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# The catalytic domain of dihydrolipoyl acetyltransferase from the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus*

## Expression, purification and reversible denaturation

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**Abstract** **A sub-gene encoding the catalytic (acetyltransferase) domain (E2pCD) comprising residues 173–427 of the dihydrolipoyl acetyltransferase (E2p) chain of the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus* was expressed in *Escherichia coli*. The product assembled to form the characteristic icosahedral (60-mer) core structure with full catalytic activity. The $K_m$ values for dihydrolipoamide and acetyl-CoA were 1.2 mM and 13 μM, respectively. Dissociation of the icosahedral E2pCD into monomers by exposure to guanidine hydrochloride and the subsequent reassociation by gradual removal of the denaturing agent demonstrated the ability of the polypeptide chain to fold and reassemble in the absence of chaperonins.**

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*Key words:* Catalytic domain; Dihydrolipoyl acetyltransferase; Multienzyme complex; Chaperonin; Reversible denaturation; Self-assembly

### 1. Introduction

The 2-oxo acid dehydrogenase complexes consist of multiple copies of three enzymes which together catalyse the oxidative decarboxylation of 2-oxo acids. The complexes are assembled around an oligomeric dihydrolipoyl acyltransferase (E2) core to which the 2-oxo acid decarboxylase (E1) and dihydrolipoyl dehydrogenase (E3) components are bound tightly, but non-covalently [1–4]. The E2 components are found in two different symmetries, octahedral or icosahedral, depending on the source. The octahedral E2 consists of 24 identical polypeptide chains arranged with 432 symmetry, generating a cube-like structure with each vertex composed of three, and each face of four, E2 chains. The icosahedral E2 consists of 60 E2 chains arranged with 532 symmetry to generate a pentagonal dodecahedron. Each vertex is again composed of three E2 chains and each face of five E2 chains. The intact multienzyme complexes have molecular masses in the range of $1–10\times10^6$ Da, and as such are easily visible in the electron microscope [5–7] where the different symmetries can be identified.

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The mechanistic core of the 2-oxo acid dehydrogenase complexes is also provided by the E2 component. The N-terminal region of each E2 chain consists of one, two or three lipoyl domains, each approximately 80 amino acids in length, depending upon the species. The lipoyl domains are tandemly arrayed, being joined by flexible linker peptides approximately 25–30 amino acids long, rich in alanine, proline and charged amino acids. The lipoyl domains are followed by another domain, about 35 amino acids in length, the main function of which lies in binding E3 dimers to the E2 core. In icosahedral PDH complexes, such as that from *Bacillus stearothermophilus* [8,9], this domain also serves as a major contributor to the pyruvate decarboxylase (E1p)-binding site [4]. The peripheral subunit-binding domain is in turn joined by another linker peptide to a large (29 kDa) C-terminal domain. This C-terminal region aggregates to form the inner (octahedral or icosahedral) core structure of the 2-oxo acid dehydrogenase complexes and also contains the active site for the acyltransferase reaction.

X-ray crystallography of the octahedral (24-mer) complex of the catalytic domain of *Azotobacter vinelandii* dihydrolipoyl acetyltransferase (E2p) has produced a high resolution structure and thrown light on the mode of assembly and catalytic activity [10,11]. In this paper, we describe the creation of a sub-gene encoding the catalytic domain from *B. stearothermophilus* E2p. We have been able to express this sub-gene in *Escherichia coli*, thereby permitting the purification and characterization of the icosahedral assembly. The icosahedral structure, with full catalytic activity, can be reassembled from monomers after denaturation in vitro with guanidine hydrochloride and subsequent removal of the denaturing agent.

### 2. Materials and methods

#### 2.1. Materials

Bacterological media were from Difco. The pKKE2 vector carrying the dihydrolipoyl acetyltransferase gene that encodes residues 1–427 of *B. stearothermophilus* E2p [12] was generated earlier (A. Borges, C.F. Hawkins and R.N. Perham, unpublished work). Plasmid pET11d and *E. coli* host strain BL21(DE3) [($F^-$, *ompT*, $hsdS_B$, ($r_b^-$, $m_b^-$), *gal*, *dcm*, (DE3)] were obtained from Novogen Inc. Dihydrolipoamide was synthesized from lipoamide by reduction with sodium borohydride [13].

