Eur. J. Biochem. *257*, 538−546 (1998)

Active site mutants of pyruvate decarboxylase from *Zymomonas mobilis*
A site-directed mutagenesis study of L112, I472, I476, E473 and N482

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(Received 18 May/6 August 1998) − EJB 98 0670/4

The homotetrameric pyruvate decarboxylase (PDC) from *Zymomonas mobilis* requires the cofactors thiamin diphosphate and Mg^{2+} for catalytic activity. We have investigated the role of various amino acid residues in the direct environment of the active site. The role of residue E473 in the catalytic activity and stability of the enzyme was probed by several mutations. All mutant enzymes were either inactive or failed to give any recombinant protein. The close interaction of E473 and N482, which can be deduced from the X-ray structure, has been probed by mutagenesis of N482 to D. This mutation has a significant influence especially on the carboligation reaction of PDC, whereas the binding of the cofactors and the thermostability were not affected. These data suggest a specific interaction of N482 and E473 which is essential for coordinating the second aldehyde molecule during carboligation.

Three hydrophobic residues (L112, I472 and I476) in the vicinity of the active centre have been investigated with respect to their potential influence on the transition states during catalysis. In contrast to L112, I472 and I476 influence the decarboxylation and carboligation reactions. The enlarged substrate-binding site of PDC*I472A* allows the decarboxylation of longer aliphatic 2-keto acids (C_4-C_6) as well as aromatic 2-keto acids besides pyruvate. Carboligations using PDC*I472A* as a catalyst yielded 2-hydroxypropiophenone, benzoin and phenylacetylcarbinol. The enantioselectivity of PAC formation is impaired by mutations of both I472 and I476. The stereochemistry is most significantly affected with the mutant enzyme PDC*I476E*, which catalyses predominantly the synthesis of (*S*)-phenylacetylcarbinol.

Keywords: 2-hydroxy ketone; pyruvate decarboxylase; site-directed mutagenesis; thiamin diphosphate; phenylacetylcarbinol.

In addition to the decarboxylation of 2-keto acids, pyruvate decarboxylases (PDC) catalyse the enantioselective formation of 2-hydroxy ketones, and the mechanisms of both reaction pathways have been investigated intensively [1−4]. The reaction cycle of thiamin-diphosphate (ThDP)-dependent decarboxylation of pyruvate and subsequent carboligation with acetaldehyde or benzaldehyde as cosubstrates is depicted in Fig. 1. It is started by the ThDP carbanion (structure 1 in Fig. 1) attacking the 2-keto group of pyruvate forming 2-lactyl-ThDP (structure 2 in Fig. 1). This double negatively charged species is stabilised by decarboxylation yielding a carbanion/enamine ('active aldehyde'; structure 3 in Fig. 1), which may either be protonated to give hydroxyethyl-ThDP (structure 4 in Fig. 1) followed by the release of acetaldehyde, or react with an aldehyde cosubstrate yielding 2-hydroxy ketones (structure 5 in Fig. 1). The release of acetaldehyde is a reversible reaction; thus, hydroxyethyl-ThDP can also be formed by addition of acetaldehyde to enzyme-bond ThDP.

Three crystal structures of PDC from yeast have been described [5−7]. Each monomer of the tetrameric enzyme consists of three domains. Whereas the first domain of one subunit and the third domain of the second subunit (and *vice versa*) form one active centre, the second domain participates in the interaction between the dimeric halves and contains the allosteric control site in yeast PDC.

The wall of the cavity leading to the active centre of PDC is lined with mostly conserved hydrophobic and hydrophilic residues (Table 1). Among these are D28, H113 and H114, which have recently been the target of site-directed mutagenesis studies [8, 9]. A further important hydrophilic residue in the vicinity of C2-ThDP is E473 (corresponding to E477 in yeast PDC), which is suggested to be involved in the various protonation and deprotonation steps during the decarboxylation reaction [1, 10−12]. Moreover, an involvement of this conserved glutamate residue in the coordination of a second aldehyde during the carboligation leading to chiral 2-hydroxy ketones was discussed [13]. E477 in pyruvate decarboxylase from *Saccharomyces cerevisiae* (PDC*S.c.*) has been exchanged for D, which led to a total loss of activity (< 1 %) [8]. In the present study, mutants of pyruvate decarboxylase from *Zymomonas mobilis* (PDC*Z.m.*) with A, N, T, D, I and V in position 473 have been generated to further investigate the function of E473.

