Stoichiometry of Binding of Mature and Truncated Forms of the Dihydrolipoamide Dehydrogenase-Binding Protein to the Dihydrolipoamide Acetyltransferase Core of the Pyruvate Dehydrogenase Complex from *Saccharomyces cerevisiae*†

Cheol-Young Maeng, Mohammed A. Yazdi, and Lester J. Reed*

Biochemical Institute and Department of Chemistry and Biochemistry, The University of Texas at Austin, Austin, Texas 78712

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ABSTRACT: The dihydrolipoamide dehydrogenase-binding protein (E_3BP), a component of the *Saccharomyces cerevisiae* and mammalian pyruvate dehydrogenase (PDH) complexes, anchors an E_3 homodimer inside each of the 12 pentagonal faces of the 60-mer dihydrolipoamide acetyltransferase (E_2). To gain further insight into the number and localization of binding sites for E_3BP on the 60-mer E_2, truncated forms of the E_3BP lacking the lipoyl and E_3-binding domains were engineered by deletion mutagenesis. The recombinant proteins contained a polyhistidine extension on the amino terminus to facilitate purification to near-homogeneity. The stoichiometry of binding of the truncation mutants to a truncated form (inner core) of E_2 (tE_2, residues 181−454), lacking the lipoyl domain and the E_1-binding domain, was determined. Mixtures containing tE_2 and excess intact or truncated forms of E_3BP were subjected to ultracentrifugation to separate the large complexes from unbound E_3BP or tE_3BP, 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 tE_2 binds about 20 copies of intact E_3BP-H, about 24 copies of tE_3BP-H144 (residues 144−380), lacking the lipoyl domain, and about 31 copies of tE_3BP-H218 (residues 218−380), lacking both the lipoyl and E_3-binding domains. The results indicate that there apparently is a binding site for E_3BP on each E_2 subunit and that steric hindrance by segments of E_3BP prevents full stoichiometric binding of E_3BP to the pentagonal dodecahedron-like E_2.

Mammalian and *Saccharomyces cerevisiae* pyruvate dehydrogenase (PDH)[1] complexes are organized about a 60-subunit pentagonal dodecahedron-like dihydrolipoamide acetyltranferase (E_2) core, to which multiple copies of pyruvate dehydrogenase (E_1) tetramers ($\alpha_2\beta_2$), dihydrolipoamide dehydrogenase (E_3) homodimers, and E_3-binding protein (E_3BP) monomers are bound by noncovalent bonds (Reed & Hackert, 1990; Patel & Roche, 1990; Perham, 1991; Mattevi *et al*., 1992). A polypeptide chain ratio of $60E_1\alpha$:$60E_1\beta$:$60E_2$:$12E_3$BP:$24E_3$ represents a full complement of subunits (Maeng *et al*., 1994). E_3BP has a multidomain substructure consisting of an amino-terminal lipoyl domain, followed by an E_3-binding domain, and then by a carboxyl-terminal E_2-binding domain (Figure 1). The domains are linked to each other by flexible segments. E_3BP plays mainly a structural role. It binds and apparently positions E_3 to the E_2 core, and this positioning is an important part of the active-site coupling mechanism (Gopalakrisnan *et al*., 1989; Lawson *et al*., 1991a; Neagle & Lindsay, 1991). E_3BP binds via its E_2-binding domain (Figure 1) to the assemblage of catalytic domains (residues ∼221−454) comprising the inner core of E_2 (Rahmatullah *et al*., 1989; Lawson *et al*., 1991a,b). The

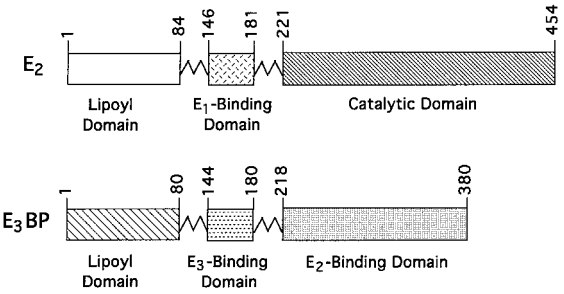

FIGURE 1: Diagrammatic representation of the structural domains of *S. cerevisiae* E_3BP and E_2. The domains are connected by hinge regions. The limits of the domains are approximate.

