1-E_2 Subcomplex. Yeast *pdx1* null mutant cells (JLY61c; Lawson et al., 1991a) were grown in 12 L of YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) for 24 h at 30 °C in a New Brunswick Model SF-116 MicroGen fermentor. *All buffers* contained 0.1% (v/v) 2-mercaptoethanol, 0.05 mM thiamin diphosphate, 5% (v/v) glycerol, and protease inhibitors (1 mM EDTA, 2 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride). All operations were carried out at about 4 °C, except as noted. Approximately 250 g (wet weight) of cells was resuspended in 750 mL of buffer A [50 mM imidazole chloride, pH 7.3, and 0.01% (v/v) Nonidet P-40]. The suspension was passed 4 times through a Manton-Gaulin Laboratory homogenizer operated at 8000 psi. Cell debris was removed by centrifugation at 20 000 rpm for 30 min in a Beckman JA-20 rotor, and the protein concentration was adjusted to about 10 mg/mL by adding buffer A. The solution was brought to 25 °C, and the E_1-E_2 subcomplex was precipitated by dropwise addition, with stirring, of 0.075 volume of 50% (w/v) poly(ethylene glycol) 8000. After 15 min, the precipitate was collected by centrifugation at 16 000 rpm for 15 min at 25 °C. The pellets were resuspended, by means of a glass homogenizer equipped with a motor-driven Teflon pestle, in 350 mL of ice-cold buffer A. To the suspension was added 250 mL of buffer A and sufficient NH_4Cl to make a final concentration of 0.2 M. The suspension was stirred for 1 h and then centrifuged at 20 000 rpm for 20 min. To the supernatant fluid was added dropwise, with stirring, 0.025 volume of 2% (w/v) streptomycin sulfate. After 30 min, the precipitate was removed by centrifugation at 20 000 rpm for 30 min. The supernatant fluid was diluted with an equal volume of buffer B (buffer A without NP-40) and applied to a Q-Sepharose column (5 × 10 cm) that had been equilibrated with buffer B. The column was washed with 200 mL of buffer B. The flow-through and wash were combined, and the pH was lowered to 6.3 by dropwise addition, with stirring, of 10% acetic acid. After 30 min, the precipitate was collected by centrifugation and resuspended in 400 mL of buffer A containing 0.2 M NH_4Cl. The suspension was stirred for 1 h and then centrifuged at 20 000 rpm for 20 min. The supernatant fluid was diluted 5-fold with buffer B and applied to a column (2.3 × 4 cm) of hydroxylapatite (Calbiochem fast flow) equilibrated with buffer B. The column was washed successively with approximately 100 mL each of buffer B and 50, 100, 150, 200, and 230 M potassium phosphate buffer, pH 7.3. The E_1-E_2 subcomplex was eluted with 250 mM potassium phosphate, pH 7.3, containing 6% (w/v) ammonium sulfate. Active fractions were pooled and concentrated by vacuum dialysis and then in a Centricon-30 concentrator. The sample was subjected to fast protein liquid chromatography on a Superose 6 column equilibrated with 50 mM potassium phosphate, pH 7.3 at 25 °C. The yield

of highly purified E_1-E_2 subcomplex was 1−2 mg; specific activity about 300 units of E_2 activity per milligram of protein.

Expression of E_3BP and E_3 in E. coli. *E. coli* strain JM105 was transformed with pN-E3X. Standard methods for the transformation of competent *E. coli* cells were used (Cohen et al., 1971). Transformants were selected on media containing 50 μg/mL ampicillin. Fresh transformants were grown at 22, 30, or 37 °C to an A_{600} of ~1.0. Expression was induced by addition of IPTG to a final concentration of 0.05−1.0 mM. Incubation was continued for 2−10 h. Appreciably more E_3 then E_3BP was expressed, as determined by SDS−PAGE and immunoblot analysis. Conditions optimal for expression of soluble E_3BP without excessive expression of E_3 were growth of fresh transformants at 30 °C to an A_{600} of ~1.0, followed by induction with 0.05 mM IPTG for 3 h at 30 °C.