The crystal structure of yeast PDC reveals that the oxygen of the amide side chain of N488 (N482 in PDC*Z.m.*) is 0.29 nm

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Abbreviations. BFD, benzoylformate decarboxylase; ee, enantiomeric excess; 2-HPP, 2-hydroxypropiophenone (2-hydroxy-1-phenyl-propane-1-one); PAC, phenylacetylcarbinol (1-hydroxy-1-phenyl-propane-2-one); PDC, pyruvate decarboxylase; PDC*Z.m.*, pyruvate decarboxylase from *Zymomonas mobilis*, PDC*S.c.*, pyruvate decarboxylase from *Saccharomyces cerevisiae*; ThDP, thiamin diphosphate; wt, wild-type.

Enzymes. Alcohol dehydrogenase (EC 1.1.1.1); benzoylformate decarboxylase (EC 4.1.1.7); indole-3-pyruvate decarboxylase (EC 4.1.1.74); pyruvate decarboxylase (EC 4.1.1.1).

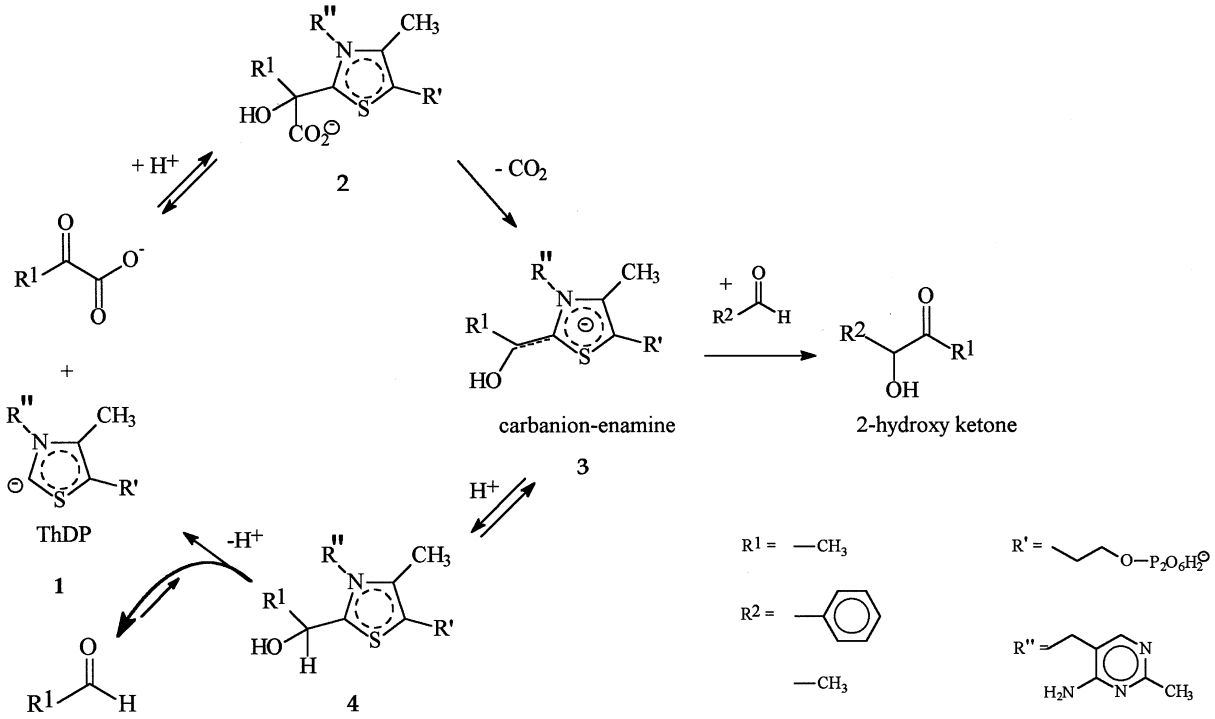


Fig. 1. Reaction cycle of PDC: decarboxylation of pyruvate to acetaldehyde and carboligation to acetoin and phenylacetylcarbinol. See text for details.

