

Mutagenesis at Asp27 of pyruvate decarboxylase from *Zymomonas mobilis* Effect on its ability to form acetoin and acetolactate

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Pyruvate decarboxylase (PDC) is one of several enzymes that require thiamin diphosphate (ThDP) and a bivalent cation as essential cofactors. The three-dimensional structure of PDC from *Zymomonas mobilis* (ZMPDC) shows that Asp27 (D27) is close to ThDP in the active site, and mutagenesis of this residue has suggested that it participates in catalysis. The normal product of the PDC reaction is acetaldehyde but it is known that the enzyme can also form acetoin as a by-product from the hydroxyethyl–ThDP reaction intermediate. This study focuses on the role of D27 in the production of acetoin and a second by-product, acetolactate. D27 in ZMPDC was altered to alanine (D27A) and this mutated protein, the wild-type, and two other previously constructed PDC mutants (D27E and D27N) were expressed and purified. Determination of the kinetic properties of D27A showed that the affinity of D27A for ThDP is decreased 30-fold, while the affinity for Mg²⁺ and the Michaelis constant for pyruvate were similar to those of the wild-type. The time-courses of their reactions were investigated. Each mutant has greatly reduced ability to produce acetaldehyde and acetoin compared with the wild-type PDC. However, the effect of these mutations on acetaldehyde production is greater than that on acetoin formation. The D27A mutant can also form acetolactate, whereas neither of the other mutants, nor the wild-type PDC, can do so. In addition, acetaldehyde formation and/or release are reversible in wild-type ZMPDC but irreversible for the mutants. The results are explained by a mechanism involving thermodynamic and geometric characteristics of the intermediates in the reaction.

Keywords: acetolactate; acetoin; pyruvate decarboxylase; site-directed mutagenesis; thiamin diphosphate.

Pyruvate decarboxylase (PDC; EC 4.1.1.1) is a thiamin diphosphate (ThDP)-dependent enzyme that catalyzes the irreversible non-oxidative decarboxylation of pyruvate to produce acetaldehyde. It also catalyzes an aldol-type condensation reaction in which the 'active acetaldehyde' intermediate bound to the enzyme is condensed with a second acetaldehyde to form acetoin. This reaction is often called the carboligase activity [1]. This condensation reaction is of considerable industrial interest for synthesis of some useful compounds such as phenylacetylcarbinol, a precursor for ephedrine and pseudo-ephedrine [2,3]. Therefore, it is very important to understand the mechanism of the reaction that produces acetoin.

Several studies have explored the carboligase activity of PDC from various species [4–8]. PDC from yeast has a higher carboligase activity than that from the bacterium *Zymomonas mobilis*, but has a lower stability. As a result, attempts have been made to increase the carboligase activity of *Z. mobilis* PDC (ZMPDC) by mutation [2,3]. Site-directed mutagenesis *in vitro* of ZMPDC has been employed to detect the roles of

various residues in cofactor binding [9–11], decarboxylase/carboligase activity and stability [5], and catalysis [12]. The mutagenic sites that have received the most attention are those that were thought to be relevant to cofactor binding, substrate activation and the natural catalytic reaction. The three-dimensional structures of several forms of yeast PDC [13–16] and ZMPDC [8] have revealed features that are involved in subunit interactions and binding of the two cofactors (ThDP and Mg²⁺) and residues that might participate in catalysis. The active-site region contains several ionizable groups of proven or potential importance (Fig. 1). One of these is the carboxy group of Asp27 (D27), which may be hydrogen-bonded to a water molecule. Two mutations at this position of ZMPDC have been carried out previously: D27E and D27N [17]. These changes either crowd the space around this region (D27E) or remove the negative charge (D27N). In each case, the specific activity of the mutant for the production of acetaldehyde is very much lower than that of wild-type PDC. However, the carboligase activity of these two mutants has not been studied.

For this carboligase reaction to occur, a second acetaldehyde molecule must enter the active site and there is a cavity that could accommodate this reactant in yeast PDC. In ZMPDC, this cavity is smaller because of the presence of more bulky amino-acid side chains [8]. Therefore, it is probable that a larger space around the active site of ZMPDC would facilitate the entrance of a second acetaldehyde molecule. Replacement of D27 in ZMPDC by alanine is a plausible substitution to increase the carboligase activity because it enlarges the space and changes the electronic microenvironment around the active site.

In this study, the D27A mutant of ZMPDC was constructed by site-directed mutagenesis *in vitro*. The basic kinetic

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Abbreviations: PDC, pyruvate decarboxylase; ThDP, thiamin diphosphate; ZMPDC, PDC from *Zymomonas mobilis*.

Enzymes: alcohol dehydrogenase (EC 1.1.1.1), lactate dehydrogenase (EC 1.1.1.27), pyruvate decarboxylase (EC 4.1.1.1).