Purification of E_3BP−E_3 Complex. *E. coli* strain JM105 harboring plasmid pN-E3X was grown in 12 L of LB medium containing 50 μg/L ampicillin and 150 μg/L DL-lipoic acid at 30 °C in a New Brunswick MicroGen fermentor until the absorbance at 600 nm was about 1.0. Expression was induced by addition of IPTG to a final concentration of 0.05 mM, and the cells were harvested 3 h after induction. All operations were carried out at 4 °C, except as noted. Approximately 70 g (wet weight) of cells was resuspended in 200 mL of buffer C [50 mM imidazole chloride, pH 7.3, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, and protease inhibitors]. The cells were broken by passing the suspension twice through a French press at 16 000 psi. Cell debris was removed by centrifugation at 20 000 rpm for 30 min in a Beckman JA-20 rotor, and the protein concentration was adjusted to about 10 mg/mL by adding buffer C. To the supernatant fluid was added dropwise, with stirring, 0.0016 volume of 5% (v/v) poly(ethylenimine), pH 6.0. After 30 min, the precipitate was removed by centrifugation. The supernatant fluid was applied to a DEAE-cellulose column (4.5 × 12 cm) that had been equilibrated with buffer C. The column was washed extensively with buffer C and then with 200 mL of buffer C containing 0.1 M NaCl, and was developed with a 600-mL linear gradient from 0.1 to 0.4 M NaCl in buffer C. The fractions containing E_3BP activity were pooled, diluted 3-fold with buffer C, and applied to a heparin−agarose column (2.3 × 17 cm) that had been equilibrated with buffer C. The column was washed extensively with buffer C and then with 500 mL of buffer C containing 0.1 M NaCl, and was developed with a 400-mL linear gradient from 0.1 to 0.4 M NaCl in buffer C. The active fractions were pooled, diluted 3-fold with buffer C, and applied to an Affi-Gel Blue gel column (2.3 × 9 cm) equilibrated with buffer C. The column was washed extensively with buffer C and then with 250 mL of buffer C containing 0.1 M NaCl, and was developed with a 300-mL gradient from 0.1 to 0.4 M NaCl in buffer C. The active fractions were pooled and concentrated by vacuum dialysis. To separate the E_3BP−E_3 complex from uncomplexed E_3, about 0.5 mL of concentrate containing about 5 mg of protein was subjected to FPLC on a preparative Superose 12 column (1.6 × 50 cm). The column was equilibrated and developed with buffer D [50 mM potassium phosphate, pH 7.3, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, and protease inhibitors]. The active fractions were pooled and concentrated in a Centricon-30 concentrator.

Separation of E_3BP from E_3. About 0.5 mL of concentrate (20 mg of protein) from the Affi-Gel Blue chromatography step was applied to a hydroxylapatite (Sigma HA-Ultrogel) column (1.2 × 20 cm) equilibrated with buffer C. The column was washed with about 200 mL each of buffers C and D, and E_3BP was eluted with buffer D containing 5 M urea. The active fractions were pooled, dialyzed against buffer D, and concentrated by vacuum dialysis. The yield of E_3BP was about 1.5 mg. To remove small amounts of impurities, some preparations of E_3BP were subjected to FPLC on a Mono Q column equilibrated with buffer D. The column was washed with 20 mL of buffer D and then with 20 mL of buffer D containing 0.15 M NaCl, and was developed with a 30-mL gradient from 0.15 to 0.3 M NaCl in buffer D. The active fractions were pooled, dialyzed against buffer D, and concentrated in a Centricon-30 concentrator.

Construction of Expression Vector for GST−tE_2 Fusion Protein. The specific oligonucleotide primers B1 and B2 (Table 1) were used to amplify from yeast genomic DNA the gene fragment encoding residues 206−454 of E_2, which contain the catalytic domain and about 14 residues of interdomain linker segment (Figure 1) (Niu et al., 1990). Each primer introduced a *Bam*HI site at the two ends of the subgene. The restriction sites were used to subclone the fragment into pGEX-2T in-frame with the GST gene to generate pGEX−tE_2 for expression in *E. coli*. Strain DH5α was cotransformed with pGST−tE_2 plus pGroESL, which encodes the *E. coli* chaperonin proteins groES and grpEL (Goloubinoff et al., 1989). Double transformants containing plasmids pGroESL and pGST−tE_2 were selected on media containing 50 μg/mL ampicillin and 30 μg/mL chloramphenicol.