#### 2.2. Recombinant DNA techniques

Small DNA fragments were isolated from agarose gels by using the Magic miniprep purification systems (Promega). Dideoxynucleotide

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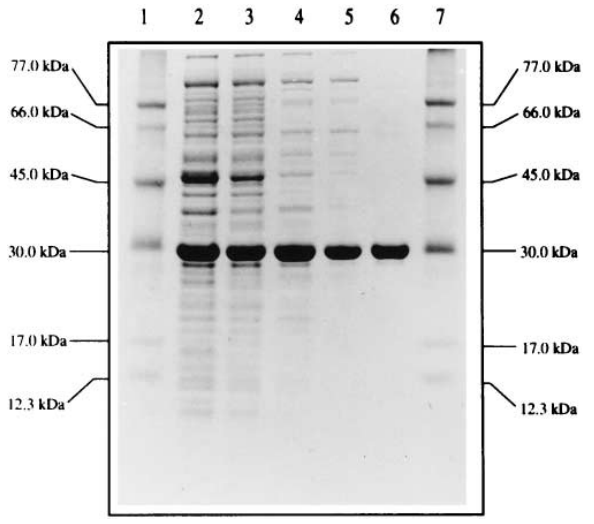



Fig. 1. Purification of E2pCD, as judged by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 7: $M_r$ standards (ovotransferrin 77 000, BSA 66 000, ovalbumin 45 000, carbonic anhydrase 29 000, myoglobin 17 000, cytochrome *c* 12 300). Lane 2: Soluble fraction from disrupted *E. coli* cells previously transformed with pET11E2pCD and grown for 3 h after induction with IPTG. Lane 3: 30–55% ammonium sulfate fraction. Lane 4: Protein eluted from Hi-load Q column. Lane 5: Protein eluted from Superose 6 column. Lane 6: Protein eluted from Mono Q column.

DNA sequencing [14] was carried out using the T7 sequencing kit from Pharmacia and [α-$^{35}$S]dATP from Amersham International. Polymerase chain reactions (PCR) [15,16] were carried out under mineral oil in a reaction buffer (100 μl) containing 100 pmol of each sense and antisense primer, and 2.5 U of Taq polymerase on a programmable PHC-3 thermal cycler (Techne Corp.). The cycling parameters were as follows: 3 min at 94°C (one cycle); and 1 min at 94°C, 2 min at 55°C and 3 min at 72°C (25 cycles). The fidelity of the amplified PCR fragments was established by DNA sequencing after sub-cloning into the vector.

*2.3. Construction of expression vector pET11E2pCD*

The DNA sequence encoding the C-terminal catalytic domain (residues 173–427) of the *B. stearothormophilus* E2p was amplified from plasmid pKKE2 by PCR using a 24-bp sense oligonucleotide (5′-GGCGGCGCAACCATGGCACCGGCC-3′; mismatches underlined) and a 27-bp antisense oligonucleotide (5′-TTCGGATCCATCGTTTTACGCCTCCATT-3′). The PCR product (798 bp) was digested with *Nco*I and *Bam*H1, purified by agarose gel electrophoresis and sub-cloned into the pET11d vector previously digested with *Nco*I and *Bam*H1 and treated with calf intestinal alkaline phosphatase.

*2.4. Gene expression and purification of E2pCD*

*E. coli* strain BL21(DE3) cells were transformed with pET11E2pCD and grown to an $A_{600}$ of 1.0 in 2×YT medium [17] supplemented with ampicillin (100 μM) before induction with IPTG (1 mM). The cells were harvested 4 h after induction and resuspended in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM PMSF, 0.02% $NaN_3$ and 0.1 mg/ml RNAse. The suspension was incubated for 30 min at room temperature and then disrupted in a French press. After centrifugation (130 000×*g* for 20 min), the supernatant was subjected to fractional precipitation with $(NH_4)_2SO_4$. The precipitate that formed between 30 and 55% saturation was dissolved in buffer A (20 mM potassium phosphate, pH 7.0, containing 0.02% $NaN_3$), dialysed against the same buffer, and applied to a Hi-load-Q column (Pharmacia) equilibrated with buffer A. E2pCD was eluted with buffer B (20 mM potassium phosphate, pH 7.0, 1 M NaCl, 0.02% $NaN_3$) applied as a 0–100% gradient over four column volumes. Fractions containing E2pCD (as identified by SDS-PAGE using a Tris/Tricine buffer system [18]) were pooled and gel-filtered on a Superose 6 HR (10/30) column (Pharmacia) equilibrated with buffer A. Fractions containing E2pCD were applied to a Mono-Q column (Pharmacia) equilibrated with buffer A, and the E2pCD was eluted with buffer B applied as a 0–100% gradient over four column volumes. The purity of the E2pCD was confirmed by N-terminal sequence analysis.