E_3-binding domain of E_3BP, in turn, binds an E_3 homodimer (Maeng *et al*., 1994).

Cryoelectron microscopy and three-dimensional image reconstruction results show clearly that E_3BP or E_3BP−E_3 complex is bound inside each of the 12 pentagonal faces of *S. cerevisiae* E_2, extending into the central cavity (Stoops *et al*., 1992; J. K. Stoops, R. H. Cheng, C.-Y. Maeng, M. A. Yazdi, T. S. Baker, J. P. Schroeter, W. Klueppelberg, S. J. Kolodziej, and L. J. Reed, unpublished data). The finding that the E_2 structure can physically accommodate only 1 E_3-BP−E_3 complex in each of its 12 faces provides a satisfactory explanation of the unique polypeptide chain ratio in the *S. cerevisiae* and mammalian PDH complexes. However, assuming that there is one E_3BP-binding site on each E_2 subunit, the 60-mer E_2, with icosahedral *532* symmetry, would be expected to bind 60 copies of E_3BP. A probable explanation of this apparent discrepancy is that steric hindrance prevents full stoichiometric binding of E_3BP. To

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* Address correspondence to this author.

[1] Abbreviations: PDH complex, pyruvate dehydrogenase complex; E_1, pyruvate dehydrogenase; E_2, dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; E_3BP, E_3-binding protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-thiogalactoside; t, truncated; Ni−NTA resin, Ni^{2+}−nitriloacetic−agarose.

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Table 1: Oligonucleotide Primers for PCR[a]

protein	primers	sequence	location
		Bam HI	
tE_2-H181	1	GCTGGATCCGAGTCATATCTAGAAAAG	2626 (F)
		Sal I	
	2	AATTTGTCGACCATTCTAACCTCACAATAGCA	3447 (R)
		Bam HI	
E_3BP-H	3	AAAGGATCCGCTGTAAAGACATTTTCAATG	442 (F)
		Sal I	
	4 [b]	AAAGCGTCGACTTTATTCAAAATGATTCTA	1581 (R)
		Bam HI	
tE_3BP-H144	5	TTAGGATCCGTGTCATTACTACTGGCTGAG	871 (F)
		Bam HI	
tE_3BP-H218	6	ATAGGATCCCAAGCTCAAACAAAAGCTGCC	1093 (F)

[a] Sequences are listed 5′ to 3′. Added restriction sites are underlined. Location refers to the nucleotide of the DNA at which hybridization to the primer begins and continues in the forward (F) or reverse (R) direction. [b] This reverse primer was also used to amplify cDNAs encoding the E_3BP truncation mutants.

address this question and to gain further insight into the localization of E_3BP within the central cavity of E_2 by cryoelectron microscopy, truncated forms of E_3BP were prepared by deletion mutagenesis, and the stoichiometry of binding of these truncation mutants to the inner core of E_2 was determined.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and Bethesda Research Laboratories. Plasmid pQE-30, *Escherichia coli* strain M15[pREP4], and Ni−NTA resin were purchased from Qiagen. [1-^{3}H]Acetyl-CoA was purchased from New England Nuclear. Imidazole chloride (grade III) and heparin−agarose were obtained from Sigma. Primers for PCR were obtained from Bio-Synthesis, Inc. (Lewisville, TX). 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. Other reagents and materials were of the highest grade available commercially.

PCR. PCR was conducted as described previously (Maeng *et al*., 1994).

Immunoblotting. Proteins were separated by SDS−PAGE (Laemmli, 1970) (12.5% acrylamide) and then transferred electrophoretically to an Immobilon-P membrane (Matsudaira, 1987). 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 a fixed amount of yeast E_1−E_2 subcomplex, excess E_3, and varying amounts of E_3-BP, and then measuring the initial rate of the CoA- and NAD^+-dependent oxidation of pyruvate by the reconstituted PDH complex (Maeng *et al*., 1994). One unit of E_3BP corresponds to the production of 1 μmol of NADH per minute by the reconstituted PDH complex. Assay of tE_2-H181 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). Protein was determined as described by Bradford (1976).