Expression, Purification, and Cleavage of Fusion Protein, and Purification of tE_2. Growth conditions for expression of soluble GST−tE_2 fusion protein and conditions for purification (Smith & Johnson, 1988) and cleavage (Guan & Dixon, 1991) of the fusion protein were varied to obtain optimal conditions for production of active soluble tE_2. A 50-mL overnight culture of the transformant was inoculated into 6 L of LB medium containing 50 μg/mL ampicillin and 30 μg/mL chloramphenicol in a New Brunswick Microgen fermentor. The culture was incubated at 37 °C for 2 h and then at 30 °C to an A_{600} of 1.0. Expression was induced by addition of IPTG to a final concentration of 0.065 mM. Incubation was continued at 30 °C for at least 4 h before harvesting. Approximately 30 g (wet weight) of cells was resuspended in 90 mL of ice-cold PBS buffer (150 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) containing 1% Triton X-100. The suspension was passed once through a French press at 12 000 psi and then was centrifuged at 20 000 rpm for 30 min in a Beckman JA-20 rotor. The extract (78 mL) was mixed gently with 10 mL of a 50% slurry (v/v) of glutathione−Sepharose 4B beads for 10 min at room temperature. The beads were collected by centrifugation and washed 5 times with ice-cold PBS buffer, once with wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl), and once with wash buffer containing 2.5 mM $CaCl_2$ (thrombin cleavage buffer). The beads were resuspended in 10 mL of thrombin cleavage buffer and incubated with thrombin (approximately 0.5% by weight of fusion protein) for 45 min at 25 °C. The supernatant fluid was separated, and the beads were washed 4 times with 0.5

Table 1: Oligonucleotide Primers for PCR[a]

primer	sequence	location
	*Eco*RI	
A1	AGTCGAATTCATGACCATTAACAAGTCA	595 (F)
	*Nco*I	
A2	ACAGCCATGGTCTGTTTCCTGTGTGCCTGT TTTCAACAATGAATAG	2028 (R)
	*Nco*I	
A3	AAATCCATGGCTGTAAAGACA	442 (F)
	*Pst*I	
A4	TGTTCTGCAGTCAAAATGATTCTAA	1581 (R)
	*Bam*HI	
B1	CCTGGATCCACCTCAAGCACTACTGCT	2701 (F)
	*Bam*HI	
B2	TTGGATCCCATTCTAACCTCACAATAG	3442 (R)

[a] Sequences are listed 5′−3′. Added restriction sites are underlined. Location refers to the nucleotide of the E_3 DNA (primers A1 and A2), the E_3BP DNA (primers A3 and A4), and the E_2 DNA (primers B1 and B2) at which hybridization to the primer begins and continues in the forward (F) or reverse (R) direction.

bed volume of thrombin cleavage buffer. The supernatant fluid and washes were combined, diluted 3-fold with 50 mM potassium phosphate buffer, pH 7.3, and applied to a 2.5 × 5 cm heparin−agarose column that had been equilibrated with the phosphate buffer. The column was washed with the phosphate buffer, and the tE_2 was eluted with phosphate buffer containing 0.5 M NaCl. Approximately 5.5 mg of highly purified tE_2 was obtained from 30 g (wet weight) of transformed cells.

Stoichiometry of Binding of E_3BP and E_3BP−E_3 to tE_2 and to E_1−E_2 Subcomplex. Solutions of E_3BP and E_3BP−E_3 complex were centrifuged at 35 000 rpm for 2 h before use. Mixtures of tE_2 (156 μg; 100 pmol) and E_3BP (101 μg; 2.4 nmol) or E_3BP−E_3 complex (360 μg; 2.4 nmol) in molar ratios of 1:24 in 1.0 mL of buffer D, and controls lacking tE_2, were centrifuged at 35 000 rpm for 2 h in the TLS55 rotor of a Beckman Optima TLX ultracentrifuge to separate the large tE_2−E_3BP and tE_2−E_3BP−E_3 complexes from unbound E_3BP and E_3BP−E_3. In reconstitution of PDH complex, mixtures containing E_1−E_2 subcomplex (100 μg; 34.4 pmol) and E_3BP−E_3 (124 μg; 826 pmol) were used. The supernatant fluids were removed, the pellets were washed once with buffer D and then covered with a layer of buffer D and allowed to dissolve slowly over a period of several hours. The complexes were resolved by SDS−PAGE. The gel was stained with Coomassie brilliant blue and destained, and the amount of protein in a band was determined by video area densitometry (Poulsen & Ziegler, 1993) relative to that of a bovine serum albumin standard in an adjacent lane on the same gel. Standard curves were obtained with known amounts of tE_2, E_3BP, E_3, and bovine serum albumin (Figure 2). Protein concentrations in the standard solutions were determined by quantitative amino acid composition analysis.

