Novel tetramer assembly of pyruvate decarboxylase from brewer's yeast observed in a new crystal form

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Abstract A new crystal form of thiamine diphosphate dependent pyruvate decarboxylase from *Saccharomyces cerevisiae* has been obtained in the presence of the activator pyruvamide. The crystallographic structure analysis reveals differences in the domain packing in the enzyme subunit and a novel assembly of the subunits in the tetramer, when compared to the structure of native PDC. The orientation of the β domains in the subunit differs by a 6.3° and 8.3° rotation, respectively, whereas the subunit-subunit interface in the dimer, formed by the α and γ domains, is essentially maintained. In the tetramer, one of the dimers rotates relative to the second dimer by approximately 30° creating a new dimer-dimer interface.

Key words: Quaternary structure; Thiamine diphosphate; Pyruvate decarboxylase; Molecular replacement; Protein crystallography

1. Introduction

The thiamine diphosphate (ThDP) dependent enzyme pyruvate decarboxylase (PDC) is a key enzyme in the alcohol fermentation process where it catalyzes the conversion of pyruvate to acetaldehyde and CO_2. All PDCs have an oligomeric structure and in most cases they are composed of four identical or almost identical subunits with a molecular mass of about 60 kDa per subunit.

The subunit of PDC consists of three distinct structural domains [1,2]. The amino- and carboxy-terminal domains (α and γ domain, respectively) are similar in structure and have the same fold as the corresponding domains of pyruvate oxidase and transketolase [3]. These two domains are involved in binding of ThDP, Mg(II) and substrate. The β domain, located in the middle of the polypeptide chain, contains a seven-stranded mixed sheet with five parallel and two antiparallel strands. This domain is involved in activation of the enzyme by the substrate and it has been proposed that it contains the binding site for activators such as pyruvamide [1,4].

Two of the subunits associate tightly to form a dimer with approximate twofold symmetry. As in other ThDP dependent enzymes, the cofactors ThDP and Mg(II) are bound between the α and γ domains from two different subunits at the subunit-subunit interface [3]. Two of these dimers assemble to a loose tetramer with approximate 222 symmetry, which can be described as a dimer of dimers [1,2,5]. The dimer-dimer interactions are formed almost entirely through residues from the β domain, the most important being the extension of the seven-stranded β-sheet in the β domains across the subunit-subunit interface to a large 14-stranded β-sheet.

An extraordinary property of all PDCs characterized so far, with the exception of the enzyme from *Zymomonas mobilis*, is the allosteric substrate activation process first described by Davies in 1967 [6]. Substrate analogues such as pyruvamide [7] are also able to activate the enzyme. Based on the X-ray structure [1,2] and biochemical data [4,8] it has been proposed that substrate activation occurs by binding to the regulatory (β) domain, specifically to Cys^{221} ,which induces a conformational transition from the inactive to the active conformation of the enzyme.

In solution, active PDC from brewer's yeast is a tetramer [5] and small-angle X-ray solution scattering experiments with the enzyme in the absence and presence of pyruvamide [5,9,10] provided evidence that large conformational changes within the tetramer indeed are likely to occur upon addition of the activator. In order to further examine possible conformational changes in PDC we have crystallized the enzyme in the presence of the activator pyruvamide. In this communication we describe the results of a crystallographic analysis of these crystals which reveal a novel assembly of the subunits in the tetramer when compared to the structure of native PDC.

2. Materials and methods

2.1. Protein purification

PDC was purified from cells of the strain WS 34/70 of brewer's yeast (*Saccharomyces cerevisiae*). The yeast cells (approximately 60 g of packed cells) were suspended in 100 mM sodium phosphate pH 6.1 containing 5 mM DTE, 1 mM EDTA, 1 mM ThDP and 5 mM $MgSO_4$ and disrupted in a bead mill. After centrifugation, the supernatant was treated with protamine sulfate and ammonium sulfate as described [11]. The crude enzyme was further purified (Dobritzsch, unpublished results) by gel filtration (Sephacryl S200 HR), hydrophobic interaction chromatography (Fraktogel EMD Phenyl column), and anion exchange chromatography (Resource Q, Pharmacia). The purified pyruvate decarboxylase had a specific activity of approximately 45–50 U/mg, comparable to the activity of other PDC species from yeast and plant seeds.