Construction of Expression Vector for tE_2-H181. The specific oligonucleotide primers 1 and 2 (Table 1) were used to amplify by PCR from yeast genomic DNA the gene fragment encoding residues 181−454 of E_2 (Niu *et al*., 1990). The primers introduced, respectively, a *Bam*HI site at the 5′ end and a *Sal*I site at the 3′ end of the subgene. The restriction sites were used to subclone the fragment into pQE-30 to generate pQE−tE_2-H181. *E. coli* strain M15[pREP4] was transformed with the construct, using standard methods for the transformation of competent *E. coli* cells (Cohen *et al*., 1971). Transformants were selected on LB medium containing 50 μg/mL ampicillin and 25 μg/mL kanamycin.

Expression and Purification of tE_2-H181. Growth conditions were varied to obtain optimal conditions for expression of active, soluble tE_2-H181. A 10-mL overnight culture of the transformant was inoculated into 1 L of LB medium containing ampicillin and kanamycin. The culture was incubated (with shaking) at 37 °C to an A_{600} of 0.8−0.9 and then cooled to room temperature (25−28 °C). Expression was induced by addition of IPTG to a final concentration of 0.4 mM, and incubation was continued for 4−5 h. The harvested cells (10−13 g wet weight) were resuspended in 40 mL of buffer A [50 mM sodium phosphate, pH 8.0, 0.5 M NaCl, and 0.1% (v/v) Triton X-100]. *All buffers* contained 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol, 2 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride. All operations were carried out at about 4 °C, except as noted. The cells were broken by passing the suspension once through a French pressure cell at 16 000 psi. Cell debris was removed by centrifugation at 20 000 rpm for 30 min in a Beckman JA-20 rotor. The extract was mixed gently with 5 mL of Ni−NTA resin (equilibrated with buffer A) for 30 min at 4 °C. The resin was collected by centrifugation and washed 4 times with 35-mL portions of buffer A, and then 3 times with 45-mL portions of buffer B (50 mM sodium phosphate, pH 7.5, and 0.3 M NaCl). The resin was washed consecutively with 45-mL portions of buffer B containing 20, 50, 100, 150, and 250 mM imidazole chloride, pH 7.5. The tE_2-H181 was eluted with buffer B containing 500 mM imidazole, pH 7.5. The active fractions were pooled and dialyzed overnight against several changes of buffer C (50 mM sodium phosphate, pH 7.5, and 0.1 mM EDTA) containing 50 mM NaCl. The solution was concentrated by vacuum dialysis and then in a Centricon-30 concentrator. The yield of highly purified protein was approximately 14 mg. To remove small amounts of impurities, some preparations of tE_2-H181 were subjected to chromatography on a heparin−agarose column (1.2 × 6 cm) equilibrated with buffer C. The column was washed extensively with buffer C and then with buffer C containing 50 mM NaCl. The column was developed with a 100-mL linear gradient from 0.05 to 1 M NaCl in buffer B. The active fractions were pooled and concentrated in a Centricon-30 concentrator.

Expression and Purification of E_3BP and Truncation Mutants. Primers 3 and 4 (Table 1) were used with pN-E_3X (Maeng *et al*., 1994) as a template to generate by PCR a fragment coding for the mature E_3BP. Primer 3 introduced a *Bam*HI site, and primer 2 introduced a *Sal*I site. These sites were used to subclone the fragment into pQE-30. The construct was used to transform *E. coli* strain M15[pREP4]. Transformants were selected on LB medium containing 50 μg/mL ampicillin and 25 μg/mL kanamycin. A 10-mL overnight culture of the transformant was inoculated into 1 L of LB medium containing ampicillin and kanamycin. The culture was incubated at 37 °C to an A_{600} of 0.7−0.9 and then cooled to room temperature (25−28 °C). Expression was induced by addition of IPTG to a final concentration of

0.2 mM, and incubation was continued for 4 h. The recombinant E_3BP-H was purified to near-homogeneity by affinity chromatography on Ni−NTA resin essentially as described above. About 12 mg of E_3BP-H was obtained from 15 g (wet weight) of transformed cells. The truncation mutants tE_3BP-H144 (residues 144−380) and tE_3BP-H218 (residues 218−380) were obtained by similar procedures. Primers 4−6 and 2 (Table 1) were used with pN-E_3X as a template to generate by PCR gene fragments encoding the mutant proteins. The fragments were subcloned into pQE-30 for expression in *E. coli* strain M15[pREP4]. Expression was induced by addition of IPTG to a final concentration of 0.1−0.2 mM, and growth was continued for 4−6 h. The expression level of tE_3BP-H218 was lower than that of tE_3BP-H144, and the former protein showed a lower affinity for the Ni−NTA resin. The recovery of highly purified tE_3BP-H144 from 12−15 g (wet weight) of transformed cells was about 10 mg. The recovery of tE_3BP-H218 from about 10 g (wet weight) of transformed cells was about 7 mg. To remove small amounts of impurities, E_3BP-H and the truncation mutants were subjected to chromatography on heparin−agarose as described above.