RESULTS

Construction of Expression Vector for E_3BP and E_3. The strategy for construction of pN-E3X, a plasmid for the coexpression of yeast E_3 and E_3BP in *E. coli*, is shown in Figure 3. Two pairs of specific oligonucleotide primers, A1 and A2, and A3 and A4 (Table 1), were used to amplify

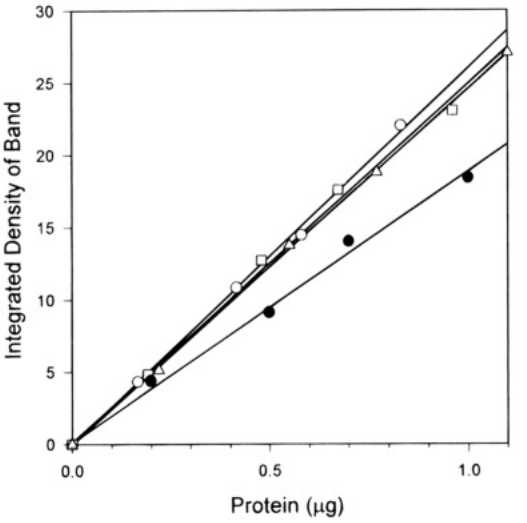

FIGURE 2: Proportionality between amount of protein subjected to SDS−PAGE and integrated density of the Coomassie blue-stained band determined by video area densitometry. (□) E_3; (○) tE_2; (△) E_3BP; (●) bovine serum albumin.

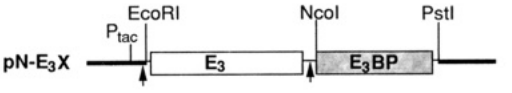

FIGURE 3: Construction of plasmid for coexpression of E_3 and E_3-BP. The genes encoding E_3 and E_3BP were linked into a single transcriptional unit separated by a 31-nucleotide segment containing a ribosome-binding site (arrowhead). The genes were expressed from the Ptac promoter of pKK223-3.

from yeast genomic DNA the gene fragments encoding the mature forms of E_3 and E_3BP, respectively (Browning et al., 1988; Behal et al., 1989). Primer A1 introduced an *Eco*RI site followed by an ATG start codon, and A2 introduced a 31-nucleotide segment containing a sequence for ribosome binding followed by a *Nco*I site. Primer A3 introduced a *Nco*I site containing an ATG start codon, and A4 introduced a *Pst*I site. The restriction sites were used to ligate the two DNA fragments into pKK223-3 to generate pN-E3X for expression in *E. coli*.

Expression and Purification of E_3BP. To minimize proteolysis and to facilitate purification of E_3BP, it was coexpressed with yeast E_3 in *E. coli* strain JM105. The E_3-BP−E_3 complex and uncomplexed E_3 copurified as anticipitated, and were separated by FPLC on a Superose 12 column (data not shown). A summary of the purification is presented in Table 2. When analyzed by SDS−PAGE (Figure 4) and by immunoblotting (data not shown), the E_3-BP−E_3 complex showed two bands, corresponding to E_3 and E_3BP. The polypeptide chain ratio of E_3BP:E_3, determined by video area densitometry, was about 1:2 (Table 3). This ratio corresponds to a subunit composition of one E_3BP monomer and one E_3 homodimer.