2.2. Crystallization

The purified enzyme was concentrated to approximately 5 mg/ml and diluted in 20 mM sodium citrate buffer pH 6.0 containing 1 mM DTE, 5 mM ThDP and 5 mM $MgSO_4$ and concentrated again by using Ultrafree-CL filter units (Millipore, exclusion size 10 kDa). The procedure was repeated 4 times to exchange buffer and desalt the samples. Pyruvamide was added to a final concentration of 320 mM. Pyruvate decarboxylase was crystallized by the hanging drop vapor diffusion method using polyethylene glycol 6000 as precipitating agent. To 4 μl of the protein sample (5 mg/ml PDC, 20 mM sodium citrate pH 6.0, 1 mM DTE, 5 mM ThDP and $MgSO_4$, 320 mM pyruvamide) the same volume of the reservoir solution was added (20 mM sodium citrate pH 5.7, 1 mM DTE, 5 mM ThDP and $MgSO_4$, 8–14% PEG). All crystallization experiments were carried

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Abbreviations: PDC, pyruvate decarboxylase; ThDP, thiamine diphosphate

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out at 4°C. The best crystals were obtained at PEG concentrations of 13–14%. The first crystals appeared after 2 days and grew to maximal size in less than one week. In order to distinguish these crystals from the crystal form described previously [1], the enzyme crystallized in the absence of pyruvamide will be designated form A, and the enzyme crystallized in the presence of the activator form B.

2.3. Crystallographic data collection

Crystals were soaked in a solution containing 15% (w/v) PEG 6000, 100 mM buffer (pH 5.7) and 17% (v/v) glycerol for 1 min and were transferred into a cryogenic nitrogen gas stream at 110 K. A MAR research imaging plate mounted on a Rigaku rotating anode, operated at 50 kV and 90 mA, was used for data collection. The data were processed by the DENZO/SCALEPACK packages [12].

X-ray data were collected to 2.7 Å resolution using two crystals. However, the completeness of the X-ray data in the highest resolution bin (2.8–2.7 Å) was approximately 30%, so that the effective resolution of the data set is more appropriately described as 2.8 Å. Nevertheless, the measured reflections between 2.8 and 2.7 Å resolution were included in the refinement and calculation of the electron density maps. Statistics of the X-ray data set are given in Table 1. Space group and cell dimensions of the crystals were determined using the auto-indexing routine in DENZO [12] and by the analysis of the diffraction pattern using the program PATTERN (Lu, unpublished).

2.4. Molecular replacement

The structure of PDC in this new crystal form was determined by molecular replacement using the structure of form A PDC [1], PDB accession code 1PYA. Orientation and positions of the molecule were determined with the AMORE program [13] with the PDC monomer as search model. A rotation function was calculated with X-ray data in the resolution interval 10–3.5 Å with an integration radius of 30 Å. Two peaks were found with correlation coefficients 0.198 and 0.134 respectively, at least two times higher than the background. A translation function, calculated using X-ray data in the same resolution range gave clear solutions for the positions of the two molecules, with correlation coefficients 0.331 and 0.303, respectively. A cross translation function was calculated in order to determine the relative positions of the two monomers. The resulting model had a correlation coefficient of 0.50 and a crystallographic *R*-factor of 0.41 at 2.7 Å resolution.