Stoichiometry of Binding of E_3BP, tE_3BP-H144, and tE_3BP-H218 to tE_2-H181. Solutions of E_3BP-H and the truncation mutants were centrifuged at 35 000 rpm in the TLS55 rotor of a Beckman Optima TLX ultracentrifuge for 2 h before use. Mixtures of tE_2-H181 (181 μg; 100 pmol) and E_3BP-H (172 μg; 4 nmol), tE_3BP-H144 (111 μg; 4 nmol), or tE_3BP-H218 (78.5 μg; 4 nmol) were prepared in a molar ratio of 1:40 in 1.0 mL of buffer C. The mixtures and controls lacking tE_2 were allowed to stand at room temperature for 10 min and then were centrifuged at 35 000 rpm for 15 min to remove any insoluble material. The supernatant fluids were removed carefully, transferred to clean tubes, and then centrifuged at 35 000 rpm for 2 h to separate the large tE_2−E_3BP and tE_2−tE_3BP complexes from the unbound E_3BP and tE_3BP. The supernatant fluids were removed; the pellets were washed once with buffer C and then covered with a layer of buffer C and allowed to dissolve slowly over a period of several hours. Several aliquots of the solution and a bovine serum albumin standard were subjected to SDS−PAGE (10% acrylamide). 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 the serum albumin standard in an adjacent lane. Standard curves were obtained as described previously (Maeng *et al*., 1994). Protein concentrations in the standard solutions were determined by quantitative amino acid composition analysis. Proportionality between the amount of protein subjected to SDS−PAGE and the integrated density of the Coomassie blue-stained band was established to be in the range 0.2−1.0 μg. Calculations of binding stoichiometry are based on amounts of protein within this range.

RESULTS AND DISCUSSION

Expression and Purification of E_3BP-H. Because E_3BP is sensitive to proteolysis, a procedure was developed to co-express the genes encoding E_3BP and E_3 in *E. coli* to produce an E_3BP−E_3 complex, thereby minimizing proteolysis of E_3BP and facilitating its purification (Maeng *et al*., 1994). The E_3BP−E_3 complex was purified to near-homogeneity and then separated into E_3BP and E_3 by chromatography on hydroxylapatite in the presence of 5 M urea. In the present investigation, a simpler procedure was developed to permit rapid purification of E_3BP. A 6 × His tag was engineered on the amino terminus of E_3BP, and the recombinant protein was purified to near-homogeneity by chromatography on Ni−NTA resin. About 12 mg of E_3BP-H was obtained from 15 g (wet weight) of transformed cells. The highly purified E_3BP-H exhibited a specific activity of 48 units/mg of protein. When analyzed by SDS−PAGE (Figure 2), the purified E_3BP-H showed a single band with an apparent M_r of ∼47 000.

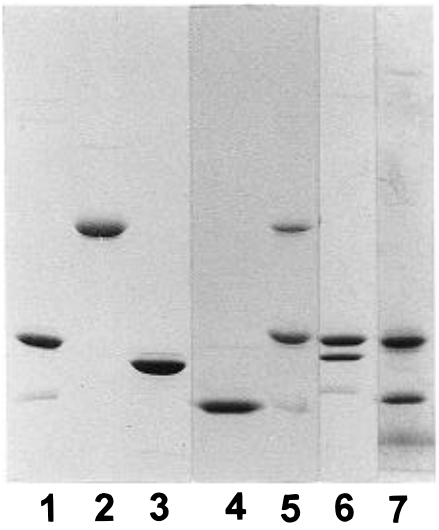

FIGURE 2: SDS−PAGE patterns of tE_2-H181 (lane 1), E_3BP-H (lane 2), tE_3BP-H144 (lane 3), tE_3BP-H218 (lane 4), tE_2-H181−E_3BP-H complex (lane 5), tE_2-H181−tE_3BP-H144 complex (lane 6), and tE_2-H181−tE_3BP-H218 complex (lane 7). Approximately 1, 1.2, 1.3, 0.9, 1.5, 1.7, and 2 μg of protein was applied to lanes 1−7, respectively. The gels were stained with Coomassie brilliant blue.