E_3BP was separated from E_3 by chromatography on hydroxylapatite in the presence of 5 M urea in 50 mM potassium phosphate buffer, pH 7.3. E_3BP eluted prior to E_3 (data not shown). When analyzed by SDS−PAGE (Figure 4), the purified rE_3BP showed a major band with M_r ~47 000 and variable amounts of two minor bands. The amino-terminal sequence of the major band was determined to be AVKTFSMPAMSP. This is the expected sequence, based on the nucleotide sequence of the *PDX1* gene (Behal et al., 1989). The lower minor band is apparently a truncated

Table 2: Purification of E_3BP-E_3 Complex[a]

	volume (mL)	protein (mg)	sp act.[b]	recovery (%)
cell extract	680	9250	0.3	100
DEAE-cellulose	136	2094	1.2	90
heparin−agarose	90	141	16.9	86
Affi-Gel Blue	136	70	29.5	74
Superose 12	31	25	41.3	37

[a] From about 70 g of *E. coli* cells (wet weight). [b] Units of E_3BP-E_3 activity per milligram of protein.

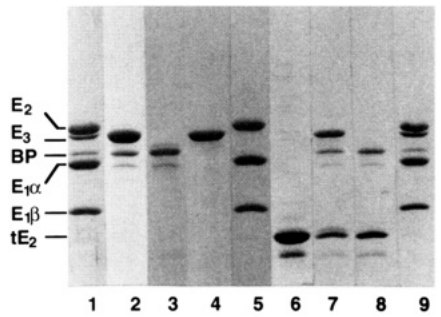

FIGURE 4: SDS−PAGE patterns of bakers' yeast PDH complex (lane 1), E_3BP-E_3 complex (lane 2), E_3BP (lane 3), E_3 (lane 4), E_1-E_2 subcomplex (lane 5), tE_2 (lane 6), $tE_2-E_3BP-E_3$ (lane 7), tE_2-E_3BP (lane 8), and reconstituted PDH complex (lane 9). Approximately 5, 3, 1.6, 1.5, 3, 1.9, 3, 3, and 4.2 μg of protein was applied to lanes 1−9, respectively. The gels were stained with Coomassie brilliant blue.

Table 3: Stoichiometry of Binding of E_3BP and E_3BP-E_3 to E_2

	polypeptide chain ratios[a]		
complex	E_2	E_3BP	E_3
tE_2-E_3BP	60	15.0 ± 0.7	
$tE_2-E_3BP-E_3$	60	13.6 ± 0.6	28.0 ± 0.9
reconstituted PDC	60	11.7 ± 0.4	24.9 ± 1.9
yeast PDC	60	11.2 ± 0.4	19.7 ± 0.9
E_3BP-E_3		1.0	2.0 ± 0.1

[a] Polypeptide chain ratios are the means of three to six determinations.

form of E_3BP (residues 40−380), as indicated by immunoblotting (data not shown) and by amino-terminal sequence analysis. The upper minor band was not present in some samples of E_3BP. It is apparently a host protein.

Reconstitution of PDH Complex. Mixtures containing highly purified E_1-E_2 subcomplex and increasing amounts of highly purified rE_3BP-E_3 generated a dose−response curve with maximum pyruvate oxidation activity similar to that of wild-type PDH complex (Figure 5). Maximum activity was obtained with a E_1-E_2:E_3BP molar ratio of about 1:12. The results were not affected by varying the time of incubation between 30 s and 2 min. These observations demonstrate that E_3BP-E_3 binds rapidly to the E_1-E_2 subcomplex to reconstitute a functional PDH complex. A mixture of E_1-E_2 subcomplex and an excess of E_3BP-E_3 (molar ratio 1:24) was centrifuged at 35 000 rpm for 2 h in a Beckman TLS55 rotor to separate the reconstituted PDH complex from unbound E_3BP-E_3. The SDS−PAGE pattern of the reconstituted complex is shown in Figure 4. The specific activity of the reconstituted complex was ~14 units/mg of protein. The specific activity of highly purified PDH complex from bakers' yeast was ~12 units/mg of protein.

