High Resolution Crystal Structure of Pyruvate Decarboxylase from Zymomonas mobilis

IMPLICATIONS FOR SUBSTRATE ACTIVATION IN PYRUVATE DECARBOXYLASES*

(Received for publication, March 10, 1998, and in revised form, May 7, 1998)

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The crystal structure of tetrameric pyruvate decarboxylase from Zymomonas mobilis has been determined at 1.9 Å resolution and refined to a crystallographic R-factor of 16.2% and Rfree of 19.7%. The subunit consists of three domains, all of the a/b type. Two of the subunits form a tight dimer with an extensive interface area. The thiamin diphosphate binding site is located at the subunit-subunit interface, and the cofactor, bound in the V conformation, interacts with residues from the N-terminal domain of one subunit and the C-terminal domain of the second subunit. The 2-fold symmetry generates the second thiamin diphosphate binding site in the dimer. Two of the dimers form a tightly packed tetramer with pseudo 222 symmetry. The interface area between the dimers is much larger in pyruvate decarboxylase from Z. mobilis than in the yeast enzyme, and structural differences in these parts result in a completely different packing of the subunits in the two enzymes. In contrast to other pyruvate decarboxylases, the enzyme from Z. mobilis is not subject to allosteric activation by the substrate. The tight packing of the dimers in the tetramer prevents large rearrangements in the quaternary structure as seen in the yeast enzyme and locks the enzyme in an activated conformation. The architecture of the cofactor binding site and the active site is similar in the two enzymes. However, the x-ray analysis reveals subtle but significant structural differences in the active site that might be responsible for variations in the biochemical properties in these enzymes.

Pyruvate decarboxylase (PDC) is a key enzyme in alcohol fermentation and depends on thiamin diposphate (ThDP) and Mg(II) ions for catalytic activity. The enzyme catalyzes the conversion of pyruvate to acetaldehyde and carbon dioxide but is also able to utilize other 2-oxo acids as substrates. The obligatory fermentative Gram-negative bacterium Zymomonas mobilis uses only hexoses as carbon sources for glycolysis and produces ethanol and carbon dioxide via the Entner-Doudoroff pathway (1). In this organism, PDC amounts to 4% of the total soluble protein and to 10% of the extractable protein after cell lysis (1). There appears to be only one gene coding for this enzyme in Z. mobilis (2).

In solution, native ZmPDC is a tetramer of four identical subunits, and each subunit consists of 568 amino acids with a molecular mass of about 60 kDa (1, 2). Every subunit binds a set of cofactors (ThDP and Mg(II) ions) very tightly but not covalently at pH 6.0, the optimum for catalytic activity. The mechanism of cofactor binding in ZmPDC is similar to that for the yeast enzyme (1, 3). The cofactors stabilize the quaternary structure in a wide range of pH from 4.6 to 8.5, but more alkaline conditions lead to complete loss of catalytic activity because of dissociation of the cofactors (4).

A common feature of pyruvate decarboxylases, with the exception of ZmPDC, is their allosteric regulation by the substrate or other activator molecules such as pyruvamide (5, 6). Crystallographic studies of yeast PDC revealed considerable differences in the tetramer assembly between enzyme species obtained in the absence (7, 8) or presence of the activator pyruvamide (9). Cross-linking and small angle x-ray solution scattering experiments also indicated significant tetramer re-assembly and conformational changes during substrate activation in yeast PDC (10–12).

Here, we present the crystal structure of recombinant PDC from Z. mobilis at 1.9 Å resolution. The crystallographic study reveals a novel, as yet unobserved tetramer assembly in pyruvate decarboxylases. Comparison of the quaternary structures of PDC from Z. mobilis and yeast suggests that the structural differences in the interface regions might be related to the differences in their kinetic behavior. In addition, the crystallographic analysis provides further insights into the structural basis of catalysis and substrate specificity in pyruvate decarboxylases.

