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Crystallization and initial X-ray diffraction analysis of human pyruvate dehydrogenase

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Human pyruvate dehydrogenase (E1) is a component enzyme of the pyruvate dehydrogenase complex. The enzyme catalyzes the irreversible decarboxylation of pyruvic acid and the rate-limiting reductive acetylation of the lipoyl moiety linked to the dihydrolipoamide acetyltransferase component of the pyruvate dehydrogenase complex. E1 is an $\alpha_2\beta_2$ tetramer (~154 kDa). Crystals of this recombinant enzyme have been grown in polyethylene glycol 3350 using a vapor-diffusion method at 295 K. The crystals are characterized as orthorhombic, space group $P2_12_12_1$, with unit-cell parameters a = 64.2, b = 126.9, c = 190.2 Å. Crystals diffracted to a minimum d spacing of 2.5 Å. The asymmetric unit contains one $\alpha_2\beta_2$ tetrameric E1 assembly; self-rotation function analysis showed a pseudo-twofold symmetry relating the two $\alpha\beta$ dimers.

1. Introduction

The mammalian multienzyme pyruvate dehydrogenase complex (PDC) plays a pivotal role in carbohydrate metabolism by linking the cytosolic non-oxidative glycolytic pathway to the more efficient energy-generating process of the tricarboxylic acid cycle in the mitochondria. Mammalian PDC is also a member of the family of large mitochondrial α-keto acid dehydrogenase complexes that include the α-ketoglutarate dehydrogenase complex (KGDC) and the branched-chain α-keto acid dehydrogenase complex (BCKADC). PDC, KGDC and BCKADC catalyze the thiamine pyrophosphate-dependent oxidative decarboxylation of pyruvic acid, α-ketoglutarate and branched-chain α-keto acids, respectively, to form the corresponding acyl-CoA and CO_2 with concomitant conversion of NAD^+ and NADH. Acetyl-CoA generated by PDC is either further oxidized for energy production or utilized for lipid synthesis.

Eukaryotic PDCs including human PDC are unique in that they contain multiple copies of six different components: pyruvate dehydrogenase (E1; 20–30 $\alpha_2\beta_2$ tetramers per complex), dihydrolipoamide acetyltransferase (E2; 60 monomers), dihydrolipoamide dehydrogenase (E3; six homodimers), E3-binding protein (E3BP; 12 monomers), E1 kinase (2–3 homodimers) and phospho-E1-phosphatase (2–3 heterodimers) (Reed, 1974; Patel & Roche, 1990). E3BP is required for the association of E3 with E2 in eukaryotic PDC. The last two components, the specific kinase and phosphatase, are responsible for regulation of PDC by phosphorylation/dephosphorylation. The 60 E2 subunits associate with 12 E3BP subunits to form the core to which the other components are non-covalently bound. The conglomerate of the eukaryotic PDC components, with a molecular mass of approximately 10^7 Da, is among the most complex multienzyme structures known. PDCs from most prokaryotes have a 24-mer cubic E2 core. Associated with this core are 12 dimers of E1 and six dimers of E3. Some Gram-positive bacteria (*e.g.* *Bacillus stearothermophilus*, *Enterococcus faecalis*) have 60-mer dodecahedral cage-like E2 cores (Kalia *et al.*, 1993; Izard *et al.*, 1999) similar to that of eukaryotic PDCs (Stoops *et al.*, 1997); however, E3BP is not present in PDCs from Gram-positive bacteria (Reed, 1974; Patel & Roche, 1990).

The first three components, E1, E2 and E3 perform the following sequential reactions, *i.e.* decarboxylation of pyruvate with the intermediate formation of 2-α-hydroxyethylidene-TPP-E1 and CO_2 (1), the reductive acetylation by E1 of the lipoyl moieties covalently bound to E2 (2), transacetylation reaction of coenzyme A catalyzed by E2 (3), oxidation of the reduced lipoyl moieties of E2 by E3-FAD (4) and transfer by E3 of the reducing equivalents from FAD to NAD^+ with the formation of NADH and H^+ (5).