Expression and Purification of Truncated E_2. Cultures of *E. coli* transformants which harbored $pGST-tE_2$ and pGroESL were grown under conditions optimal for expression of soluble active $GST-tE_2$ fusion protein, as determined by immunoblot analysis and assay of E_2 activity. The fusion protein was purified by affinity chromatography on glutathione−Sepharose 4B beads, and cleaved by treatment with thrombin while still attached to the beads. The tE_2 was further purified by chromatography on heparin−agarose. Analysis of tE_2 by FPLC with a Superose 6 column indicated that the recombinant protein eluted before thyroglobulin (M_r = 640 000) and after Blue Dextran (2 000 000) (data not shown). This observation indicated that the recombinant tE_2 is a large oligomer, consistent with a calculated molecular weight of 1 619 160 for the 60-subunit tE_2. When analyzed by SDS−PAGE (Figure 4) and by immunoblotting (data not shown), the purified tE_2 showed a major band with apparent M_r ~29 000 and a minor band with apparent M_r ~26 000. The amino-terminal sequence of the major band was determined to be SGTSSTTAGSAP. This is the expected sequence, based on the nucleotide sequence of the *LAT1* gene (Niu et al., 1988) and the fact that the vector pGEX-2T introduced two amino acids, serine and glycine, at the amino terminus of the recombinant polypeptide. The amino-terminal sequence of the minor band was SIIGERLLQSTQG. This finding indicates that some of the tE_2 subunits were cleaved by thrombin between Arg-232 and Ser-233. Analysis of stained gels by video area densitometry indicated that the ratio of tE_2 (233−454) to tE_2 (206−454) was 1:3 or 1:4.

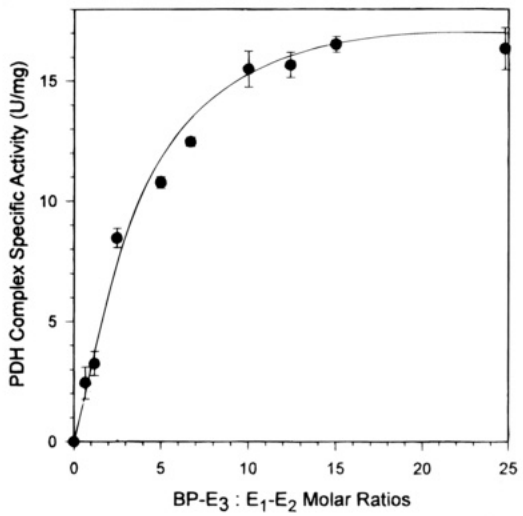

FIGURE 5: Reconstitution of PDH complex activity with E_1-E_2 subcomplex and E_3BP-E_3. Mixtures of E_1-E_2 subcomplex (0.77 μg) and increasing amounts of E_3BP-E_3 in 30 μL of buffer E were incubated at 30 °C for 1 min and then assayed for PDH complex activity.

Stoichiometry of Binding of E_3BP and E_3BP-E_3 to E_2. The stoichiometry of binding of E_3BP and E_3BP-E_3 complex to the 60-subunit E_2 was determined with truncated E_2 (residues 206−454) and with the E_1-E_2 subcomplex, which contains intact E_2. Samples of highly purified PDH complex from bakers' yeast were also analyzed. Mixtures of tE_2 or E_1-E_2 subcomplex and an excess of E_3BP or E_3BP-E_3 complex (molar ratio, 1:24) were centrifuged at 35 000 rpm for 2 h in a Beckman TLS55 swinging-bucket rotor to separate the large complexes from unbound E_3BP and E_3BP-E_3. The complexes were resolved by SDS−PAGE. After staining the gel with Coomassie blue and destaining, the polypeptide chain ratios of tE_2 or E_2:E_3BP:E_3 were determined by video area densitometry. We assumed that the proportionality between the amount of intact E_2 protein

and Coomassie blue staining was similar to that of tE_2 and E_3BP, which are very similar (Figure 2). Furthermore, the domain structure of E_3BP is very similar to that of E_2 (Figure 1). The results (Table 3) show that the E_1-E_2 subcomplex binds about 12 E_3BP monomers attached to 12 E_3 homodimers (reconstituted PDH complex). Similar results were obtained by analysis of highly purified PDH complex from bakers' yeast. The amount of bound E_3 in the latter samples was somewhat lower, due presumably to loss of E_3 during the purification procedure. Somewhat more E_3BP (~15 molecules) and E_3BP-E_3 complex (~14 molecules) bound to tE_2. In one experiment, a tE_2:E_3BP-E_3 molar ratio of 1:36 was used. The results were similar to those obtained with the 1:24 ratio.