Table 1. Comparison of amino acid residues lining the cavity leading to the active centre of PDC. Residues mutated are underlined. n.d., not determined.

	PDC*S.c.*	PDC*Z.m.*	Function	References
Hydrophobic residues	I480	I476	stability enantioselectivity	this study
	I476	I472	substrate range enantioselectivity	this study
	F393	F393	n.d.	
	V410	M410	n.d.	
	T388	T388	n.d.	
	A392	W392	carboligase activity	[16, 29, 31]
Hydrophilic residues	D28	D27	catalytic activity	[8]
	H114	H113	cofactor binding	[9]
	H115	H114	cofactor binding	[9]
	E477	E473	catalytic activity	this study, [8]

distant to the carboxylic side chain of the mechanistically important E477 (E473 in PDC*Z.m.*). This configuration seems to be stabilised by hydrogen bonds formed by neighbouring amino acids, and therefore the pK_a of E477 (E473 in PDC*Z.m.*) is probably increased. To investigate whether this interaction is essential for the correct function of E473, N482 in PDC*Z.m.* was exchanged for A, S and D.

Three conserved hydrophobic residues (L112, I472 and I476; Fig. 2) are located close to C2-ThDP and thus may interact with transition states during catalysis. The side chains of I472 and I476 in PDC*Z.m.* (corresponding to I476 and I480 in yeast PDC) are about 0.5 nm and 0.8 nm from the active centre, respectively. Thus, these isoleucine residues are likely to influence the substrate range of the decarboxylation reaction and the enantioselectivity of the ligation reaction. This thesis has been tested with a set of mutants by varying the side-chain size.

L112 is located in a flexible loop which could not be resolved in the X-ray structures of yeast PDC [5–7]. Since the subsequent histidine residues at positions 113 and 114 are close to ThDP, a steric influence of L112 has been discussed [9]. Like the histidine residues this leucine residue is conserved in all pyruvate decarboxylases, except that of *Aspergillus parasiticus* (Fig. 2). To examine for a possible interaction during catalysis, L112 was exchanged for alanine.

The enzymes have been characterised with respect to the decarboxylation of 2-keto acids, predominantly pyruvate, the kinetic constants of the decarboxylation reaction, their thermostability and their ability to bind cofactor. The conformational stability has been studied by stepwise unfolding in urea, and the carboligation was investigated with respect to the ligation products using acetaldehyde and benzaldehyde as educts.

EXPERIMENTAL PROCEDURES

Construction of the expression vector pPDC-His$_6$. For the construction of the C-terminal hexa-histidine fusion proteins the