2.5. Crystallographic refinement

The X-plor program [14] was used for the crystallographic refinement. A test set of 4% randomly selected reflections were omitted from the refinement and used to monitor *R*-free [15]. The model was first refined as a rigid body which reduced *R*-free to 0.36. At this stage, an anisotropic correction to the X-ray data was introduced which resulted in an decrease of *R*-free to 0.34. Refinement continued by simulated annealing in the resolution interval 6.0–2.7 Å. Non-crystallographic symmetry (NCS) restraints were imposed in the refinement. However, as indicated by the electron density maps and the *R*-free value, the two monomers in the asymmetric unit were not completely identical in terms of domain-domain packing. Therefore, NCS restraints were imposed on the individual domains rather than the whole subunit. In a few cases, when indicated by the electron density map, NCS restraints for individual residues were removed. Parameters as defined by Engh and Huber [16] were used. Refinement runs were followed by manual intervention using the O program [17] and 2Fo-Fc electron density maps calculated in the resolution interval 15–2.7 Å. The model was analyzed with the program PROCHECK [18]. Details of the refinement and the current model are summarized in Table 2. Structural comparisons were made using O [17].

3. Results and discussion

3.1. Structure determination

The crystals of PDC obtained in the presence of the activator pyruvamide belong to the monoclinic space group C2,

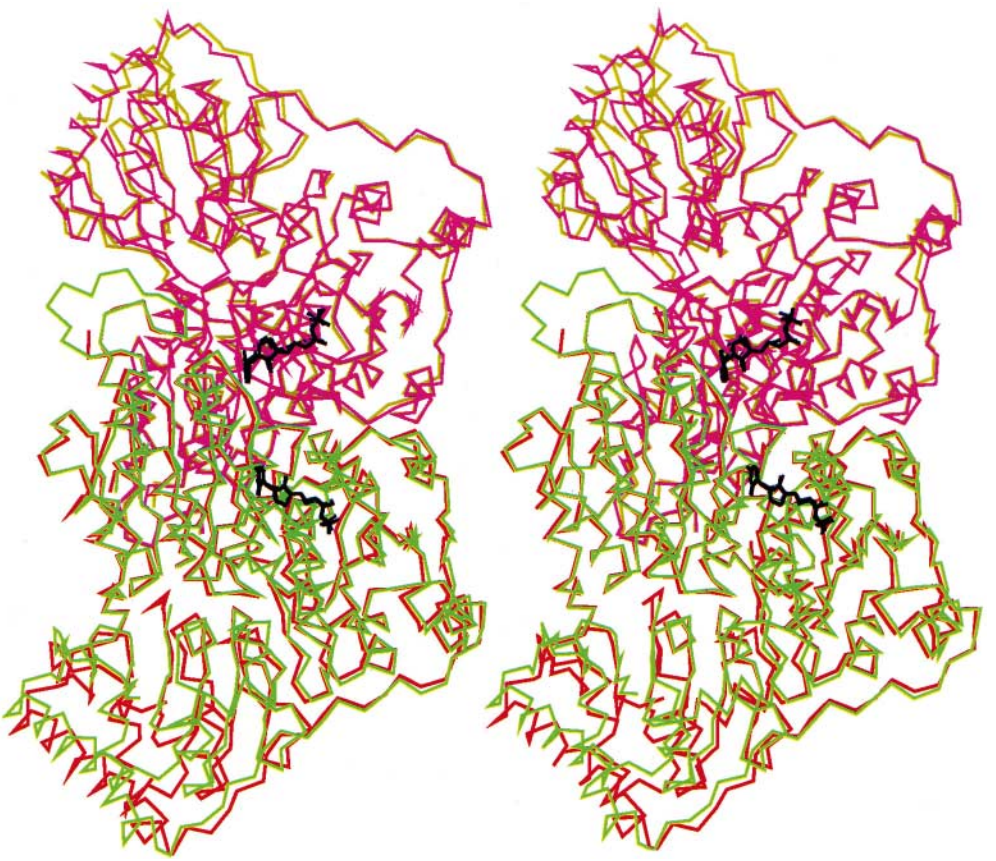

Fig. 1. Comparison of the three-dimensional structures of form A and B PDC dimers. The color code for form A subunits is yellow and red and for form B subunits pink and green. The superpositions were based on the α- and γ domains of the two monomers.