$$CH_3COCOOH + \text{E1-TPP} \rightarrow CH_3C(OH)\text{=TPP-E1} + CO_2 \quad (1)$$

$$CH_3C(OH)\text{=TPP-E1} + \text{E2-lipoyl(S)}_2 \rightleftharpoons \text{E1-TPP} + \text{E2-lipoyl(SH)}SCOCH_3 \quad (2)$$

$$\text{E2-lipoyl(SH)}SCOCH_3 + \text{CoASH} \rightleftharpoons \text{E2-lipoyl(SH)}_2 + CH_3COSCoA \quad (3)$$

$$\text{E2-lipoyl(SH)}_2 + \text{E3-FAD} \rightleftharpoons \text{E2-lipoyl(S)}_2 + \text{E3-FADH}_2 \quad (4)$$

$$\text{E3-FADH}_2 + NAD^+ \rightleftharpoons \text{E3-FAD} + \text{NADH} + H^+. \quad (5)$$

Three-dimensional structure has not been solved for any human PDC components so far. We studied the human E1, an $\alpha_2\beta_2$ heterotetramer with a molecular mass of 154 kDa (α-subunit 41 kDa and β-subunit 36 kDa), which catalyzes the irreversible decarboxylation of pyruvic acid and the rate-limiting reductive acetylation of the lipoyl moieties linked to E2 (reactions 1 and 2). E1 has two active sites, formed by both α- and β-subunits, each containing one thiamine pyrophosphate (TPP) molecule (Butler *et al.*, 1977; Khailova & Korochkina, 1982). These active sites are proposed to work by an 'alternating sites' catalytic mechanism (Khailova & Korochkina, 1985). E1 is proposed to be bound to E2 through its β-subunit. E1, like other TPP-requiring enzymes, has a putative TPP-binding motif, GDGX25-27N in the α-subunit (Hawkins *et al.*, 1989). Each E1α subunit has three specific serine residues which are subject to a phosphorylation (inactivation)/dephosphorylation (activation) mechanism (Yeaman *et al.*, 1978). Phosphorylation of only one serine out of six potential sites in tetrameric E1 renders the E1 (and hence PDC) inactive (Yeaman *et al.*, 1978; Sugden & Randle, 1978). Complete dephosphorylation of all potential sites restores the catalytic activity of E1. This covalent modification plays a central role in regulating glucose metabolism in the fed and fasting states, as well as in pathological conditions such as diabetes and obesity.

Genetic defects in PDC reported in more than 150 patients further support the importance of this complex in carbohydrate metabolism (Kerr *et al.*, 1996). The majority of PDC-deficient patients show defects associated with the E1 component and all of those defects occur in the α-subunit. Approximately half of these defects are point mutations in the coding region of the gene and hence indicate their involvement in the normal function and/or structure of E1.

At this time, three-dimensional structures of three E1 component proteins of α-keto acid dehydrogenase complexes, including 2-oxoisovalerate dehydrogenase (E1) from *Pseudomonas putida*, BCKADC-E1 from humans and PDC-E1 from *Escherichia coli*, have been determined (Æversson, Seger *et al.*, 2000; Æversson, Chuang *et al.*, 2000; Arjunan *et al.*, 2000). The first two E1s form heterotetrameric assemblies similar to that of E1 from the human PDC and each contains two TPP-binding sites located on subunit interfaces. In contrast, E1 from *E. coli* PDC functions as a homodimer rather than a heterotetramer and has essentially no sequence homology with α-keto acid dehydrogenases having both α- and β-subunits (Arjunan *et al.*, 2000). Comparison of the sequence of human PDC-E1 with that of E1 of human BCKADC showed high percentages of identity and homology. The sequence alignment of corresponding α-subunits showed 26% identity and 41% homology, respectively, and 35% identity and 52% homology, respectively, for the β-subunits. This significant sequence homology will likely serve as a model for the determination of the structure of human PDC-E1 using molecular-replacement methods. The crystallization and preliminary X-ray analysis of recombinant human E1 reported here represents the first prerequisite steps towards determining the tetrameric structure of this enzyme and the role of key amino acids involved in its function.