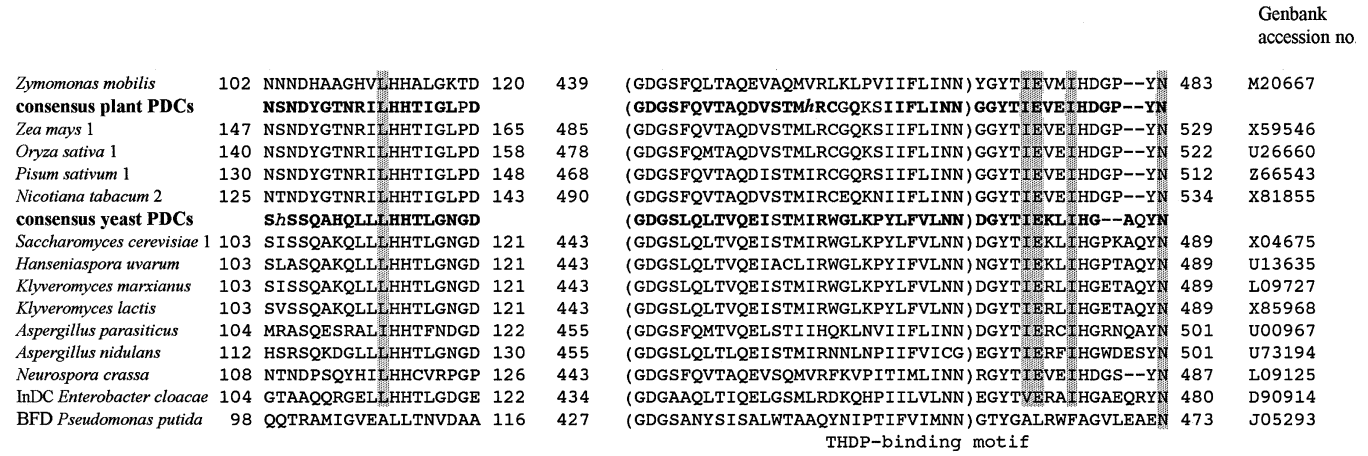


Fig. 2. Partial sequence alignment of various ThDP-dependent enzymes. *h*: L, V or I. Conserved amino acid residues mutagenised in this study are marked in grey. The ThDP-binding motif is given in parentheses [31]. InDC, indole-3-pyruvate decarboxylase; BFD, benzoylformate decarboxylase.

pQE60 vector (Qiagen) was used. In order to ligate the PDC gene to pQE60, two new restriction sites were introduced into the PDC gene by PCR with pPDC as a template [14]. pPDC was constructed from pBTac2 (Boehringer) and the PDC gene from the original plasmid pZY134b [15, 16]. At the 5′-end an *Nco*I site was introduced using the primer 5′-GAAACAGAAT**CCATGG**AGTAA-3′, and at the 3′-end of the PDC gene the stop codon was removed and a *Bam*HI site was introduced using the primer 5′-ACATGC**GGATCC**GAGGAGCTTGTTAACAG-GC-3′.

Primers were synthesised using an oligonucleotide synthesiser (Pharmacia Biotech). Following cleavage from the column and purification using a NAP column (Pharmacia Biotech) the oligonucleotides were used as primers for PCR and the resulting modified PDC gene was ligated into the vector pUC18 using the Sure-Clone-Kit (Pharmacia Biotech). After amplification of the modified PDC gene in *Escherichia coli* XL1Blue and preparation of the vector DNA, the vector pUC18/PDC was restricted with *Nco*I and *Bam*HI and the PDC-fragment was ligated into the vector pQE60. The vector was restricted with *Nco*I and *Bgl*II instead of *Bam*HI, because the *Nco*I and *Bam*HI restriction sites are located side by side in pQE60 and thus may not be co-digested. Since PDC could not be overexpressed in this vector, the resulting vector pQE60/PDC-His_6 was restricted with *Eco*RV and *Hin*dIII in order to transfer the PDC fragment 882−1733, which includes the codons for six histidine residues, into the likewise restricted expression vector pPDC. The resulting vector was called pPDC-His_6.

Construction of mutants. All point mutations were introduced by PCR using the overlap extention technique [17]. Fragments carrying the mutation and flanked by appropriate restriction sites were generated, restricted and cloned into the expression vector pPDC-His_6. The following restriction sites in pPDC-His_6 were used: *Eco*RI (upstream ATG), *Bss*HII (bp 660), *Eco*RV (bp 889), and *Stu*I (bp 1524).

Mutations of I472, E473, I476 and N482 were introduced into an *Eco*RV/*Hin*dIII fragment. All fragments were amplified using the following outer primers: (s) 5′-GGTTGGACG**GATAT**CCCTGATCC-3′ (*Eco*RV site in bold face) and (as) 5′-AG-T**AAGCTT**CTAGAGGAGCTTGTTAAC-3′ (*Hin*dIII site in bold face; stop codon underlined).

The I472 (codon 1414−1416) mutagenesis primers were 5′-GGTTACACC**BYY**GGAGTTATGA-3′ (s) and 5′-TCATAA-CTTC**SKT**GGTGTAACC-3′ (as). Clones encoding A, L, F, S and C were identified by sequencing.

The E473 (codon 1417−1419) mutagenesis primers were 5′-TACACCATC**RHT**GTTATGATCCAT-3′ (s) and 5′-GGATCA-TAAC**ADY**GATGGTGTAAC-3′ (as). Clones encoding D, N, T, A, I and V were identified by sequencing.