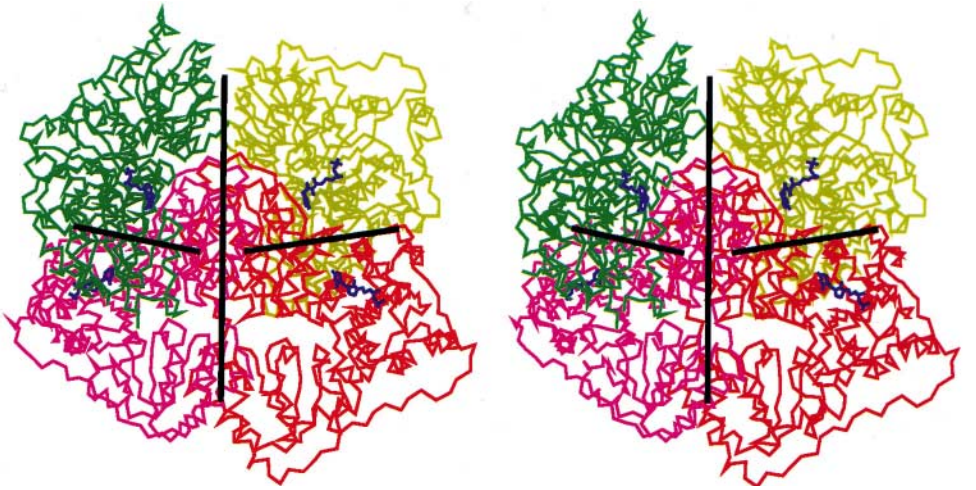

Fig. 2. Stereoview of the PDC tetramer in the new crystal form. The crystallographic two-fold axis which relates the two dimers in the tetramer runs vertically in the figure and the two non-crystallographic two-fold axes relating the monomers in the dimer run perpendicular to the crystallographic two-fold axis. The four subunits are color coded differently. Four ThDP molecules (blue) are included to indicate the location of the active sites.