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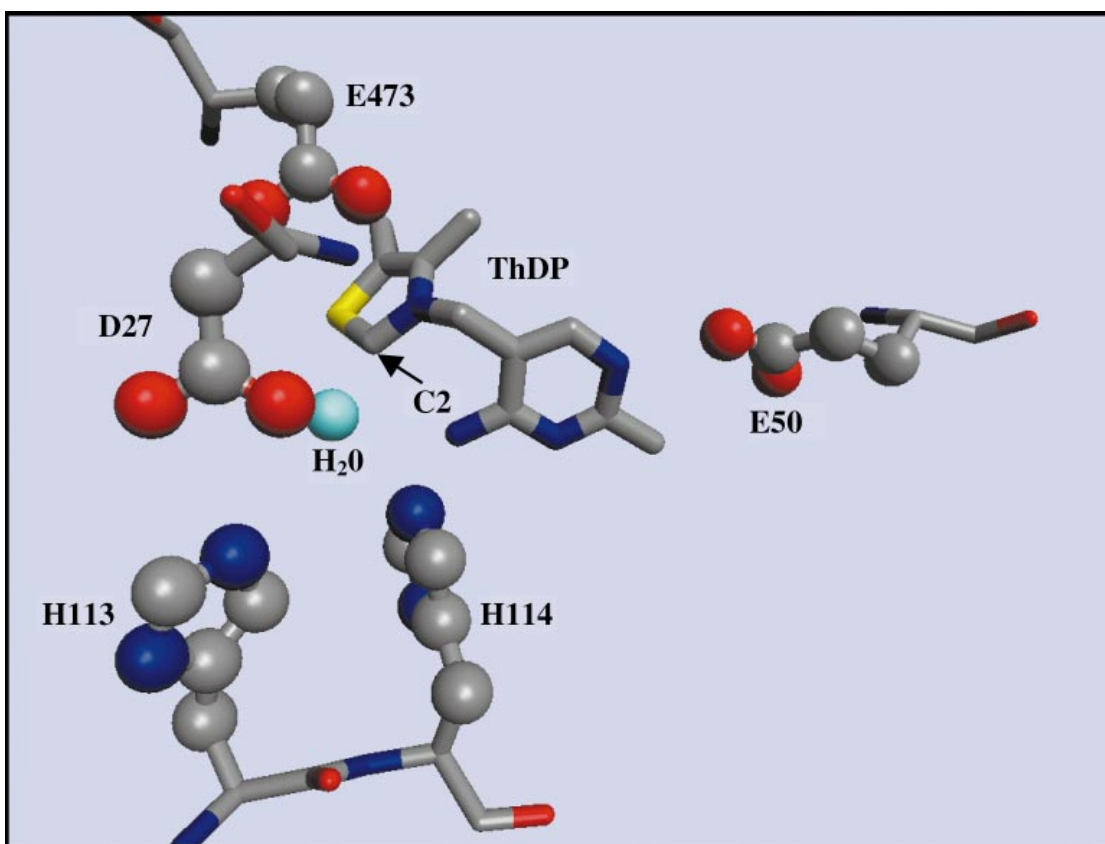


Fig. 1. Simplified representation of the active site of ZMPDC. The structure was drawn using the co-ordinates of [8] with the hydroxyethyl diphosphate side chain of ThDP, and most amino-acid residues not germane to the present study eliminated. The published structure is missing C2 of ThDP and its expected position was calculated from the positions of the remaining atoms of the thiazole ring.

properties of this mutant with respect to substrate and cofactors were measured, as were the abilities of wild-type PDC and the three D27 mutants to produce acetaldehyde, acetoin and acetolactate.

MATERIALS AND METHODS

Restriction endonucleases and molecular biology products

Restriction enzymes, T4 DNA ligase and T4 DNA polymerase were purchased from New England Biolabs (Beverly, MA, USA) and deoxyribonucleotides from Perkin-Elmer (Norwalk, CT, USA). For sequencing, we used the Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin-Elmer Applied Biosystems. Mutagenic primers and the ampicillin repair primer were synthesized by Sigma-Aldrich.

Bacterial strains and plasmids

Escherichia coli strain JM109 was from Promega (Madison, WI, USA). The plasmid pPLZM [10] was constructed by subcloning a 1.8-kb *EcoRI*–*SphI* fragment of pIDT1A, containing the entire PDC gene from *Z. mobilis*, into the expression vector pPL450 (a gift from N. E. Dixon, Research School of Chemistry, Australian National University, Canberra, Australia) [18]. This plasmid for heat-inducible PDC expression has been described previously [10]. All *E. coli* cultures containing the wild-type and the mutant constructions were maintained on Luria broth plates with 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin [19]. For long-term storage, 15% glycerol was added to liquid cultures, which

were then stored at $-70\text{ }^{\circ}\text{C}$. Plasmid DNA preparations, restriction endonuclease digestions, ligations and transformations were carried out according to standard protocols [19].

In vitro site-directed mutagenesis

The D27A mutant was constructed by using the ‘Altered Sites’ *in vitro* mutagenesis system (Promega, Sydney, Australia), as described previously [9]. The mutagenic primer was 30 bases in length with a G + C content of 67%. Two alterations from the wild-type sequence (GCGGGCGAC) spanning residues 25–27 (AGD) were introduced in this primer. One was the codon change from GAC to GCC to replace D with A at position 27 of ZMPDC. The other was a silent change from GCG to GCC at codon 25 to add an *NaeI* restriction endonuclease recognition site (GCCGGC) to facilitate the screening of transformants. An *EcoRI*–*NcoI* fragment of the PDC gene with the desired mutations was subcloned into appropriately digested pPLZM to construct the full-length mutated gene for expression. The introduction of the desired base changes was confirmed by DNA sequencing.