DISCUSSION

In view of recent evidence that protein X plays a structural role as an E_3-binding protein (Powers-Greenwood et al., 1989; Gopalakrishnan et al., 1989; Lawson et al., 1991a,b; Neagle & Lindsay, 1991), it seems appropriate to replace the ambiguous designation protein X by the functional designation E_3-binding protein (E_3BP). The carboxyl-terminal domain of E_3BP (residues ~218–380) is bound to the pentagonal dodecahedron-like inner core (assemblage of catalytic domains) of E_2 (Rahmatullah et al., 1989; Lawson, 1991a,b). Because of difficulties encountered in separating E_3BP from E_2 in a functional state, we undertook overexpression of yeast E_3BP in *E. coli*. Because E_3BP does not possess catalytic activity, it was necessary to design an indirect assay for this protein. The rationale was to reconstitute a functional PDH complex from fixed amounts of E_1-E_2 subcomplex, isolated from a *pdx1* null mutant (Lawson et al., 1991a), and E_3, and varying amounts of E_3-BP, and then measure the CoA- and NAD^+-linked pyruvate oxidation activity of the reconstituted PDH complexes. Although soluble functional yeast E_3BP was expressed in *E. coli* (data not shown), attempts to purify the recombinant protein to homogeneity were unsuccessful, apparently due to the sensitivity of E_3BP to proteolysis (as indicated by immunoblot analysis). This problem was solved by coexpressing E_3BP and E_3 in *E. coli* to produce an E_3BP-E_3 complex. The E_3BP-E_3 complex was more resistant than uncomplexed E_3BP to proteolysis. The E_3BP-E_3 complex was purified to near-homogeneity. E_3BP was separated from E_3 by chromatography of the E_3BP-E_3 complex on hydroxylapatite in the presence of 5 M urea. Similar conditions were used previously to separate E_3 from the E_2 component of the *E. coli* (Koike et al., 1963) and yeast (Kresze & Ronft, 1981) PDH complexes. E_3BP-E_3 combined rapidly with E_1-E_2 subcomplex to reconstitute a functional PDH complex, with pyruvate oxidation activity similar to that of PDH complex from bakers' yeast.

Structural considerations predict that the icosahedral E_2, with *532* symmetry, should have 20, 30, or 60 equivalent binding sites for E_3BP depending on whether the interactions of E_3BP with E_2 involve the 3-fold, 2-fold, or 5-fold axes of the E_2 structure, respectively. The availability of recombinant yeast E_3BP, E_3BP-E_3 complex, E_1-E_2 subcomplex (lacking E_3BP and E_3), and a truncated form of E_2 (residues 206–454, lacking the lipoyl domain and the E_1-binding domain) provided a unique opportunity to gain insight into the binding stoichiometry.

Mixtures containing tE_2 or E_1-E_2 subcomplex and excess E_3BP or E_3BP-E_3 were subjected to ultracentrifugation to separate the large complexes from unbound E_3BP and E_3-$BP-E_3$, and the complexes were subjected to SDS–PAGE. After staining with Coomassie brilliant blue and destaining, the gels were analyzed with a video area densitometer. The results show that the E_1-E_2 subcomplex, which contains intact E_2, binds about 12 E_3BP monomers attached to 12 E_3 homodimers. Similar results were obtained by analysis of highly purified PDH complex from bakers' yeast. Somewhat more E_3BP (~15 molecules) and E_3BP-E_3 complex (~14 molecules) bind to the truncated E_2. Structrual considerations suggest that 1 E_3BP molecule, bearing an E_3 homodimer, is bound in each of the 12 faces of the pentagonal dodecahedron-like E_2. This positioning presumably optimizes access of E_3 to the mobile lipoyl-bearing domains of E_2 subunits and may be an important aspect of the active-site coupling mechanism. Direct evidence that E_3BP and the E_3BP-E_3 complex are indeed localized inside the 12 faces of tE_2 and that E_3BP apparently interacts with tE_2 near the 3-fold axis was obtained recently by three-dimensional reconstruction of cryoelectron microscopy images of tE_2 and tE_2-E_3BP and $tE_2-E_3BP-E_3$ complexes (J. K. Stoops, R. H. Cheng, C.-Y. Maeng, M. A. Yazdi, T. S. Baker, J. P. Schroeter, U. Klueppelberg, S. J. Kolodziej, and L. J. Reed, unpublished data).