MATERIALS AND METHODS

Expression and Purification—The gene coding for ZmPDC was expressed in the Escherichia coli SG13009 prep4 strain containing the Z. mobilis gene ATCC 29191. This strain was kindly provided by Dr. Martina Pohl (Institut für Enzymtechnologie, Heinrich-Heine-Universität Düsseldorf). Expression of the PDC gene was induced by addition of 0.5 mM isopropyl β-D-thiogalactoside to the late exponential growth phase. The cells were harvested by centrifugation and disrupted in a French Press (SLM Instruments, Inc.). Ammonium sulfate precipitation was carried out in two steps (30 and 42% w/v ammonium sulfate, respectively). The precipitate from the last step, which contains crude pyruvate decarboxylase, was suspended in 10 mM Mes/NaOH, pH 6.5, 1 mM MgSO4, 0.2 mM ThDP, 1 mM dithioerythritol and dialyzed overnight.
against the same buffer. The enzyme solution was applied to Fraktogel EMD TMAE (S) (Merck, column 2.6 × 10 cm) equilibrated with the same buffer without ThDP and MgSO₄ (flow rate 1 ml/min). The protein was eluted with a linear ammonium sulfate gradient (0–40 mM in 100 ml) at 4 °C. The purest fractions showed PDC activity of 100–120 units/ml with about 95% homogeneity in SDS-polyacrylamide gel electrophoresis. These fractions were collected and concentrated to 30–40 mg/ml by ultrafiltration. Simultaneously, the buffer was changed to 10 mM sodium citrate pH 6.0. The concentrated protein solution was stored at −20 °C without significant loss of activity.

**Crystallization**—ZmPDC was crystallized using the hanging drop vapor diffusion method. Droplets were set up for crystallization by mixing 1 μl of solution containing 13 mg/ml protein and 1 mM dithioerythritol with 4 μl of the reservoir solution containing 100 mM Mes/NaOH, pH 6.5, and 24% (v/v) PEG 1500. Tiny crystals were obtained within 10 days at 20 °C. Streak seeding was used to improve crystal size. The above protein solution containing 5 mM ThDP, 5 mM MgSO₄, and 1 mM dithioerythritol was mixed with reservoir solution of 100 mM sodium citrate, pH 6.0, and 19–22% (v/v) PEG 1500 and pre-equilibrated for 2 days. After seeding, crystals appeared within a few hours and grew to a maximum size of 0.7 × 0.5 × 0.2 mm in 3 days.

**Data Collection**—The crystals were soaked in a solution containing 100 mM sodium citrate, pH 6.0, 22.5% (v/v) PEG 1500 and 17% (v/v) glycerol for 5 min and transferred into a cryogenic nitrogen gas stream at 110 K. The x-ray diffraction data sets were collected on a MAR research image plate mounted on a Rigaku rotating anode, operating at 50 kV and 90 mA. Data processing was carried out by the DENZO/SCALEPACK packages (13). The crystals belong to the triclinic space group P1 with cell dimensions a = 69.9 Å, b = 92.0 Å, and c = 98.0 Å, α = 103.7°, β = 94.5°, γ = 112.3°. There are four ZmPDC monomers in one asymmetric unit, resulting in a packing density of 2.7 Å³/Da. Details of the data collection are given in Table I.

**Structure Solution by Molecular Replacement**—The structure of ZmPDC was determined by molecular replacement using a model of form B ScPDC refined at 2.4 Å (9), which shares only 28% sequence identity with ZmPDC. Orientation and positions of the molecules were determined using the AMORE program (14) with a yeast PDC dimer as a search model. Both self- and cross-rotation functions were calculated with x-ray data in the resolution interval 10–3 Å with an integration radius of 30 Å. Two orientation solutions were found with correlation coefficients 0.125 and 0.118, respectively (1.8 times the highest noise peak). With the position of one dimer fixed in the P1 space group, a cross-translation function using the data in the 10–3 Å resolution range determined the relative position of the other dimer with a correlation coefficient of 0.173 (1.3 times the highest noise peak) and an R-factor of 0.52.

**Model Building and Crystallographic Refinement**—A test set of 4% of the reflections was excluded before starting any crystallographic refinement to monitor the R│obs value. Rigid body refinement was carried out with XPLOR (15) using data in the 15–2.4 Å resolution interval. The resulting model (R│obs = 0.50) was used for NCS averaging with the Dm program (16) in the CCP4 package (17) and the Rave packages (18). Based on the averaged electron density maps, the model was rebuilt using the O program (19), and the ZmPDC sequence was fitted to the electron density map. Atomic positions and B-factors of the model were refined with noncrystallographic symmetry restraints using REFMAC (20), and the model was rebuilt according to the resulting averaged electron density map. Iterations of the procedure were performed with the resolution gradually extended to 1.86 Å. When the R│obs value had dropped to 27% and most of protein atoms were defined, 2Fo − Fc and Fc − Fo maps were used for further inspection of the model. NCS restraints were released for several residues as indicated by the electron density map. Most water molecules were added automatically using the PEAKMAX program in CCP4 (17), the PEAKCHECK program (written by J. Smith), and the WATMCS program. NCS restraints were introduced for most of the water molecules in the refinement. Additional solvent molecules that did not follow NCS were added by visual examination of the electron density map in the final stage. Errors in the model were found automatically from F│obs − Fo maps by the DIFLIST program and corrected on the display using O. At this stage, it became obvious that the bound cofactor had undergone chemical degradation in the ZmPDC crystals, and a model of a ThDP analogue with an open thiazolium ring was introduced. Citrate molecules were modelled as well as double conformations for some of the amino acid residues. Statistics of the refinement and the final protein model are given in Table II.