Expression and Purification of tE_2-H181. Previously, tE_2-181 was expressed in *E. coli* strain JM105 via the expression vector pKK233-2 (Niu *et al*., 1990). Because of difficulties in obtaining adequate amounts of the homogeneous recombinant protein, a different procedure was developed to express and purify t$E_2$181. A 6 × His tag was engineered on the amino terminus of t$E_2$181, and a two-plasmid system was used for high-level expression of the protein. The recombinant protein was purified to near-homogeneity by affinity chromatography on Ni−NTA resin, followed by chromatography on heparin−agarose. About 14 mg of tE_2-H181 was obtained from 10−13 g (wet weight) of transformed cells. When analyzed by SDS−PAGE (Figure 2), tE_2-H181 showed a major band with M_r ∼30 000 and a minor band with M_r ∼19 000. The minor band is apparently a truncated form of tE_2-H181, as indicated by immunoblotting (data not shown).

Stoichiometry of Binding of E_3BP-H, tE_3BP-H144, and tE_3BP-H218 to tE_2-H181. Previous studies (Maeng *et al*., 1994; J. K. Stoops, T. S. Baker, L. J. Reed *et al*., unpublished data) showed that an E_3BP monomer anchors an E_3 homodimer inside each of the 12 faces of the 60-mer dodecahedral E_2. Although E_2 can physically accommodate only 1 E_3BP−E_3 complex in each of its 12 faces, there should be potentially 60 binding sites for E_3BP, i.e., 1 E_3BP-binding site per each E_2 subunit. However, previous studies (Maeng *et al*., 1994) showed that t$E_2$206 can bind only about 15 E_3BP monomers. A probable explanation of this discrepancy is that steric

Table 2: Stoichiometry of Binding of E_3BP-H, tE_3BP-H144, and tE_3-H218 to tE_2-H181[a]

	polypeptide chain ratios[b]	
complex	tE_2	E_3BP/tE_3BP
tE_2-H181−E_3BP-H	60	20.0 ± 0.6
tE_2-H181−tE_3BP-H144	60	23.8 ± 1.4
tE_2-H181−tE_3BP-H218	60	31.0 ± 1.8

[a] Each protein had a 6 × His tag at its amino terminus. [b] Polypeptide chain ratios are the means of three determinations ± standard deviations.

hindrance by the lipoyl and E_3-binding domains of E_3BP prevents full stoichiometric binding of E_3BP to E_2. To address this question, truncated forms of E_3BP lacking these domains were prepared by deletion mutagenesis, and binding studies were conducted with these tE_3BP mutants and a truncated form of E_2 (tE_2-H181) lacking the lipoyl and E_1-binding domains. Mixtures of tE_2-H181 and an excess of E_3BP-H, tE_3BP-H144, or tE_3BP-H218 (molar ratio 1:40) were centrifuged at 35 000 rpm for 2 h in a Beckman TLS55 swinging-bucket rotor to separate the bound and unbound forms of E_3BP. The pellets, which contained tE_2-H181 complexed with E_3BP-H, tE_3BP-H144, or tE_3BP-H218, were resolved by SDS−PAGE. After staining the gels with Coomassie blue and destaining, the polypeptide chain ratios of tE_2:E_3BP or tE_3BP were determined by video area densitometry. The results (Table 2) show that tE_2-H181 binds about 20 copies of E_2BP-H, about 24 copies of tE_3-BP-H144, lacking the lipoyl domain, and about 31 copies of tE_3BP-H218, lacking both the lipoyl and E_3-binding domains. These data demonstrate that steric hindrance by segments of E_3BP does indeed prevent full stoichiometric binding of E_3BP to the pentagonal dodecahedron-like E_2 and support the proposal that there is a binding site for E_3BP on each E_2 subunit. Apparently, the geometric constraints inside the E_2 scaffold determine the stoichiometry of binding of E_3BP and E_3.

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