*2.5. Measurement of E2p catalytic activity*

The catalytic activity of E2p was assayed (increase in $A_{240}$ corresponding to formation of acetyldihydrolipoamide) at 30°C under the following conditions [19]: 85 mM Tris-HCl, pH 8.0, 10 mM acetyl phosphate, 0.24–10.4 mM dihydrolipoamide, 5–200 μM CoA, phosphotransacetylase (2 U/ml).

*2.6. Electron microscopy of E2pCD*

A drop of the protein sample (0.1 mg/ml) was placed on a Formvar and carbon-coated copper grid, and the excess removed by touching with filter paper. The grid was dried and negatively stained with 1% (w/v) phosphotungstic acid buffered with 100 mM potassium phosphate, pH 7.0, and inspected in a Philips EM 301 electron microscope.

*2.7. Dissociation and reconstitution of E2pCD*

Samples of *B. stearothermophilus* E2pCD or bovine serum albumin (BSA) were incubated for 1 h in buffer A (50 mM potassium phosphate, pH 7.0) containing a range of concentrations (50 mM to 6 M) of guanidine hydrochloride (GuHCl). Each sample of E2pCD was gel-filtered through a Superose 6 (HR) column pre-equilibrated with the relevant incubation buffer. Comparison of the elution positions for BSA and E2pCD was used to calibrate the dissociation of the (60-mer) E2pCD core complex ($M_r$ = 1.8 MDa) into monomers ($M_r$ = 28 kDa).

A separate sample of *B. stearothermophilus* E2pCD was subsequently incubated for 1 h in buffer A (50 mM potassium phosphate, pH 7.0) containing 6 M GuHCl and 1 mM DTT and gel-filtered through a Superose 6 (HR) column in the same solution. The pooled fractions containing E2pCD were dialysed successively against buff-

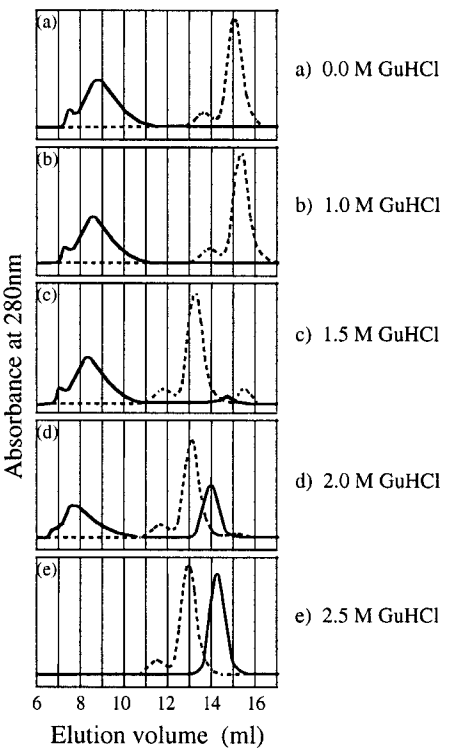



Fig. 2. Dissociation of icosahedral *B. stearothermophilus* E2pCD induced by GuHCl, as judged by gel filtration. Samples of *B. stearothermophilus* E2pCD (1 nmol) dissolved in 50 mM potassium phosphate, pH 7.0, containing increasing concentrations of GuHCl were subjected to gel filtration on a Superose 6 (HR) column equilibrated with the relevant GuHCl-containing buffer (solid line). BSA examined under identical conditions was used as a molecular mass marker (dotted line).

er A containing 1 mM DTT and supplemented in turn with 4, 3, 2, 1.5, 1.0, 0.7, 0.5, 0.3, 0.2 and 0.1 M GuHCl (2 h for each dialysis). The final dialysis was against buffer A without DTT. The resulting solution was then gel-filtered through a Superose 6 (HR) column equilibrated with buffer A to determine the elution position of the reconstituted E2pCD.