**Activity Measurements**—Enzymatic activity was assayed at 30 °C and 13 mg/ml with a NADH/ADH coupled test in 100 mM sodium citrate, pH 6.1, according to Holzer et al. (25). One unit of activity is the quantity of enzyme that catalyzes the formation of 1 μmol of product/min. The protein concentration was determined spectrophotometrically at 280 nm (ε = 275 320 M⁻¹ cm⁻¹).

**RESULTS**

**Structure Determination and Model Quality**—The crystal structure of ZmPDC was determined by molecular replacement. Electron density maps calculated from crude models at different stages of the refinement allowed tracing of the polypeptide chains, even for those parts where the ZmPDC chain is quite different in structure compared with ScPDC. The final model of ZmPDC in the asymmetric unit is a homotetramer of four monomers related by pseudo 222 symmetry. The model contains 4 × 565 amino acids, (comprising residues 2–566), four chemically modified ThDP molecules, four Mg(II) ions and four citrate molecules, and a total of 2569 water molecules. Most of these solvent molecules (2448) fully or partially follow the pseudo symmetry.

The structure of ZmPDC was refined to an R-factor of 16.2% and an R│obs value of 19.7% using all the data between 15–1.86 Å with good stereochemistry (for detailed statistics see Table II). The electron density map was of very good quality (Fig. 1) and allowed location of almost all protein atoms, except the N-terminal methionine and the two C-terminal residues. Electron densities were poor or weak for side chain atoms of several residues, including Glu227 and Asp530 in all subunits, Lys523 in three and Glu520 in one of the subunits. All of these residues are exposed to solvent on the protein surface. Double conformations were found for two residues in all the subunits, Lys553 located on the protein surface and Ile472 at the active site.

In total, four amino acid sequences of ZmPDC have been published (26–29). Although only one ZmPDC gene has been

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2. “Form A” and “form B” ScPDC denote yeast pyruvate decarboxylase crystallized in the absence or presence of the allosteric activator, pyruvamidce.

3. D. Dobritzsch, S. König, G. Schneider, and G. Lu, unpublished observation.

4. G. Lu, http://gamma.mbb.ki.se/~guoguang/watncs.html.

5. G. Lu, manuscript in preparation.
identified so far (2), all these sequences differ from each other at a few positions. None of these sequences completely matched the electron density map, and the amino acid sequence of our final model (listed in Fig. 4) is slightly different from all the templates. The amino acid sequence determined by protein sequencing (29) required the fewest changes to be consistent with the electron density map (Glu200 → Asp, Asn256 → Leu, and Ala341 → Ser).

In the present model, 92.1% of the amino acid residues are located in the most favored regions of the Ramachandran plot. One residue, Ser74, is located in the disallowed region in all four subunits. This residue is, however, well defined in its electron density.

**Overall Structure**—ZmPDC is a homotetramer with an overall size of approximately 85 × 98 × 118 Å. Each monomer can be divided into three domains, denoted as PYR (residues 1–188), R (residues 189–354), and PP (residues 355–568) domains,6 each with an open α/β topology (Fig. 2). The nomenclature of the secondary structural elements is shown in Figs. 2 and 4B. No significant structural differences were found between the subunits, except for the side chains of about 20 residues, which are involved in the crystal packing.

Two of the monomers are tightly bound to each other and are related by 2-fold symmetry. The extensive interface in the dimer comprises about 4400 Å², corresponding to 19.4% of the surface area of the monomer. Most of the amino acid residues building up this interface are from the PYR and PP domains, but residues 286–290 of the R domain also are part of this region. In total, 7 pairs of salt bridges, 66 pairs of hydrogen bonds, and extensive hydrophobic interactions are formed between the two subunits.

Two such dimers further form a tetramer with pseudo 222 symmetry, with an interface area of 4400 Å² (12.4% of the dimer surface). All three domains of each subunit are involved in these interfaces. The interactions between the two dimers include 25 sets of salt bridges and 64 sets of hydrogen bonds. At the dimer-dimer interface some narrow but extensive cavities are formed, part of which are occupied by a large number of ordered water molecules. The interactions between the two dimers are not as tight as at the monomer-monomer interface so that the ZmPDC tetramer can be described as a dimer of dimers, similar to ScPDC (7, 30).