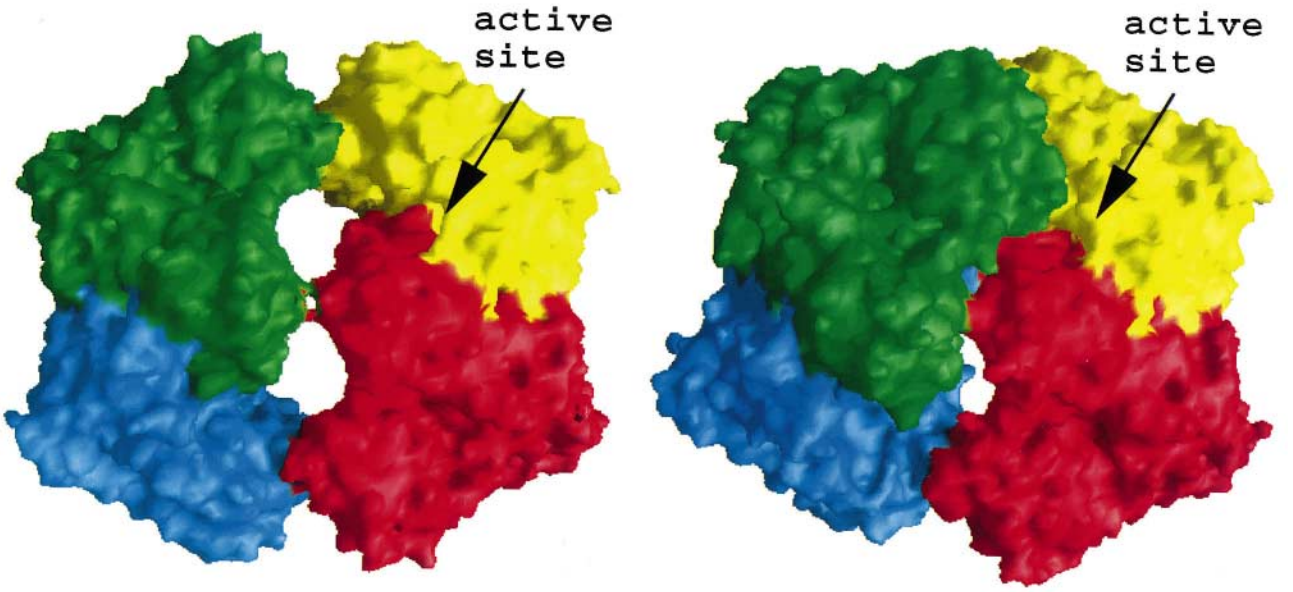

Fig. 3. Tetramer assembly in PDC. The four subunits are color coded differently. The blue and green subunits form one of the dimers, and the yellow and red represent the second dimer. Left: Form A PDC [1,2]. Right: PDC in crystals grown in the presence of pyruvamide. One of the four active sites is indicated by an arrow. The figure was generated with GRASP [21].

with cell dimensions a = 145.1 Å, b = 119.7 Å, c = 81.3 Å, β = 120.2°. There are two subunits in the asymmetric unit, resulting in a packing density of 2.5 $Å^3$/Da.

There is continuous and well-defined electron density for most parts of the molecule, except for a few loop regions (see below) which could not be modelled. Furthermore, no electron density for the N-terminal residue and C-terminal residues 557–562 was observed in any of the monomers in the asymmetric unit.

3.2. *Structure of the subunit and dimer packing*

The overall fold of the subunit of form B PDC is identical to the subunit structure described previously [1,2]. In the form A structure, loop regions comprising residues 106–113 and 292–303 are disordered in all four subunits of the tetramer [1]. In the form B crystals, the loop comprising residues 292–303 in one of the subunits has defined electron density. The other disordered loop in form A crystals, comprising residues 106–113, is better defined in one of the subunits in the form B structure, but residues 105–108 show only weak electron density and their positions are poorly defined. Both loop regions are in contact with each other and are involved in dimer-dimer interactions close to one of the active sites (see below).

The orientation of the three domains in the two subunits in the asymmetric unit is very similar. When superimposing the α domains in each monomer, the rotation needed for optimal superposition of the γ domains is less than a rotation of 0.3° and a translation of 0.1 Å. In the case of the β domains, a rotation of 1.3° and a translation of 0.3 Å is required for

Table 1
Data collection statistics

	Overall
Resolution	2.8 Å
Measured reflections	210802
Unique reflections	27789
Completeness (%)	94.1
R-merge	0.119

optimal superposition of these domains. This slight difference in packing of the β domains in the two subunits might or might not be significant at the present resolution. However, it would explain the observed differences in side chain conformation at the interface between the β domain and the other two domains in the dimer.

More significant differences in domain orientation were found when the form B structure was compared with the three-dimensional structure of form A PDC [1]. While the orientation of the α domain with respect to the γ domain is very similar in the two structures, the packing of the β domain to the rest of the subunit is clearly different (Fig. 1). When the α or γ domains of the two PDC structures were superimposed, the β domains differed by a 6.3° and 8.2° rotation, respectively, between the corresponding monomers of the two forms of PDC. This difference in domain packing, which is significant at the present resolution, results in an average r.m.s. difference of 1.8–2.0 Å for the Cα atoms of the β domain in the two structures.

In the asymmetric unit, two independent subunits of PDC form a dimer, related by a non-crystallographic two-fold axis. There are numerous packing interactions which create a tight monomer-monomer interface, consisting of an area of 3374 Å^2 (14% of the monomer surface). This interface, which is exclusively formed between the α and γ domains, is maintained in the dimer found in the form B crystals of PDC (Fig. 1).