It is surprising that the E_3 homodimer binds one rather than two E_3BP monomers. A similar finding was reported recently for the association of E_3 with the E_3-binding domain of the E_2 component of the pyruvate dehydrogenase complex from *Bacillus stearothermophilus* (Hipps et al., 1994). Presumably, steric hindrance or a conformational change in the E_3 dimer induced by association with one E_3-binding domain prevents the association of a second binding domain.

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REFERENCES

Behal, R. H., Browning, K. S., Hall, T. B., & Reed, L. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8732–8736.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Browning, K. S., Uhlinger, D. J., & Reed, L. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1831–1834.

Cohen, S. N., Chang, A. C. Y., & Hsu, L. (1971) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2110–2114.

DeMarcucci, O., & Lindsay, J. G. (1985) *Eur. J. Biochem. 149*, 641–648.

Goloubinoff, P., Christeller, J. T., Gatenby, A. A., & Lorimer, G. H. (1989) *Nature 342*, 884–889.

Gopalakrishnan, S., Rahmatullah, M., Radke, G. A., Powers-Greenwood, S., & Roche, T. E. (1989) *Biochem. Biophys. Res. Commun. 160*, 715–721.

Guan, K. L., & Dixon, J. E. (1991) *Anal. Biochem. 192*, 262–267.

Hipps, D. S., Packman, L. C., Allen, M. D., Fuller, C., Sakaguchi, K., Appella, E., & Perham, R. N. (1994) *Biochem. J. 297*, 137–143.

Jilka, J. M., Rahmatullah, M., Kazemi, M., & Roche, T. E. (1986) *J. Biol. Chem. 261*, 1858−1867.
Koike, M., Reed, L. J., & Carroll, W. R. (1963) *J. Biol. Chem. 238*, 30−39.
Kresze, G.-B., & Ronft, H. (1981) *Eur. J. Biochem. 119*, 581−587.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lawson, J. E., Behal, R. H., & Reed, L. J. (1991a) *Biochemistry 30*, 2834−2839.
Lawson, J. E., Niu, X.-D., & Reed, L. J. (1991b) *Biochemistry 30*, 11249−11254.
Li, L., Radke, G. A., Ono, K., & Roche, T. E. (1992) *Arch. Biochem. Biophys. 296*, 497−504.
Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035−10038.
Mattevi, A., Schierbeek, A. J., & Hol, W. G. J. (1991) *J. Mol. Biol. 220*, 975−994.
Neagle, J. C., & Lindsay, J. G. (1991) *Biochem. J. 278*, 423−427.
Niu, X.-D., Browning, K. S., Behal, R. H., & Reed, L. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7546−7550.
Niu, X.-D., Stoops, J. K., & Reed, L. J. (1990) *Biochemistry 29*, 8614−8619.
Patel, M. S., & Roche, T. E. (1990) *FASEB J. 4*, 3224−3233.
Perham, R. N. (1991) *Biochemistry 20*, 8501−8512.
Poulsen, L. L., & Ziegler, D. M. (1993) "Video densitometer," U.S. Patent 5 194 949.
Powers-Greenwood, S. L., Rahmatullah, M., Radke, G. A., & Roche, T. E. (1989) *J. Biol. Chem. 264*, 3655−3657.
Rahmatullah, M., Gopalakrishnan, S., Radke, G. A., & Roche, T. E. (1989) *J. Biol. Chem. 264*, 1245−1251.
Reed, L. J., & Hackert, M. L. (1990) *J. Biol. Chem. 265*, 8971−8974.
Smith, D. B., & Johnson, K. S. (1988) *Gene 67*, 31−40.
Watanabe, H., Green, G. D. J., & Shaw, E. (1979) *Biochem. Biophys. Res. Commun. 89*, 1354−1360.
Williams, C. H., Jr. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. 3, pp 121−211, CRC Press, Boca Raton, FL.