**ThDP Binding**—The four identical subunits, in combination with the pseudo 222 symmetry of the tetramer, generate four cofactor and substrate binding sites in the ZmPDC molecule. The ThDP and substrate binding sites are located in narrow clefts at the interfaces formed by the PYR domains from one subunit and the PP domains of another subunit. The ThDP binding site is deeply buried inside the molecule, about 15 Å away from the protein surface. The cofactor is bound in the V conformation as found in other ThDP-dependent enzymes such as ScPDC (7), transketolase (31), and POX (22). The pyrimidine ring of ThDP interacts with the PYR domain of one subunit, whereas the residual part interacts with the PP domain of another subunit. The Mg(II) ion anchors the diphosphate group of ThDP to the protein, and it forms an octahedral coordination sphere with two oxygen atoms of the diphosphate group of ThDP, the side chain oxygen atoms of Asp440 and Asn467, the main chain oxygen atom of Gly469, and a water molecule (Fig. 3).

During the refinement, it became clear that the ThDP molecule bound to the ZmPDC crystals was a chemically modified

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6 To facilitate comparison, we are using the nomenclature defined by Muller et al. (34) to identify the various domains in ThDP-dependent enzymes.
analogue of ThDP rather than the cofactor itself. The electron density maps showed no electron density between the assumed position of the C2 carbon atom of the thiazolium ring and the neighboring sulfur and nitrogen atoms, indicating that the bond between these atoms was broken (Fig. 1, bottom). In addition, negative difference electron density was observed at the position of the C2 carbon atom, indicating that the thiazolium ring had been opened and that the C2 carbon atom had been lost. Positive difference electron density was found close to the 4′ amino group of the pyrimidine ring, which has been interpreted as a water molecule tightly bound to the amino group via a hydrogen bond. The well defined electron density for the remaining parts of ThDP, the diphosphate and pyrimidyl moieties, does not indicate any significant further chemical modification or degradation (Fig. 1, bottom). In addition, negative difference electron density was observed at the position of the C2 carbon atom, indicating that the thiazolium ring had been opened and that the C2 carbon atom had been lost. Positive difference electron density was found close to the 4′ amino group of the pyrimidine ring, which has been interpreted as a water molecule tightly bound to the amino group via a hydrogen bond. The well defined electron density for the remaining parts of ThDP, the diphosphate and pyrimidyl moieties, does not indicate any significant further chemical modification or degradation (Fig. 1, bottom) and suggests that the bound species might be \(N\)-(2-methyl-4-amino-5-pyrimidyl)-N-(1-methyl-2-thiol but(1)en-4-diphosphatidyl) amide. Analysis by mass spectrometry of the ThDP analogue isolated by HPLC from redissolved crystals revealed a mass difference consistent with the loss of a methine carbon atom. The apparent selectivity in binding the acyclic analogue of ThDP most likely reflects the inability of cofactor exchange in the crystals rather than preferential binding. In the crystals, ThDP is buried deeply in its binding site, and cofactor release would require large conformational changes, which are prevented by the crystal packing.

Analysis of redissolved crystals of ZmPDC showed that the catalytic activity had been lost. Only when redissolved enzyme was incubated with fresh ThDP under conditions where cofactor exchange can take place could we restore about 55% of the original activity. The modification of bound ThDP is not induced by x-ray radiation during data collection, because loss of catalytic activity is also observed in crystals not exposed to x-ray radiation. It thus appears that during crystallization of ZmPDC, a partial degradation of ThDP occurs leading to an inactive cofactor analogue.

The tendency to undergo hydrolytic cleavage of the thiazolium ring is an established feature of thiamin and related compounds (32). For instance, ring opening can proceed through the formation of a pseudobase by nucleophilic attack of hydroxide on the C2 carbon atom (33). In ZmPDC crystals, the initial nucleophilic attack on the C2 carbon could be delivered by the water molecule that is in hydrogen bonding distance to the 4′-amino group. The nucleophilicity of this water molecule could be increased through interactions with the neighboring side chain of His\(^{114}\), which probably is uncharged (see below) and can act as a proton abstractor. The chemical steps that lead to the loss of the C2 carbon atom after ring opening are less clear and require further study. In view of the observed loss of activity in redissolved crystals (restored by the addition of fresh ThDP) and the compelling evidence that thiamin catalysis proceeds though the cyclic thiazolium ion and not an ring-opened form of thiamin (see for example Ref. 33), we consider the acyclic analogue of ThDP at the active site an artifact and not a species of mechanistic relevance.