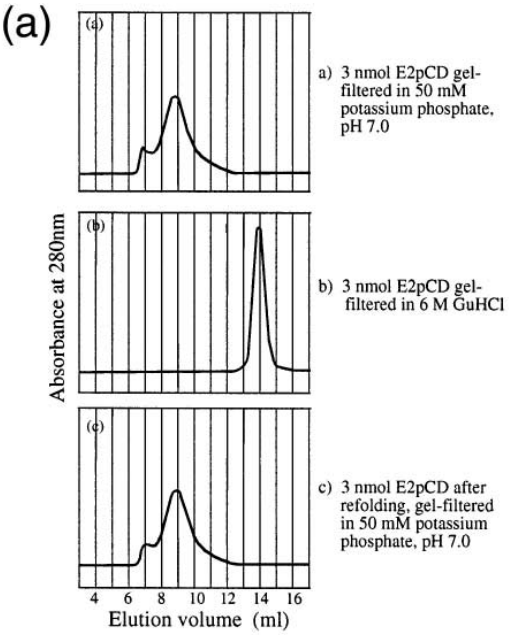
## 3. Results

Expression in *E. coli* of a sub-gene encoding residues 173–427 of the *B. stearothermophilus* E2p polypeptide chain was successfully achieved, enabling the *B. stearothermophilus* E2pCD complex to be purified in high yield (Fig. 1). The protein displayed the high molecular mass, as judged subsequently by gel filtration (Fig. 2) and the appearance in the electron microscope (Fig. 3), expected of an icosahedral (60-mer) aggregate.

The catalytic activity of the truncated E2p component, which should contain the acetyltransferase active site, was measured in the reverse physiological direction [19]. Double-reciprocal plots (1/v vs. 1/S) of the initial velocity data were linear, converging at a point close to the *x*-axis. Secondary plots of the intercepts on the *y*-axis against the reciprocal of the fixed substrate concentration were also linear. The $K_m$ values for dihydrolipoamide and acetyl-CoA were estimated as 1.2 mM and 13.0 μM, respectively. The convergence of the double-reciprocal plots at a point on the *x*-axis suggests the bi-substrate reaction proceeds via a sequential mechanism. The bovine kidney E2p component was previously shown to follow a random sequential mechanism [20].

The ability of E2pCD to bind the E1p and E3 components of the *B. stearothermophilus* PDH complex was assessed by gel-filtering E2pCD with various admixtures of recombinant E1p and E3 [[28]; A. Borges, C.F. Hawkins and R.N. Perham, unpublished work] in 20 mM postassium phosphate buffer, pH 7.0, on a Superose 6 (HR) column. No evidence of interaction was observed, confirming the conclusions from limited proteolysis of the native PDH complex, which suggested that the peripheral subunit-binding domain of *B. stearothermophilus* E2p plays a major part in binding both E1p and E3 [4].

Samples of E2pCD were incubated for 1 h in 50 mM potassium phosphate, pH 7.0, containing various concentrations of GuHCl. Each sample was then gel filtered on a Superose 6 gel-filtration column in the same solution (Fig. 2), with BSA (molecular mass 66 kDa) used as a standard. In the absence of GuHCl, E2pCD was eluted as a high molecular mass species,



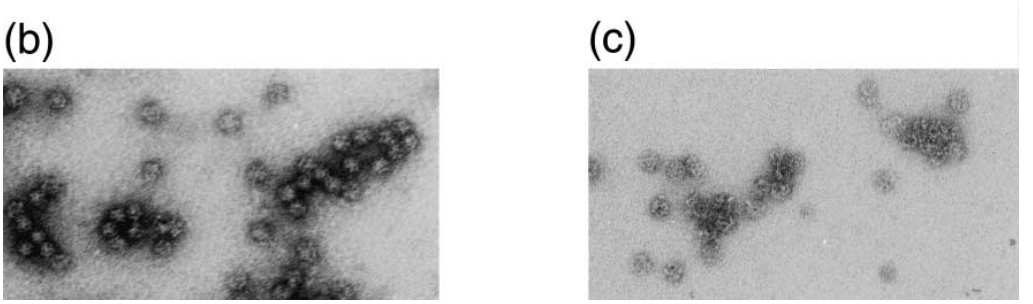




Fig. 3. Reassembly of icosahedral *B. stearothermophilus* E2pCD after dissociation with GuHCl. a: *B. stearothermophilus* E2pCD was incubated in 50 mM potassium phosphate, pH 7.0, containing 6 M GuHCl and 1 mM DTT for 1 h and the dissociation of the 60-mer structure was analysed by gel filtration on a Superose 6 (HR) column in the same solution. After gradual dialysis back into 50 mM potassium phosphate, pH 7.0, the E2pCD was gel filtered in the same buffer to assess the reassociation into an icosahedral complex. b: Electron micrograph of the E2pCD before dissociation in GuHCl. c: Electron micrograph of the E2pCD after dissociation in GuHCl and reassembly.