Expression and purification of proteins

For expression of PDC on a 2-L scale, the cells were grown in 2YT medium containing 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin at 30 $^{\circ}\text{C}$ with shaking at 300 r.p.m. When the cell culture reached an A_{600} of 0.5, the temperature was increased rapidly to 42 $^{\circ}\text{C}$ and this induction temperature was maintained for 3 h. The cells were harvested by centrifugation at 4 $^{\circ}\text{C}$ for 15 min at 2500 g and

the cell pellet was stored at -20°C . The purification of PDC was based on the protocol described previously [20] except that a Sephacryl S-300 (AMRAD Pharmacia Biotech, Melbourne, Australia) gel-filtration step on a 2×90 cm column was added as the final stage of purification. The purified enzyme was freeze-dried and stored at -20°C .

Protein analysis

During purification of PDC, SDS/PAGE was performed to estimate the purity of various PDC fractions obtained in the chromatographic steps. Protein concentration was determined as described by Bradford [21], using BSA for calibration.

Assay of PDC activity

In this work, three activities of PDC were investigated: production of acetaldehyde, production of acetoin, and production of acetolactate. The activity unit (U) is defined as the amount of enzyme that catalyzes the formation of $1\ \mu\text{mol}$ product per minute. During enzyme purification and for kinetic characterization, PDC activity to produce acetaldehyde was assayed in a coupled enzyme assay at 30°C . The rate of acetaldehyde production from pyruvate (usually at a concentration of $5\ \text{mM}$) was determined in a continuous assay by measuring the oxidation of NADH in the presence of alcohol dehydrogenase as described previously [22].

Investigation of the time-course

The concentrations of pyruvate, acetaldehyde, acetoin and acetolactate were assayed in samples removed at intervals as described below. Reactions were initiated by adding PDC to the reaction mixture containing $5\ \text{mM}$ substrate (pyruvate or acetaldehyde), $5\ \text{mM}$ MgCl_2 and $0.1\ \text{mM}$ ThDP (or $1\ \text{mM}$ ThDP for D27A) in $50\ \text{mM}$ Mes buffer (pH 6.5). This mixture was incubated at 30°C for the desired period. A $30\text{-}\mu\text{L}$ sample of reaction mixture was removed and mixed with $6\ \mu\text{L}$ 4% (v/v) HCl to stop the reaction. For acetaldehyde measurements, $15\ \mu\text{L}$ of this sample was added to $350\ \mu\text{L}$ $100\ \text{mM}$ Tris/HCl (pH 9.0) containing $10\ \mu\text{L}$ $25\ \text{mM}$ NADH and $20\ \mu\text{L}$ (22 U) alcohol dehydrogenase. After incubation at 25°C for 30 min, $100\ \mu\text{L}$ of this mixture was placed in a microtiter plate, and the A_{340} measured in a Spectramax 250 microtiter plate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA). A similar protocol was used for pyruvate measurements except that the buffer was $17.5\ \text{mM}$ Tris/HCl (pH 9.0), $14\ \text{U}$ lactate dehydrogenase replaced alcohol dehydrogenase, and the incubation was at 37°C . For all reactions the amount of PDC added was sufficient to measure reactions to completion within 2–6 h.

For assay of acetoin and acetolactate, $60\ \mu\text{L}$ of the reaction mixture was mixed with $6\ \mu\text{L}$ 10% (v/v) H_2SO_4 to stop the reaction at the desired time. Acetoin production was measured by an adaptation of the colorimetric method of Westerfeld [23] by addition of $25\ \mu\text{L}$ 5% α -naphthol (freshly prepared in $4\ \text{M}$ NaOH) and $25\ \mu\text{L}$ 0.5% creatine to $27.5\ \mu\text{L}$ of the sample solution. After incubation at 30°C for 10 min, $50\ \mu\text{L}$ of the red solution was pipetted into a microtiter plate and the absorbance measured at $525\ \text{nm}$. The incubation temperature used was lower than that described originally [23] to avoid conversion of acetolactate to acetoin. Concentrations were estimated by use of a standard curve prepared with authentic acetoin, correcting for blanks that contained solvent only. Color development was not observed in control reaction mixtures from which only enzyme was omitted, whether the substrate was pyruvate or

acetaldehyde. Acetolactate was measured by converting acetolactate to acetoin in an acidic environment [24]. The sample containing 1% (v/v) H_2SO_4 was incubated at 60°C for 15 min to convert acetolactate to acetoin, which was estimated as described above. The amount of acetolactate is the difference between the amount of acetoin with and without 60°C incubation. Acetolactate gives the red pigment only if it is first converted to acetoin as described above.

Measurement of saturation curves for pyruvate and cofactors

The K_m for pyruvate was estimated by measuring the activity as a function of the concentration of substrate, which was varied over the range $0\text{--}7.5\ \text{mM}$. Cofactor saturation curves were obtained by measuring the activity of the reconstituted holoenzyme. Removal of cofactors to yield the apoenzyme was by our published procedure [20]. The apoenzyme was incubated for 15 min at 30°C with a saturating concentration of one cofactor while the concentration of the other was varied. The reaction was started by the addition of a pyruvate/NADH/alcohol dehydrogenase mixture, and the data obtained were analyzed as described below.