**Citrate Binding Sites**—During the crystallographic refinement, strong electron density that did not represent any protein atoms was found at the dimer-dimer interface. The shape of the electron density and the high concentration of buffer ions lead us to interpret this residual electron density as citrate molecules. The four citrate ions might contribute to the tetramer assembly by electrostatic interactions and hydrogen bonds to protein side chains. The three carboxyl groups of each citrate molecule form salt bridges with five residues from two subunits, His\(^{150}\), Lys\(^{153}\), and Arg\(^{157}\) from one subunit and Arg\(^{310}\) and Arg\(^{318}\) from another subunit. In addition, several water molecules link citrate and protein atoms through hydrogen bonds, providing further stabilizing interactions.

**Comparison with Yeast Pyruvate Decarboxylase**—The overall topology of the subunit of ZmPDC is very similar to that of...
Three-dimensional Structure of Pyruvate Decarboxylase

A

PYR-domain

PP-domain

PYR-domain

R-domain

B

| Strain    | PVRa1 | PVRb1 | PVRc1 | PVRb2 | PVRd1 | PVRb3 | PVRb4 | PVRa2 | PVRa3 | PVRa4 | PVRa5 | PVRa6 | PVRa7 | PVRa8 |
|-----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| ZmPDC     | MSSTYGLA SKK IRGQQGK LVRGQLR LNL KTMSNIK MNYKHQTVCH 49   |
| ScPDC     | SEISVFYL PIR EKVYQSL VEGFPLD LLCVTL SIVYV P68 MNMAGNAN 50   |
| FOX       | TNILGAVI KVIKQAGG YHIPLSNSG SLEKMYI LIR 108               |
| ZmPDC     | EVCPCGPR PRK QQAAA AVTTQVYL SLFNBGTV AEYRIPMHL 98         |
| ScPDC     | HSAKDAVL QMF AEYRTT PFTYQPSL AERNLKPL 99                  |
| FOX       | AVNNSTAS QVGGAPI GLFYNYQY LIGTLQ A109                     |
| ZmPDC     | SIFQTQRQ SQK RNYGTVL STHATYK YPTTFPAP 149                 |
| ScPDC     | SIFQTQRQ SQK RNYGTVL STHATYK YPTTFPAP 150                 |
| FOX       | AVNNSTAS QVGGAPI GLFYNYQY LIGTLQ A150                     |
| ZmPDC     | RADLRK DQK PVYKTVL STHATYK YPTTFPAP 149                 |
| ScPDC     | RADLRK DQK PVYKTVL STHATYK YPTTFPAP 150                 |
| FOX       | AVNNSTAS QVGGAPI GLFYNYQY LIGTLQ A150                     |
| ZmPDC     | DSYTWVTDI PEFKPLVNL EFERQFTYK KETQAL Q194                |
| ScPDC     | DSYTWVTDI PEFKPLVNL EFERQFTYK KETQAL Q195                |
| FOX       | AVNNSTAS QVGGAPI GLFYNYQY LIGTLQ A196                    |
| ZmPDC     | LAGV.....FS KDELDPPK SLNAGELKKA A.PADDRPV KTLAAKNV 288   |
| ScPDC     | LAGV.....FS KDELDPPK SLNAGELKKA A.PADDRPV KTLAAKNV 290   |
| FOX       | AVNNSTAS QVGGAPI GLFYNYQY LIGTLQ A292                    |
| ZmPDC     | ALLPFQDVP ELDKPGTPLG KTVMLKGG WTYQVEMVF 424               |
| ScPDC     | ALLPFQDVP ELDKPGTPLG KTVMLKGG WTYQVEMVF 425               |
| FOX       | AVNNSTAS QVGGAPI GLFYNYQY LIGTLQ A431                    |
| ZmPDC     | TAVAPI.....Y RREYITVDP KRRKIFPGS QLQVEMVF 470               |
| ScPDC     | TAVAPI.....Y RREYITVDP KRRKIFPGS QLQVEMVF 472               |
| FOX       | AVNNSTAS QVGGAPI GLFYNYQY LIGTLQ A474                    |
| ZmPDC     | GRELAEAK VALAVTDC P PVRNFRK DRYELKTVW VPLLAAKRR 562       |
| ScPDC     | GRELAEAK VALAVTDC P PVRNFRK DRYELKTVW VPLLAAKRR 564       |
| FOX       | AVNNSTAS QVGGAPI GLFYNYQY LIGTLQ A567                    |
| ZmPDC     | PVWKKV       . . . . . . . . . . . . . . . . . . . . . . 568   |
| ScPDC     | KAPKQHTEAQ DLQFLSTLQ QGOLDD                               |
| FOX       | AVNNSTAS QVGGAPI GLFYNYQY LIGTLQ A593                    |
ScPDC. However, the orientation between the three domains is slightly different to the orientation observed in the two forms of ScPDC, and these relative shifts correspond to rotations of approximately 6–8° (Table III). Furthermore, considerable differences were found in the number of secondary structural elements. When superposing the individual domains between the two enzymes, it was found that 7 of the 24 α-helices in ZmPDC differ considerably with respect to length and orientation from their counterparts in ScPDC (Fig. 4). In general, structural differences are significantly higher for the R domains than those found for the other two domains. The twist of the β-sheet in this domain is quite different in the two enzyme species, probably because of large differences of amino acid compositions and structures in the loop regions.