3.3. Tetramer assembly

In form A PDC, the tetramer is formed through interactions across the crystallographic two-fold axis between two PDC dimers. Similarly, in form B crystals the tetramer is built up from two dimers which interact through a two-fold crystallographic symmetry axis (Fig. 2). However, there are large differences in the assembly of the tetramer in the two crystal forms. In form A PDC, the interactions between the dimers are rather weak and the tetramer has a rather loose packing (Fig. 3). In form B PDC, the packing of the dimers in the tetramer is different. When one dimer of form A PDC was superimposed on one dimer of form B PDC using least-squares methods, the second dimer had to be rotated by 30.1° and translated by 1.5 Å along the rotation axis in order to superpose with the corresponding second dimer in the tetramer (Fig. 3).

This novel mode of assembly of PDC subunits in the tetramer has a number of consequences. Firstly, it introduces an asymmetry in the PDC tetramer when going from form A to form B crystals. The angle between the axis relating the two dimers and the non-crystallographic two-fold axes relating the monomers in the dimer is 81° and the closest distance between the two axes is 6 Å. This mode of assembly of the tetramer is far from the 222 symmetry that was found in the structure of form A PDC [1], and each subunit in the dimer has therefore very different environments in the dimer-dimer interfaces. One of these interfaces is preserved in the new tetramer assembly and consists of residues from the β domain. In particular, the extension of the seven-stranded β-sheet in the β domain across the dimer-dimer interface to the adjacent β domain is preserved in this interface region. Due to the distinct packing mode of the two dimers, the other interface is different. At this new type of interface, residues from both the β domain and α domain are involved. In particular, residues from the loop regions which were disordered in the native structure and at least partly ordered in the form B crystals interact with each other across this interface close to the active site.

A second consequence of this new mode of packing of the PDC subunits is a tighter tetramer, reflected in an increase of the dimer-dimer interaction area from about 900 Å^2 (2.3% of the total dimer surface area) to 1550 Å^2 (3.8%).

A third consequence is an asymmetry for the active sites in the tetramer. In form A PDC, which represents a more open form of the tetramer, all four active site are equivalent and easily accessible. In the form B tetramer, two of the active sites are accessible, with a similar conformation and environment as in form A PDC. However, two of the active sites are closed and not accessible from the outside.

3.4. Pyruvamide binding

No electron density at the proposed regulatory site, i.e. Cys^{221} [4], could be observed and we conclude that in these crystals pyruvamide does not bind to this site. At the present resolution of the structure analysis, an unambiguous assignment of residual electron density to bound pyruvamide at other sites is not possible and has to await extension of the X-ray data set and refinement to higher resolution. We note, however, that the form B crystals cannot be obtained when pyruvamide is omitted from the crystallization mixture.

3.5. Conclusions

The most significant finding of the structure analysis of the new crystal form of PDC, obtained in the presence of the activator pyruvamide, is a distinct assembly of the subunits in the PDC tetramer. The structure analysis at 3.5 Å resolution of PDC crystallized in the presence of the activator ketomalonate in yet another space group revealed a tetramer assembly different from the native enzyme [19]. Also in this case, a rotation of the two dimers with respect to each other

Table 2
Refinement statistics

Data used for the refinement	6.0–2.8 Å
Number of reflections	22919
R value	0.213
R-free	0.280
Anisotropic correction parameters	B_{11} = 17.0454 B_{22} = 11.8610 B_{33} = 3.5683 B_{13} = 4.0636
r.m.s. bond length	0.014 Å
r.m.s. bond angle	1.9°
r.m.s dihedral	25.5°
r.m.s. improper	1.8°
*B*mean	30.7 Å^2
Ramachandran plot: % of non-glycine residues in disallowed regions	0.2

was found; however, the packing seems significantly different from the packing described here.

An important conclusion to be drawn is that various modes of tetramer assembly are possible for PDC which in turn leads to the question which (if any) of the at least three distinct tetramers observed so far represents the active enzyme. While others [19,20] have concluded, based on crystallographic [1,2], small-angle X-ray solution scattering data [5] or modelling [20], that the open form of the tetramer is the active form of the enzyme, it seems that more detailed studies are necessary to definitely establish the significance of these variations in tetramer assembly and reveal the structural basis for substrate activation.

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