Data analysis

Kinetic parameters were determined by fitting the appropriate equation to the data by non-linear regression using INPLOT (GraphPad Software, San Diego, CA, USA). The substrate saturation curve was fitted by the Michaelis–Menten equation to obtain K_m values, and cofactor saturation curves were fitted by Eqn (1) to obtain the cofactor activation constant K_c , where $[C]$ is the cofactor concentration and v_0 is the residual activity with the cofactor omitted.

$$v = V_m[C]/(K_c + [C]) + v_0 \quad (1)$$

RESULTS

Mutation, expression and purification

The mutation that replaced aspartate by alanine at position 27 was confirmed by DNA sequencing and restriction digestion, and no unintended base changes were detected (data not shown). Other mutants (D27E and D27N) had been constructed previously [17]. Each of the wild-type and mutant enzymes was purified successfully. All were eluted at approximately the same position during gel-filtration chromatography, indicating that association to form tetramers was not affected by the mutations. The final products were assessed by SDS/PAGE and appeared to be close to purity (data not shown).

Kinetic properties of mutants

We have shown previously [17] that the D27N and D27E mutants have a very low specific activity (measured at a pyruvate concentration of $5\ \text{mM}$) in comparison with wild-type. D27A also showed low activity (0.117% of wild-type). The substrate saturation curve is hyperbolic (Fig. 2A) yielding a K_m for pyruvate of $0.34 \pm 0.02\ \text{mM}$ which is about half the value observed for the wild-type, and between the values measured for D27N ($0.48 \pm 0.04\ \text{mM}$) and D27E ($0.25 \pm 0.01\ \text{mM}$). The cofactor activation curves were also hyperbolic (Fig. 2B,C) and yielded an activation constant for Mg^{2+} ($9.64 \pm 0.65\ \mu\text{M}$) that is indistinguishable from that of wild-type ($8.19 \pm 0.61\ \mu\text{M}$).

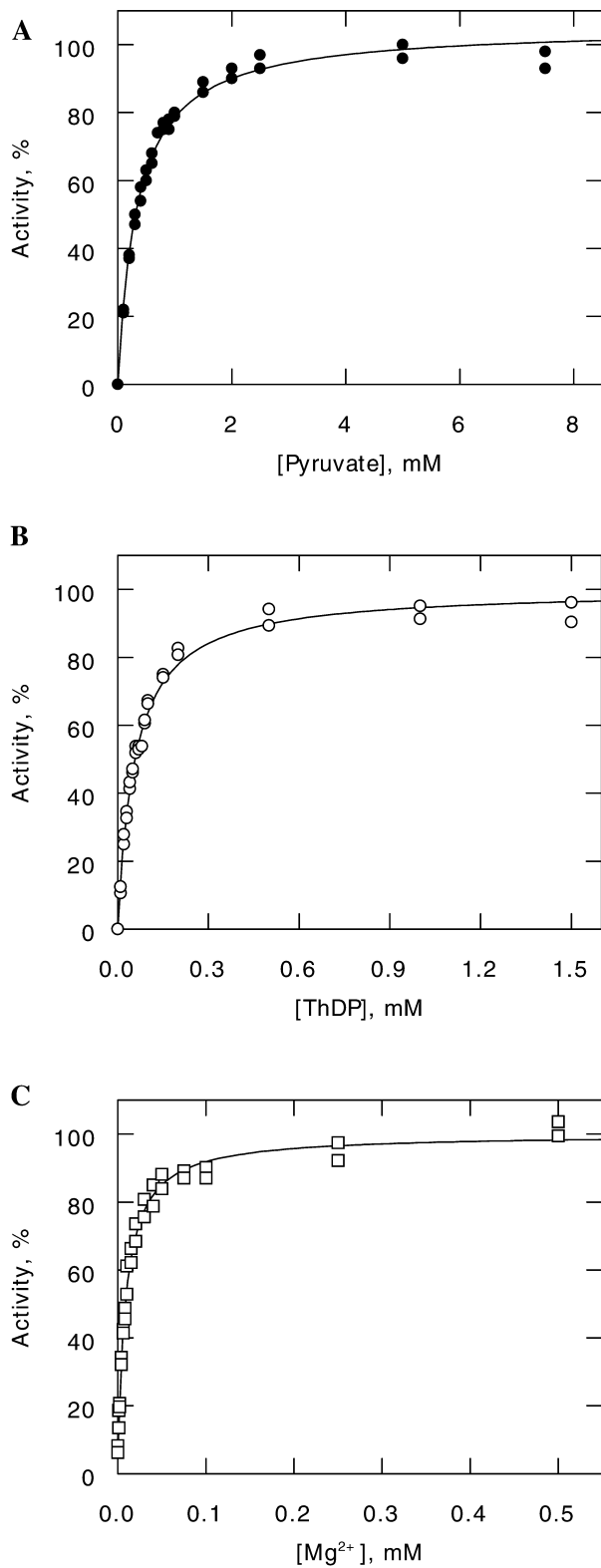


Fig. 2. Substrate and cofactor saturation curves of the D27A mutant of ZMPDC. Reactions were measured as described in the text at various concentrations of: (A) pyruvate; (B) ThDP; or (C) Mg²⁺. The curves represent the best fit to the data of the Michaelis–Menten equation (A) or Eqn (1) (B and C). Rates have been normalized to the estimated maximum velocity, which is set as 100%.