consistent with an icosahedral aggregate of the polypeptide chain (Fig. 2a). BSA began to unfold when incubated in 1.5 M GuHCl, as evidenced by an increase in the hydrodynamic radius and earlier elution from the column (Fig. 2c). E2pCD also began dissociating/unfolding at 1.5 M GuHCl, with a small peak appearing in a position consistent with a lower molecular mass than that of unfolded BSA. When the concentration of GuHCl reached 2.5 M, all the 60-mer complex was found to be dissociated into the smaller component (Fig. 2e), with no further change in elution profile when the concentration of GuHCl was raised to 6 M.

The apparent molecular mass of the dissociated E2pCD was estimated to be approximately 30 kDa, corresponding to the monomeric polypeptide chain. The formation of an intermediate trimeric species in the analytical ultracentrifuge has been described [21,22], but no corresponding peak was observed in the present experiments. Previous fluorimetric studies [23] of the icosahedral bovine heart PDH complex have also suggested that the 60-mer and trimeric E2p species dissociate into monomers, with 50% loss of tertiary structure at 1.9 M GuHCl and 50% loss of secondary structure at 2.8 M GuHCl.

The ability of E2pCD to reassociate from the unfolded state was also examined (Fig. 3a). E2pCD was incubated for 1 h in 50 mM potassium phosphate, pH 7.0, containing 1 mM DTT and 6 M GuHCl. The unfolded E2pCD was gel filtered through a Superose 6 column in the same solution and the fractions containing the unfolded E2pCD chain were collected. The pooled fractions were dialysed gradually into 50 mM potassium phosphate buffer, pH 7.0, as described in Section 2, and the protein was then subjected to gel filtration on a Superose 6 column equilibrated with the same buffer. It was found to have assembled into a complex that was eluted in a position identical to that of native E2pCD.

Electron microscopy of the refolded E2pCD (Fig. 3c) revealed the presence of icosahedral complexes identical to those observed before dissociation (Fig. 3b) and the acetyltransferase activity of the refolded E2pCD was found to have regained 95% of its original value. Interestingly both the native and refolded E2pCD complexes appear capable of forming small amounts of structures larger than the 60-mer complexes, as evidenced by a small peak emerging near the exclusion volume of the column. This suggests some degree of aggregation of E2pCD in low ionic strength buffers, an observation also made in the electron microscope (Fig. 3b).

## 4. Discussion

Previous studies of dissociation and refolding of the E2 components of various other PDH complexes have also reported full recovery of activity after unfolding [21,24], but some have obtained only partial activity [25], or no activity at all [22] after denaturation. In *Saccharomyces cerevisiae*, the folding of the mitochondrial E2p precursors in vivo is thought to be mediated by interaction with chaperonins homologous to dnaK, dnaJ, GroEL, GorES and grpE found in *E. coli* [26]. It has also been reported that in vitro assembly of the heterotetrameric ($\alpha_2\beta_2$) E1 component and octahedral (24-mer) E2 core domain (E2bCD, residues 161–421) of the bovine branched chain 2-oxo acid dehydrogenase complex relies on chaperonins GroEL and GroES [22,27]. However, it is evident from the experiments reported above that chaperonins are not absolutely essential for the in vitro refolding and assembly of the *B. stearothermophilus* E2pCD core complex, nor are they essential for the in vitro assembly of its E1 ($\alpha_2\beta_2$) component [28]. These differences between homologous E2p cores from different species will repay further study.

Crystallization of an intact E2 component from a 2-oxo acid dehydrogenase multienzyme complex has so far proved unsuccessful, probably because of the presence of flexible linkers between the domains in the E2 chains. However, overexpression of a sub-gene encoding the catalytic domain of the *A. vinelandii* E2p component has enabled the X-ray crystallographic analysis of an octahedral E2 core [10,11]. Overexpression of sub-gene encoding the catalytic domain of the *B. stearothermophilus* E2p component described in this paper demonstrates the self-assembly of an icosahedral core and paves the way to the determination of its crystal structure.

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