The I476 (codon 1426−1428) mutagenesis primers were 5′-CGAAGTTATG**SHR**CATGATGGTCCGT-3′ (s) and 5′-ACC-GACCATCATG**YDS**CATAACTTCG-3′ (as). Clones encoding L, V, A and E were identified by sequencing.

The N482 (codon 1444−1446) mutagenesis primers were 5′-GGTCCGTAC**GMM**AACATCAAGA-3′ (s) and 5′-TCTTG-ATGTT**KKC**GTACGGACC-3′ (as).

Clones encoding D, E; S and A were identified by sequencing.

The resulting PCR fragments were subcloned in pUC18 for sequencing, subsequently digested with *Eco*RV and *Stu*I and ligated into the likewise restricted vector pPDC-His_6.

The mutation of L112 (codon 334−336) was introduced into an *Eco*RI/*Eco*RV fragment by overlap extention using the following outer primers:

5′-GCAGCGCGCAGGCAGCTGCCGACG-3′ (s) and 5′-ACGCGGTTCAGCGAGAACCAGT-3′ (as), and the following mutagenesis primers: 5′-GGTCATGTG**GCG**CATCACGC-3′ (s) and 5′-AGCGTGATG**CGC**CACATGACC-3′ (as). Both strands of the complete mutant genes were sequenced using an A.L.F.-DNA-Sequencer (Pharmacia Biotech).

Expression of His-tagged PDC mutants. *E. coli* SG13009 prep4 cells (Stratagene) were transformed with the pPDC-His_6 vectors carrying the mutations. Cells were grown in Luria-Bertani medium [18] in presence of 100 μg/ml ampicillin and 25 μg/ml neomycin at 37 °C until an optical density (595 nm) of 0.6−0.8 was reached. Induction of protein expression was performed by addition of 1 mM isopropylthio-β-D-galactoside. Cells were harvested after a further 20 h by centrifugation and disrupted by mixing with glass beads (∅0.3 mm). All steps were performed in 50 mM Mes/KOH, pH 6.5, containing 5 mM $MgSO_4$ and 0.1 mM ThDP.

SDS/PAGE and Western-blot hybridisation. About 20−25% of the soluble cell protein was identified as recombinant proteins by SDS/PAGE (12.5%) [19]. The proteins were blotted onto a nitrocellulose membrane (Roth) according to the protocol

of Kyhse-Anderson [20] and detected with polyclonal rabbit anti-PDC Ig using an ECl detection kit (Amersham).

Purification of PDC mutants on Ni-NTA resin. The crude extract was applied to a Ni-NTA column (Qiagen; bed volume, 3 ml). The column was equilibrated with 50 ml 50 mM Mes/KOH, pH 6.8. The same buffer was used to remove unbound proteins in the first washing step. Subsequently, weakly bound proteins were eluted with 50 mM Mes and 20 mM imidazole, pH 7.0. The elution of the His-tagged enzyme was achieved by 250 mM imidazole in 50 mM Mes, pH 7.6. The eluted enzyme was free of ThDP. A desalting step was performed to remove imidazole using a PD10 column (Pharmacia Biotech), which was equilibrated with 10 mM Mes/KOH, pH 6.5, in the presence or absence of 5 mM $MgSO_4$ and 0.1 mM ThDP, depending on the further purpose. The enzyme samples were lyophilised and stored at −20 °C. Prior to use, the samples were reconstituted with 50 mM Mes/KOH buffer, pH 6.5, containing 5 mM $MgSO_4$ and 0.1 mM ThDP. Purified enzymes were used for kinetic and stability investigations and for biotransformations on an analytical scale.

Protein concentration. Determination of protein concentration was performed according to Bradford [21] using BSA for calibration.

Assay of decarboxylase activity. The decarboxylation of pyruvate was studied using a coupled enzymatic test as described elsewere [22]. One unit is defined as the amount of enzyme that catalyses the decarboxylation of 1 μmol pyruvate per minute at pH 6.5 and 30 °C.

For investigation of the substrate range the assay mixture contained the following components: 500 μl 2-keto acid (sodium salt; 40 mM), 100 μl NADH (2.5 mg/ml), 300 μl 50 mM Mes/KOH, pH 6.5, 5 mM $MgSO_4$ and 0.1 mM ThDP, 50 μl horse liver ADH (5 mg/ml; Aldrich), and 50 μl PDC solution. To analyse kinetic data, initial enzymatic activities were measured in the range of 0.1−30 mM pyruvate or 0.1−50 mM of other 2-keto acids. K_m and V_{max} values were calculated by a non-linear fitting of the hyperbolic curves according to Michaelis-Menten using the program Origin (Microcal).