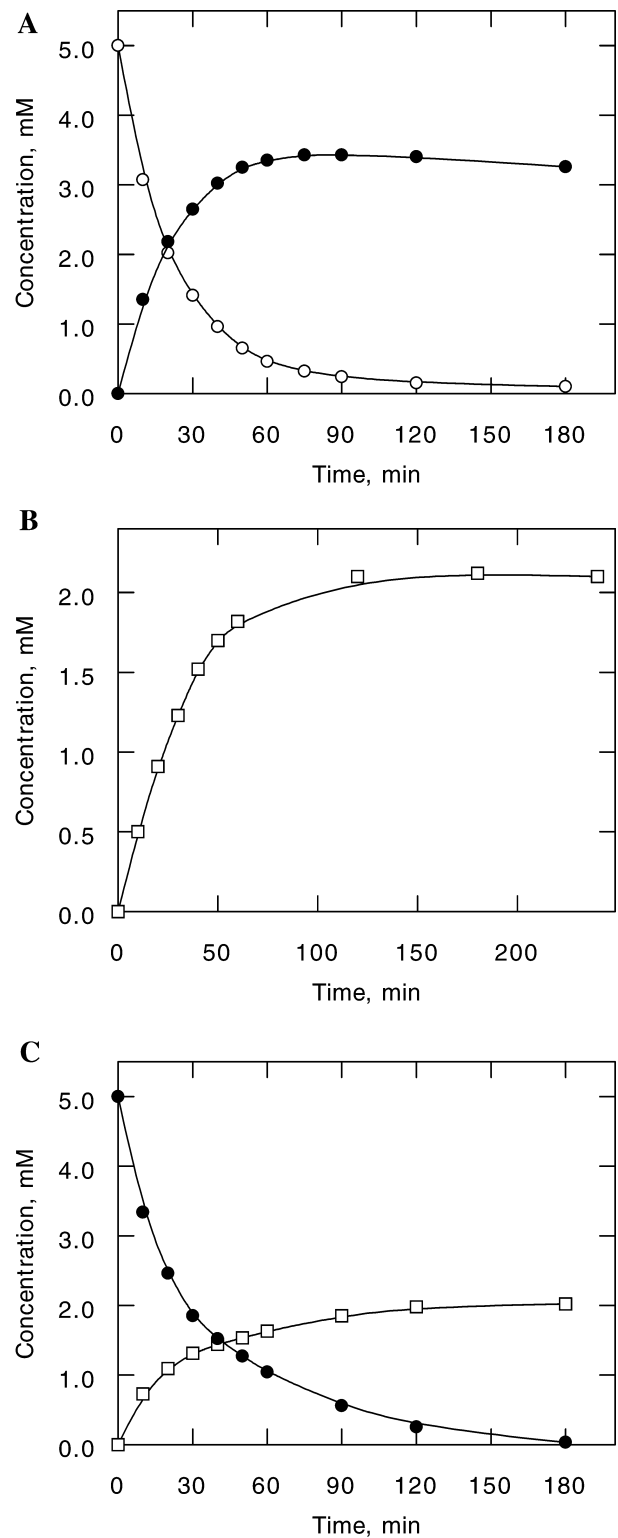


Fig. 3. Time-course of the reactions catalyzed by wild-type ZMPDC. The curves in (A) show the utilization of 5 mM pyruvate (O) and the formation of acetaldehyde (●) at an enzyme concentration of 2 $\mu\text{g}\cdot\text{mL}^{-1}$. In (B), acetoin formation (□) is shown under the same conditions as in (A), except for the use of 0.5 $\text{mg}\cdot\text{mL}^{-1}$ enzyme. In (C), 1.0 $\text{mg}\cdot\text{mL}^{-1}$ enzyme was added to start a reaction to convert 5 mM acetaldehyde (●) to acetoin (□).

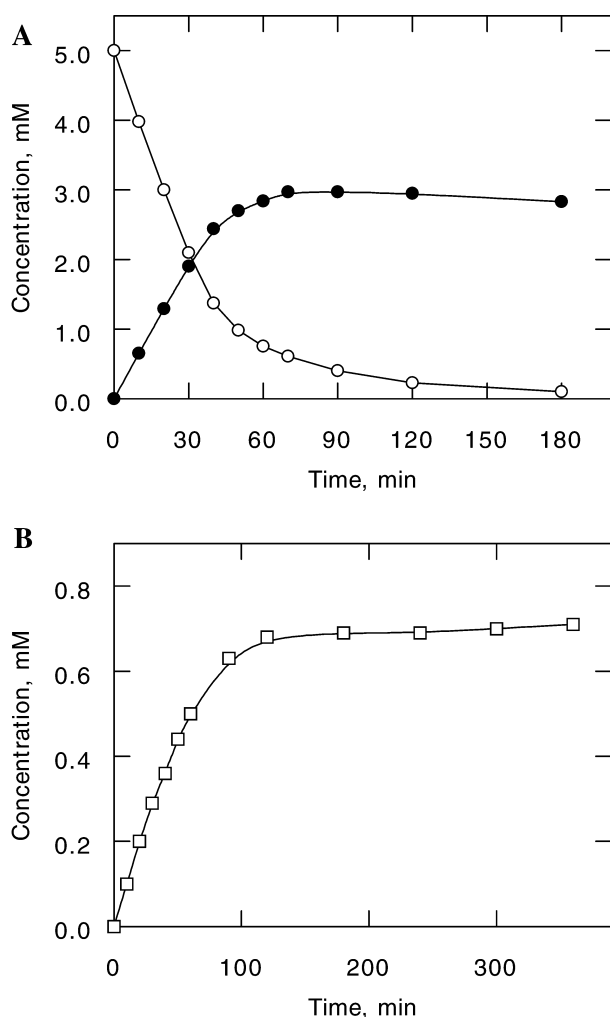


Fig. 4. Time-course of the reactions catalyzed by the D27E mutant ZMPDC. The curves in (A) show the utilization of 5 mM pyruvate (○) and the formation of acetaldehyde (●), and (B) shows acetoin formation (□). In both cases, the enzyme concentration was $1.7 \text{ mg}\cdot\text{mL}^{-1}$.