2. Methods and results

2.1. Overexpression and purification of human E1

Human E1 was overexpressed and purified as described previously with several modifications (Korotchkina *et al.*, 1995). E1 was overexpressed in *E. coli* M15 harboring pDMI.1 and pQE-9-HisE1β/E1α containing E1α and E1β cDNAs without the leader sequences (Korotchkina *et al.*, 1995). E1β had a polyhistidine extension at the N-terminus for affinity purification. Human E1 was overexpressed in 12 l Luria–Bertani medium with 100 µg ml^{-1} ampicillin, 25 µg ml^{-1} kanamycin. Bacterial culture was induced overnight at 298 K with 200 µg ml^{-1} isopropyl-β-D-thiogalactopyranoside. Cells were harvested by centrifugation at 6000*g* for 30 min at 313 K and washed once with buffer *A* (50 m*M* potassium phosphate buffer pH 7.5, 300 m*M* KCl, 5 m*M* β-mercaptoethanol containing the following protease inhibitors: 0.1 m*M* phenylmethylsulfonyl fluoride, 0.1 m*M* benzamidine, 1 µg ml^{-1} leupeptin). The washed pellet was resuspended in buffer *A* and the preparation was treated with lysozyme and passed through a French press (3.45 MPa) to open the cells. The suspension was then centrifuged at 20 000*g* for 30 min at 313 K and the supernatant was applied to a nickel–nitriloacetate–agarose column. The column was first washed with buffer *A* for 2 h and then washed overnight with buffer *A* containing 50 m*M* imidazole. E1 was eluted with a linear gradient of 50–250 m*M* imidazole in buffer *A*. Fractions containing E1 were concentrated using Millipore concentrators, washed with 50 m*M* potassium phosphate buffer pH 7.0 and loaded on a DEAE–Sephadex A-25 column prequilibrated with 50 m*M* potassium phosphate buffer pH 7.0. E1 was eluted with 50 m*M* potassium phosphate buffer pH 7.0 containing 100 m*M* NaCl. Protein fractions were dialyzed overnight against 50 m*M* potassium phosphate buffer pH 7.0, 1 m*M* dithiothreitol, 0.2 m*M* $MgCl_2$, 0.2 m*M* TPP (buffer *B*), concentrated and loaded on a Superose 200 HPLC column. E1 was eluted with 50 m*M* potassium phosphate buffer pH 7.0 containing 150 m*M* NaCl, concentrated to 12–18 mg ml^{-1}, dialyzed against buffer *B* and frozen prior to crystallization. E1 preparations were >95% pure as judged by densitometry of SDS–PAGE gels.

2.2. Crystallization

Prior to crystallization trials, purified protein preparations were examined by dynamic light scattering using a DynaPro Molecular Sizing instrument (Protein Solutions, Inc.). Protein samples were filtered through a 0.02 µm filter and their concentration adjusted to 1–2 mg ml^{-1} in 50 m*M* phosphate buffer pH 7.0 before measurements. At first, light-scattering assessments of E1 prepared without TPP showed that the size distribution of particles was broad and irregular, with molecular weights between 30 and 1400 Da (FWHM), indicating the presence of single E1 subunits, dimers and tetramers as well as large assemblies. Elimination of N-terminal His-tag amino acids further increased the polydispersity of E1 preparations. The results thus indicated a need for a decrease in polydispersity and thus stabilization of the protein preparation for successful crystallization. This was achieved by the addition of 0.2 m*M* TPP and 0.2 m*M* $MgCl_2$. The E1-TPP with His-tag amino acids showed a size distribution of the particles with molecular weights between 70 and 160 Da (FWHM), which corresponded to dimeric and tetrameric arrangements of the protein.

Following the results of light-scattering assessments, crystallization trials included only co-crystallization of E1 with TPP and magnesium ions. Initial protein crystallization conditions were screened using an in-house developed crystallization matrix, based on the original described by Jancarik & Kim (1991), that employs four common precipitants: ammonium sulfate, sodium/potassium phosphate, 2-methyl-2-propanol and PEG 4000; screening was carried out using the vapor-diffusion method at 277, 283 and 295 K. The initial experiments yielded granular precipitates by employing ammonium sulfate, sodium/potassium phosphate and PEG 4000 as precipitants. Following the lead precipitants, the first E1 crystals (mostly as clusters of thin plates) were obtained from 20–25% ammonium sulfate in 50 m*M* phosphate buffer pH 7.0 with the protein concentration in the range 6–12 mg ml^{-1}. These crystals did not develop into single crystals suitable for X-ray diffractometry.