Urea-induced denaturation. The stepwise unfolding of PDC using urea as a denaturant is described elsewhere [22]. The shift of the tryptophan emission maximum from 333−335 nm (native enzyme) to 350 nm (unfolded protein) was taken as an indication for stepwise denaturation.

Stability of the holoenzymes in cofactor-free buffer. The stability of the holoenzymes was studied by incubating 0.1 mg/ml PDC or mutant PDC in 50 mM Mes/KOH, pH 6.5, for 20 h at 25 °C. To follow the time-course of inactivation, 50-μl samples were removed and used in the photometric assay. To measure residual activity, cofactors were ommitted from the assay buffer.

Thermostability. The thermostability of wild-type (wt) PDC and the mutant enzymes was measured as described elsewhere [23].

Analysis of acetoin. For quantitation of acetoin, the colorimetric method of Westerfield [24] was applied. The assay was performed in microtitre plates. One unit of PDC and 16.9 mM pyruvate were incubated for 1 h at 37 °C. The reaction was stopped by heating the sample at 94 °C for 2 min.

Subsequently, 100 μl of each sample was mixed with 30 μl 0.5 % creatine, 20 μl α-naphthol (5 % in ethanol) and 30 μl 40 % KOH on a microtitre plate. After incubation (20 min), the concentration of the resulting red complex was spectroscopically detected at 563 nm using a microtitre plate reader (Thermomax Microplate Reader, MWG-Biotech). The assay was calibrated in the range of 0−1 mM acetoin (Fluka).

Quantitation and chiral detection of acetoin was performed by gas chromatography on a chiral column as described previously [16] with the column temperature set to 80 °C. The retention times were 7.9 min ((*R*)-acetoin) and 9.0 min. ((*S*)-acetoin). Both enantiomers were identified using polarimetry (Perkin Elmer) based on a specific rotation of $[\alpha]_D^{25} + 82 \pm 3°$ for (*S*)-acetoin (according to Crout and Morrey [25]).

Enzymatic synthesis and analysis of PAC, 2-HPP and benzoin. Ten to 50 μg wt PDC or PDC mutant was incubated in 0.5 ml 50 mM Mes/KOH, pH 6.5, containing 5 mM $MgSO_4$ and 0.1 mM ThDP, in the presence of 35 mM pyruvate and 70 mM benzaldehyde for 1−24 h at 30 °C. The pH of the reaction mixture was constant during the reaction time. After heat-inactivation of the enzymes, the amounts of PAC, 2-HPP and benzoin were measured using an analytical HPLC system (Gynkotek) equipped with an ultraviolet monitor (263 nm). Separation was performed on a C18-Hypersil ODS 5 μm column (250 mm×4.6 mm) using 0.5 %/20 % acetic acid/acetonitrile (by vol.) as eluent. The flow rate was 1.5 ml/min. The retention times were: PAC, 10.5 min; 2-HPP, 12.8 min; and benzoin, 77.6 min. The enantiomeric excess of PAC and 2-HPP was investigated by gas chromatography on a chiral column according to Bruhn et al. [16] using a column temperature of 110 °C. The enantiomeric excess of benzoin was analysed by chiral HPLC at 254 nm using a Chiracel-OB column (DIACEL) and 90 % heptane/10 % 2-propanol as eluent (flow rate, 1 ml/min). Racemic benzoin (Aldrich) was used as a standard. The retention times were: (*S*)-benzoin, 13.5 min and (*R*)-benzoin, 16.0 min. Identification of (*R*)-benzoin obtained from the enzymatic reaction was performed using ^{1}H-NMR spectroscopy.

RESULTS

All mutants were expressed as C-terminal hexahistidine fusion proteins. The hexahistidine tag has no negative influences on the enzymatic properties, which was tested by comparative studies using wt PDC*Z.m.* and the mutant PDC*W392A* in the presence and absence of a His tag [14].