The activation constant for ThDP ($56.5 \pm 2.20 \mu\text{M}$) is elevated substantially compared with that of the wild-type ($1.97 \pm 0.15 \mu\text{M}$). Overall, the D27A mutant is generally similar to D27N and D27E, and the major difference from the

Table 1. Effect of additives on re-initiation of product formation.

Reactions were initiated by adding D27E to an assay containing 5 mM pyruvate or 5 mM acetaldehyde as the starting substrate, as shown in the first column. After the reaction had proceeded for 3 h, the addition in the second column was made. The concentration of acetoin was assayed before and after the addition and any change is noted in the third column. Similar results were obtained for D27N and D27A, except that, for the latter, acetoin increases were accompanied by increases in acetolactate concentration.

Starting substrate	Addition at 3 h	Acetoin concentration
Pyruvate	D27E	No change
Pyruvate	Pyruvate	Increase
Pyruvate	Wild-type	Increase
Acetaldehyde	Pyruvate	Increase

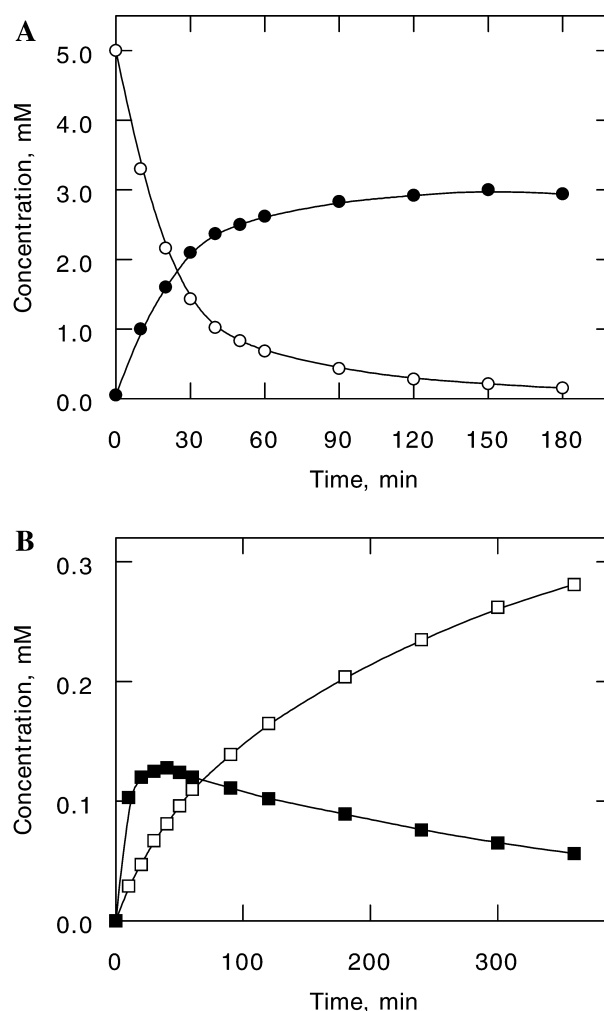


Fig. 5. Time-course of the reactions catalyzed by the D27A mutant of ZMPDC. The curves in (A) show the utilization of 5 mM pyruvate (○) and the formation of acetaldehyde (●), and (B) shows acetoin (□) and acetolactate (■) formation. In both cases, the enzyme concentration was $1.7 \text{ mg}\cdot\text{mL}^{-1}$.

wild-type is the very low specific activity. As we have noted previously [17], the simplest explanation for these observations is that D27 is involved in the decarboxylation step of the normal reaction.

Reaction time-courses for production of acetaldehyde, acetoin and acetolactate catalyzed by wild-type and mutants of PDC

The reactions catalyzed by wild-type and mutant PDC were investigated by following the conversion of 5 mM pyruvate to acetaldehyde, to acetoin and to acetolactate. With a low concentration of wild-type PDC ($2 \mu\text{g}\cdot\text{mL}^{-1}$), most of the pyruvate was consumed within 2 h, and this was mirrored by the appearance of acetaldehyde (Fig. 3A). The amount of acetaldehyde observed was less than that of pyruvate consumed, which we ascribe to the volatility of the product. The decline of acetaldehyde after 90 min is also due to loss to the atmosphere. No acetoin was detected in this experiment. However, at a 250-fold higher enzyme concentration, at which pyruvate would be converted completely to acetaldehyde within 1 min, substantial amounts of acetoin were formed (Fig. 3B).

Table 2. Comparison of the activities of PDC wild-type and mutants. For each enzyme, the specific activity in U·mg⁻¹ for production of acetaldehyde, acetoin and acetolactate is shown. The second set of values, in parentheses, is the activity expressed as a percentage of that for acetaldehyde production. Note that, for acetoin production, these activities are based on a variable concentration of acetaldehyde, initially zero but already appreciable by 10 min, the first sampling time beyond zero time.