The best diffracting crystals were obtained using a hanging-drop technique at 295 K where 4 µl of protein in 50 m*M* potassium phosphate buffer pH 7.0, at a concentration of 12 mg ml^{-1}, 0.2 m*M* TPP, 0.2 m*M* $MgCl_2$ and 1 m*M* dithiothreitol were mixed with an equal volume of reservoir precipitant solution containing 50 m*M* potassium phosphate buffer pH 6.0, 100 m*M* ammonium sulfate, 1 m*M* sodium azide and 11–14% polyethylene glycol 3350 (Hampton Research) as a precipitant. Crystals usually appeared within 48 h and continued to grow for several more days (Fig. 1). Crystals were time-sensitive in that diffraction occurred within a finite window of crystallization time. After approximately one week, crystals lost birefringence and diffractive ability. Although the microscopic appearance of these post-diffraction crystals was unchanged, they lacked the rigid mechanical properties of crystals and became deformable. The SDS–PAGE gel analysis of those crystals collected from crystallization droplets and then dissolved in phosphate buffer did not show evidence of proteolytic cleavage. Therefore, the observed deformability suggests the action of some other mechanism and is the subject of ongoing investigation.

2.3. X-ray diffraction analysis

Prior to data collection, a crystal of E1 was soaked in a cryoprotectant solution containing 80%(*v*/*v*) crystallization mother liquor and 20%(*v*/*v*) glycerol for approximately 1 min and then transferred to a nylon CryoLoop (Hampton Research) and flash-frozen in liquid nitrogen. The frozen crystal was subsequently mounted in a continuous cold stream and 0.4° oscillation images were collected at 100 K using beamline BM-19 at the Advanced Photon Source, Argonne, IL, USA. The crystal showed the symmetry of an orthogonal space group, $P2_12_12_1$, and the refined values of the unit-cell parameters were a = 64.2, b = 126.9, c = 190.2 Å. The processing of data was completed using the program *HKL*2000 and the statistics of the data processing (Otwinowski & Minor, 1997) are shown in Table 1.

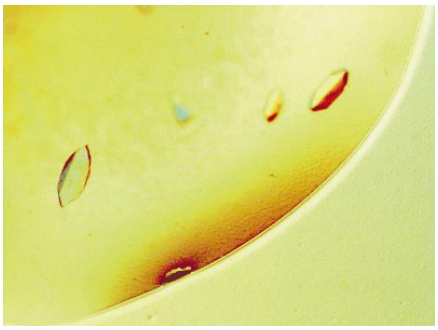

Figure 1
Photomicrograph of single crystals of human E1 with thiamine pyrophosphate and magnesium ions grown in the presence of 12% polyethylene glycol 3350 in 50 m*M* potassium phosphate buffer at pH 7.0. The largest crystal shown is 300 × 100 × 100 µm.

3. Discussion

On the basis of space-group symmetry, the unit-cell volume and the molecular weight of the α- and β-subunits, a heterotetrameric protein molecule per asymmetric unit was proposed. The V_M is calculated to be 2.23 Å^3 Da^{-1} assuming one molecule per asymmetric unit, indicating the solvent content to be 45% according to the equation of Matthews (1968). In order to establish the relationship between the two $\alpha\beta$ dimers that constitute tetrameric E1 in the asymmetric unit, we used the real-space self-rotation function implemented in the *CNS* suite (Brunger *et al.*, 1998). In the $\kappa = 180°$ section, which was calculated based on reflections in the 15–6 Å resolution range and an integration radius of 40 Å, the highest peak at $\psi = 0$ and $\varphi = 0°$ represented the crystallographic twofold symmetry axis. The second highest peak height, about 25% of the strongest peak, at ψ = 54 and φ = 86°, probably corresponds to a non-crystallographic twofold axis relating the two $\alpha\beta$ dimers in the asymmetric unit.

The fact that the asymmetric unit of the human E1 crystal consists of the entire $\alpha_2\beta_2$ tetramer is consistent with the biological observation that in solution the active form of the enzyme containing TPP in the binding sites is an $\alpha_2\beta_2$ tetramer. A search for a molecular-replacement solution as well as efforts to crystallize selenomethionine-substituted E1 are currently under way.

Table 1
Data-collection statistics for crystal of human E1.

Values within parentheses are for the reflections observed in the highest resolution shell (2.54–2.50 Å).

No. of measured reflections	351314
No. of unique reflections	54666
Resolution (Å)	20.0–2.5
Completeness (%)	100 (99.9)
R_{sym}† (%)	11.8 (41.5)
Average $I/\sigma(I)$	10.1 (5.0)

† $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ and $\langle I(h)\rangle$ are the ith and mean measurements of the intensity of reflection h, respectively.

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