Large structural differences between ZmPDC and ScPDC also occur in the two polypeptide segments linking the domains and in the loops between the secondary structure elements in the three domains. Many of these regions are part of the dimer-dimer interface and thus participate in tetramer assembly. These parts of the polypeptide chain vary in length between the two enzymes and in addition, a few unique secondary structure elements (PYRα5, PYRβα, Rα6, and Rα7) are found in ZmPDC that do not have their counterparts in ScPDC.

Of particular interest are two segments of the polypeptide chain, residues 101–112 between PYRβ4 and PYRα6 of the PYR domain and residues 285–302 between Rβ4 and Rα5 of the R domain. Most residues of the corresponding parts in ScPDC (residues 103–113 and 287–304) are disordered in the form A crystals (7, 8) but have a defined conformation in two of the four subunits in form B crystals (9). In ZmPDC, these loops are well defined in electron density and show a conformation different from that seen in form B ScPDC. In both enzyme species, the loop from the PYR domain of one monomer (residues 101–112) interacts with the loop of the R domain of the second monomer (residues 287–304) to form part of the active site in the functional dimer. In addition, residues 101–112 participate in the dimer-dimer interactions. However, the significant differences in sequence and structure of these regions of the polypeptide chain in ScPDC and ZmPDC result in changes in the dimer-dimer packing and architecture of the substrate binding site.

In form A ScPDC all active sites are accessible from the outside solution, in part because of the disorder of these two regions, whereas two of the active sites are partly closed in form B ScPDC, with the only access to the solvent through a

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**Fig. 5. Modes of tetramer assembly in pyruvate decarboxylases and pyruvate oxidase.** One functional dimer (colored red and pink) of ZmPDC was superposed with a corresponding dimer of the other enzymes (also shown in red and pink). The resulting rotations/translations were then applied to the second dimer (shown in green and yellow). POX, pyruvate oxidase from *Lactobacillus plantarum*, form A and form B ScPDC, and yeast pyruvate decarboxylase crystallized in the absence and presence of the activator pyruvamide.

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**Fig. 4. A**, superposition of the Cα trace of ZmPDC (blue) and ScPDC (green). Bound ThDP is shown in red. The orientation is same as in Fig. 2. **B**, sequence alignment of ZmPDC, ScPDC, and POX from *Lactobacillus plantarum* based on the superposition of their three-dimensional structures. Residues structurally equivalent are shown in green, and other residues are in black. Structurally equivalent residues conserved in at least two enzymes are shown in red, and other conserved residues are in black (bold letters). Boxes highlight residues conserved in the three enzymes.
narrow cavity. In ZmPDC, the more extensive interactions between these regions and the position of the C-terminal helix, PPa10 make the active site inaccessible. This helix, comprising residues 545–560, is considerably longer and runs across the active site, thus completely blocking access of solvent to the active site.

Two subunits of ZmPDC form a tight dimer, with the interface formed by the PYR and PP domains. Differences between the dimers in ZmPDC and ScPDC were found with respect to the orientations of the individual domains and the size of the interaction areas (Table III). ZmPDC has a more extensive contact area between the two subunits. In ZmPDC, 19% of the total surface area is buried in the monomer-monomer interface compared with 13–15% in the yeast PDCs.

ZmPDC has a unique mode of tetramer assembly different from that observed in other homologous enzymes. When the PYR and PP domains of one functional dimer of yeast PDC and POX are superimposed, the second dimer of POX and form A of ScPDC differs from ZmPDC by a rotation about the monomer-monomer 2-fold axis of 30° and 78°, respectively (Table III). The form B ScPDC, which differs from the native yeast enzyme by a 32° rotation about an axis approximately perpendicular to the 2-fold axis relating the two subunits, is related to ZmPDC by a rotation of about 83° around an arbitrary axis. A comparative view of the different tetramers is shown in Fig. 5.