Enzyme	Activity		
	Acetaldehyde	Acetoin	Acetolactate
Wild-type	67.5 (100)	0.1 (0.15)	0 (0)
D27E	0.038 (100)	0.006 (15.8)	0 (0)
D27N	0.032 (100)	0.0007 (2.2)	0 (0)
D27A	0.079 (100)	0.0017 (2.2)	0.0061 (7.7)

Allowing for the requirement for 2 mol pyruvate to form each mol acetoin, there was 82% conversion after 2 h. Again, the failure to achieve 100% conversion is probably due to acetaldehyde evaporation. No acetolactate was detected in these experiments. Together, these data show that wild-type PDC converts pyruvate to acetaldehyde and then this is subsequently converted to acetoin. The ability of wild-type PDC to convert acetaldehyde to acetoin was confirmed directly by experiments in which pyruvate was replaced with acetaldehyde (Fig. 3C).

The mutants D27E and D27N present a different picture, as exemplified by the former (Fig. 4). The time-courses of pyruvate utilization and acetaldehyde formation (Fig. 4A) are similar to those of the wild-type except that much more enzyme (1.7 mg·mL⁻¹) was required. With this same amount of enzyme, and on the same time-scale, significant amounts of acetoin were formed (Fig. 4B). As with wild-type, no acetolactate was detected. However, acetoin formation ceased once pyruvate was exhausted at about 2 h even though there was ample acetaldehyde present. This phenomenon was investigated further by the experiment summarized in Table 1. With pyruvate as the starting substrate, addition of further D27E after 3 h of reaction resulted in no further production of acetoin, thereby ruling out the possibility that the enzyme had simply lost its activity. However, addition of either wild-type PDC or more pyruvate resulted in the re-initiation of acetoin production. Finally, when D27E was supplied with 5 mM acetaldehyde instead of pyruvate, no acetoin production was observed until pyruvate was also added. Thus, unlike wild-type PDC, D27E and D27N appeared to be able to form detectable amounts of acetoin only by diverting the 'active acetaldehyde' intermediate of the reaction into its production. If these mutant enzymes can form this intermediate by reaction of acetaldehyde with the free enzyme, the rate is too slow to observe at an enzyme concentration of 1.7 mg·mL⁻¹.

D27A differs from the other mutants and wild-type in that it is the only one that produces acetolactate (Fig. 5), in addition to acetoin. This compound is formed in a rapid burst that ceases at about the time that pyruvate is exhausted. This cessation is entirely expected because pyruvate is a necessary reactant for acetolactate formation.

The activities of each of the enzymes with respect to formation of each of the products are summarized in Table 2. None of the mutants is as effective as the wild-type in producing acetoin. However, relative to their intrinsic activities for acetaldehyde production, and recognizing that the discontinuous assays for acetoin production give approximate

rates only, D27N and D27A are each 15 times better than the wild-type while D27E is more than 100 times better. D27A is the only PDC known to be capable of producing acetolactate, and does so about 3.5 times faster than it produces acetoin.

DISCUSSION

By observing the reaction course catalyzed by wild-type PDC, we found that complete conversion of pyruvate to acetoin could be achieved if sufficient enzyme was used. The process occurs in two phases. First, pyruvate is very quickly converted to acetaldehyde; this is then condensed with 'active acetaldehyde' in the enzyme to form acetoin. Thus PDC actually uses acetaldehyde as the substrate to form acetoin. These reactions are consistent with the scheme shown in Fig. 6. The ThDP ylide (1) reacts with pyruvate to form the lactyl-ThDP intermediate (2) which then undergoes decarboxylation to yield the equivalent resonance C2- α -carbanion (3A) and enamine (3B) adducts of ThDP, or 'active acetaldehyde'. Protonation of 3A/3B yields hydroxyethyl-ThDP (4), which releases acetaldehyde in a reversible reaction to regenerate the ThDP ylide (1). The acetaldehyde product and 3A/3B intermediate react to form acetoin via compound 5. If a large amount of enzyme is provided with acetaldehyde but no pyruvate, the enamine/C2- α -carbanion intermediate (3B/3A) is generated from the ThDP ylide (1) via hydroxyethyl-ThDP (4), and again the acetaldehyde condenses with 3A/3B to form acetoin via 5.

The D27N, D27E and D27A mutants are also capable of forming acetoin by the condensation of 3A/3B with acetaldehyde. However, at the enzyme concentrations used, these variants appear to be unable to form 3A/3B from the ThDP ylide (1) plus acetaldehyde. For example, if D27E did catalyze the direct condensation of acetaldehyde with the ThDP ylide, and the ratio of the rates of acetoin formation from acetaldehyde and from pyruvate was comparable with the same ratio for the wild-type enzyme, then it would be predicted that more than 1 mM acetoin would have been formed upon incubation of acetaldehyde with D27E (Table 1). Such an amount is at least 100-fold greater than the detection limit; in fact no acetoin was detected. This suggests that, for the mutants, protonation of 3A/3B and/or acetaldehyde release from 4 is irreversible and the variants are unable to form acetoin without provision of pyruvate.

D27A is the only PDC variant studied here that is capable of forming acetolactate, and there are two potential routes by which this may occur. The first is similar to that described for acetoin formation, with pyruvate replacing acetaldehyde in the conversion of 3A/3B to 5. The second is a reaction of 2 with acetaldehyde to give an intermediate similar to 5 which would then release acetolactate. The second route would be unlikely chemistry and is inconsistent with the observed early burst of acetolactate formation (Fig. 4); this route would predict that there would be a lag in acetolactate production while acetaldehyde accumulated to compete effectively with lactyl-ThDP decarboxylation.

We have proposed previously [17] that the main effect of D27 mutations is to reduce the rate of the decarboxylation step and this hypothesis can now be refined. If one of the functions of D27 were to stabilize the resonance structures 3A and 3B, mutations would have two effects. First, they would make these intermediates more difficult to form from lactyl-ThDP, thereby slowing the decarboxylation step as deduced earlier. Secondly, they would make it more difficult to form the resonance enamine/2C- α -carbanion intermediates 3B/3A by reaction of enzyme-bound ThDP with acetaldehyde; this is one of the

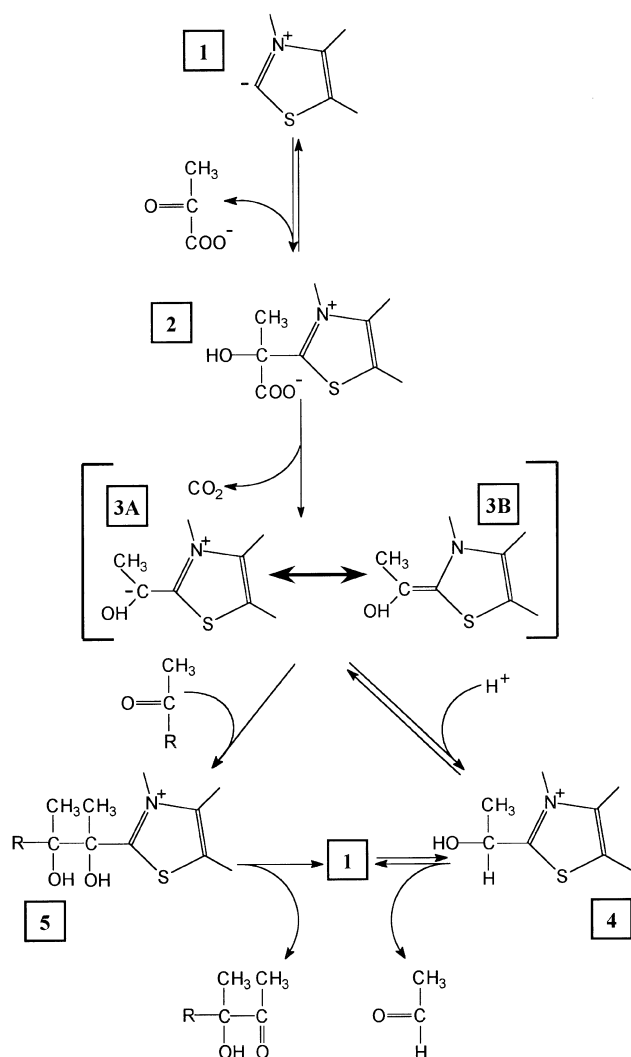


Fig. 6. Proposed reaction mechanism for conversion of pyruvate to acetaldehyde, acetoin and acetolactate catalyzed by PDC. The reaction starts with the ylide form of ThDP (1) which reacts with pyruvate to form lactyl-ThDP (2). This then undergoes decarboxylation yielding the 'active acetaldehyde' intermediate, shown as the resonating C2- α -carbanion (3A) and enamine (3B) adducts of ThDP. Further reactions of 3A/3B are able to proceed by alternative routes. In the normal PDC reaction, protonation yields hydroxyethyl-ThDP (4) which releases acetaldehyde to regenerate the ThDP ylide (1). In the carboligase reaction, condensation with acetaldehyde ($\text{R} = \text{H}$) or pyruvate ($\text{R} = \text{COO}^-$) yields the intermediate (5) which then releases acetoin or acetolactate, respectively. This diagram is adapted from Jordan [25]. Only the thiazole ring of ThDP is shown.

results observed here. Another consequence of mutations of D27 could be to slow the protonation of 3A/3B to yield hydroxyethyl-ThDP, thereby allowing this intermediate to accumulate and favoring reaction of acetaldehyde with 3A/3B to form acetoin. This consequence would be consistent with the observation that the rate of acetaldehyde production is slowed by the mutations relatively more than is the rate of acetoin production.

In the course of determining the carboligase activities of the wild-type and three mutants, we have found that D27A can catalyze production not only of acetoin but also of acetolactate. Moreover, this activity is about 3.5 times as high as that for acetoin formation (Table 2). This result can be rationalized by the facts that this mutation results in the largest space around

this residue, and the reaction of pyruvate with 3A/3B requires the enzyme to accommodate a substrate that is larger than acetaldehyde by the presence of a carboxy group. One might also anticipate that the relaxation of steric constraints might also make D27A more able to accept acetaldehyde to form acetoin, but this is not observed. For example, in comparison with both the absolute and relative acetoin formation activity of D27E, D27A is substantially less effective. It may be significant that, of the wild-type, D27E and D27N, the last of these is least active in acetoin formation and is the only one that lacks a carboxy group at this position. Possibly the carboxy group of pyruvate acts as a substitute in D27A, thereby allowing this substrate to bind and acetolactate to be formed.

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