The differences in tetramer assembly found in pyruvate decarboxylases have a number of implications. Most significantly, similar to POX, ZmPDC has more extensive dimer-dimer interactions than the yeast PDCs. The interface area of ZmPDC is about 4400 Å² (corresponding to 12% of the dimer surface area), much higher than that in form B (1900 Å²; 5.1%) and form A (1300 Å²; 3.6%) ScPDC. This is consistent with small angle x-ray scattering studies, which indicated for ZmPDC a more compact tetramer in solution than that of other PDCs.7

7 S. König, D. Svergun, and M. H. J. Koch, unpublished results.

The dimer-dimer interactions in ZmPDC include many salt bridges and hydrogen bonds of both main chain and side chain atoms from all three domains. In form A ScPDC, the dimer-dimer interactions occur mainly through a small number of hydrogen bonds between main chain atoms of the R domains, whereas in form B ScPDC, the loop region 287–304 also contributes to the interface region. In ZmPDC, the orientation of the R domain is the same in all four subunits. In contrast, the orientation of this domain in yeast PDCs is different in the various crystal forms (9).

The ThDP binding site is highly similar in PDC from Z. mobilis and yeast. Most parts of the cofactor superpose well in ZmPDC and ScPDC (less than 0.6 Å shift), except for the degraded thiazolium ring in ZmPDC. In the latter case, deviations from atomic positions are larger than 1 Å. Among the 21 amino acids within van der Waals distance (<3.8 Å) to the cofactor, 18 residues are conserved in the yeast and Z. mobilis enzyme (Fig. 6), and five of these amino acids are invariant in ThDP-dependent enzymes. Asp440 and Asn467 provide ligands to the Mg(II) ion anchoring the diphosphate of ThDP to the protein, and the side chain of Glu50 forms the hydrogen bond to the N1 nitrogen atom of the pyrimidine ring, central to the mechanism of C2 deprotonation in ThDP enzymes (34, 35).

DISCUSSION

Tetramer Assembly and Substrate Activation—In contrast to other pyruvate decarboxylases, ZmPDC does not show allosteric activation by the substrate (1, 36). In ScPDC, substrate analogues such as pyruvamide (5) can act as substitutes in the activation process. Chemical modification (37) implicated cysteinyl side chains in the activation process. Crystallography (7, 8) and site-directed mutagenesis (38) subsequently suggested Cys221 of the R domain of ScPDC as the activator binding site, possibly through the formation of a covalent hemithioketal with the activator molecule. A signal transducing pathway from the R domain to the active site of the enzyme has been proposed through which the formation of this adduct could be
translated into conformational changes at the active site (8). Indeed, crystalization of yeast PDC in the presence of the activator pyruvamide (9) and ketomalonate (39), respectively, revealed large conformational changes in the enzyme, involving both loop closure at the active site and tetramer reassembly. Surprisingly, in neither study was electron density for an activator molecule found close to Cys^{-}. Despite ambiguities about which (if any) of the structures of the yeast enzyme represents the activated enzyme, it is nevertheless clear that ScPDC is able to readily undergo large conformational changes and that these changes are triggered by molecules acting as activators (7–11, 30, 39).

There is, however, a striking difference in the extent of the interface between the dimers in both forms of the yeast enzyme on the one hand and the dimers in ZmPDC on the other hand. The extensive interface region in ZmPDC, which also may account for the higher stability of ZmPDC compared with the yeast enzyme (4), makes large conformational changes during catalysis very unlikely. The only conformational changes required in ZmPDC during catalysis invoke the C-terminal helix, which has to swing out of the way to allow access of the substrate to the active site. After binding of the substrate, this helix might close the active site to create a hydrophobic environment, which would facilitate the enzymatic reaction. However, because this helix is at the surface of the enzyme, it does not need to involve other subunits. The activity of ZmPDC is higher than that of yeast PDCs by about 3-fold (1), and this difference in turnover could be related to the magnitude of the conformational changes occurring in the two enzymes during catalysis.

Active Site and Catalysis—Although the enzyme is observed in a closed form with restricted access to the active site, there is a sizeable cavity close to the thiazolium ring of the cofactor that is assumed to be the binding site for pyruvate. Modeling of the central intermediates, 2-(2-hydroxypropionyl) ThDP and 2-(1-hydroxyethyl) ThDP, respectively, taking into account stereochemical considerations as outlined by Lobell and Crout (40), shows that these reaction intermediates fit well into this pocket (Fig. 7). The substrate atoms are surrounded by a number of amino acid side chains, Asp^{27}, His^{113}, His^{114}, Tyr^{290}, Thr^{388}, and Glu^{477} (conserved residues underlined), which might be involved in substrate binding and catalysis. Based on modeling studies (40) it was suggested that Glu^{477} of ScPDC (the residue corresponding to Glu^{473} in ZmPDC) plays a key role in both pyruvate decarboxylation, leading to the intermediate 2-(1-hydroxyethyl) ThDP, and the subsequent protonation of this compound, resulting in the release of the product, acetaldehyde. In the first case Glu^{477} is thought to stabilize the dianion formed after nucleophilic attack of the thiazolium carbon on pyruvate through a hydrogen bond, which would require the side chain of Glu^{477} to be protonated (40). In ZmPDC, in addition to the side chain of Glu^{473}, the hydroxyl group of Tyr^{290} points toward the carboxyl group of 2-(2-hydroxypropionyl) ThDP and seems sufficiently close to form a hydrogen bond and contribute to the stabilization of the negative charge of the carboxyl group.

Another significant difference in the active site structure between the two enzymes involves the side chain of residue His^{114}. In ZmPDC, the imidazole ring is close to the side chain of His^{114} (3.5 Å). One of the nitrogen atoms of the imidazole ring of His^{114} forms a hydrogen bond to the side chain of Asp^{27}, and the second ring nitrogen is within hydrogen bonding distance to the side chain of Asp^{286} (Fig. 6). In the yeast enzyme, the side chain of the His^{114} (which corresponds to His^{113} in ZmPDC) points in a different direction, with a closest distance of 4.8 Å to the side chain of Asp^{286}, the residue corresponding to Asp^{27} in ZmPDC.

Recently, Schenk et al. (41) have replaced these histidines in ZmPDC with glutamine residues using site-directed mutagenesis. Whereas the mutation His^{114}→Gln has only a small influence on the $K_m$ for substrate and cofactors and shows 37% of the wild type activity, the His^{114}→Gln mutant is completely inactive in the overall reaction. A possible explanation is suggested from the conformation and environment of this residue in the active site of ZmPDC. The interactions of both nitrogen atoms of the imidazole ring of His^{114} with aspartic acid residues stabilize a positive charge on His^{114}. The close vicinity of this positive charge to the side chain of His^{114} in turn might shift the pK of this residue such that it is in its uncharged form. Because this side chain is very close to the 4-amino nitrogen of the pyrimidine ring, a positive charge on the His^{114} side chain would not be catalytically advantageous because it could interfere with the proton relay system, which abstracts the C2 proton of ThDP, a process that at some point involves a positive charge on the 4-amino group. The glutamine mutation at position 113 will remove the positive charge at His^{114} with the consequence of His^{114} being in its ionized form. This results in a positive charge close to the amino group of the pyrimidine ring and counteracts proton abstraction from the C2 atom in the first step of ThDP catalysis. This scenario is also supported by the observation that the inactive His^{114}→Gln mutant is able to release acetaldehyde from 2-(1-hydroxyethyl)-TDP (41), which indicates that the mutant is defective in one of the initial steps of catalysis.
Carboligase Activity—A side reaction catalyzed by pyruvate decarboxylases is the carboligase activity, where the activated acetaldehyde bound to ThDP is condensed to a second aldehyde molecule. This acetoin-type condensation is of considerable industrial interest, for example for the synthesis of (R)-1-hydroxy-1-phenylpropan-2-one, an intermediate in the synthesis of L-ephedrine. The carboligase activity of PDC from yeast is 20-fold higher compared with the bacterial enzyme (42); however, because of the higher stability of the latter, it would be desirable to increase the carboligase activity of ZmPDC. The differences in activity are most likely because of steric hindrance at the active site in ZmPDC. When the solvent-accessible surface is calculated for all available PDC structures with a model of the key intermediate, 2-(1-hydroxyethyl) ThDP included, an accessible cavity was found at the active site in ScPDC, which could bind the second aldehyde substrate. In ZmPDC, this cavity is filled by bulky amino acid side chains, e.g. Tyr<sup>290</sup> and Trp<sup>192</sup>. The latter has been mutated into an alanine, which results in a 5-fold increase of the carboligase activity in ZmPDC (43). Further improvement for the carboligase activity can be expected by increasing the size of this cavity, for example by replacement of the side chain of Tyr<sup>290</sup>.

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