Probing the role of E473 and N482. The crucial role of E477 in yeast PDC (corresponding to E473 in PDC*Z.m.*) has been established by kinetic and modelling studies [1, 10−12]. These studies suggest that the highly conserved glutamate residue is involved in the stabilisation of species 2 in Fig. 1 as well as in coordinating the aldehyde cosubstrate during carboligation. For both tasks the glutamate side chain has to be protonated. Furthermore, Lobell and Crout suggest that this glutamate is involved in the protonation of the carbanion-enamine (species 3 in Fig. 1) prior to the release of acetaldehyde via a bound water molecule [11, 12].

In the present study, mutants of PDC*Z.m.* with D, N, T, A, I and V in position 473 have been generated. The mutants with D and N in position 473 showed normal expression (20 % of the soluble cell protein) but no catalytic activity (Table 2). Only week expression (< 2 %) could be detected for the threonine and alanine mutants. By contrast, no expression was found in *E. coli* with mutants containing I or V at position 473 using Western-blot techniques (data not shown). These results demonstrate the mechanistic and structural importance of this conserved glutamate residue.

The importance of the interaction between the side chains of N482 and E473, which has been deduced from the crystal structure of yeast PDC, has been studied by mutation of N482 to D, A and S. Only the mutant enzyme PDC*N482D* could be obtained in sufficient amounts for further characterisation. The mutation

Table 2. Activities and stabilities of various mutant enzymes of PDC*Z.m.* n.d., not determined.

Enzyme	K_m pyruvate	Decarboxylation V_{max}	Carboligation PAC	PAC ee	Carboligation acetoin	Thermostability T_m	$t_{1/2}$ in cofactor-free buffer	Dissociation midpoint of the tetramer [urea]
	mM	U/mg	%			°C	min	M
wt PDC	1.1 ± 0.1	120−150	100	>98 (*R*)	100	64	>1200	3.5
PDC*L112A*	1.1 ± 0.1	50−55	100	>98 (*R*)	100	53	>1200	n.d.
PDC*I472A*	9.1 ± 0.17	50−60	60	70 (*R*)	100	n.d.	>1200	3.0
PDC*E473N*	n.d.	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PDC*E473D*	n.d.	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PDC*I476L*	4.7 ± 0.2	80−90	90	95 (*R*)	60	60	<60	3.0
PDC*I476V*	8.9 ± 0.2	30−35	120	95 (*R*)	36	58	<10	3.2
PDC*I476A*	6.8 ± 0.15	0.7	4	95 (*R*)	0.07	52	<10	2.6
PDC*I476E*	n.d.	1−2	6	60 (*S*)	0.1	50	n.d.	2.2
PDC*N482D*	3.4 ± 0.17	7−12	4	95 (*R*)	0.07	62	960	1.8

reduced the decarboxylase activity of the mutant enzyme to 10 % and the K_m value for pyruvate is about three times higher than that of the wt enzyme. In contrast to other mutants of PDC*Z.m.* with low catalytic activity, the thermostability and cofactor binding of PDC*N482D* was not affected (Table 2). However, the mutant enzyme is sensible towards urea (Table 2). These data suggest that the overall structure and the amino acids involved in cofactor binding are not significantly affected by the mutation. Most remarkably, the potential of the mutant PDC*N482D* to catalyse the formation of 2-hydroxy ketones is strongly impaired. Less than 0.1 % of the acetoin, and only 4 % of (*R*)-PAC are synthesised by the mutant enzyme relative to wt PDC (Table 2). These results suggest a significant interaction of N482 with a mechanistically important amino acid, probably E473, in PDC*Z.m.*

Probing steric influences of hydrophobic side chains. *Mutation of L112.* L112 was mutated to alanine in order to study the influence of this side chain on cofactor binding and the decarboxylation and carboligation reaction. The resulting mutein showed normal expression behaviour in *E. coli*; its kinetic data are comparable to those of the wt enzyme and even the carboligation reaction was not influenced (Table 2). The high enantiomeric excess of (*R*)-PAC (>98 %) synthesised by the mutant enzyme demonstrates that the stereochemistry of the carboligation was not influenced by the mutation. In order to study whether the active centre of the mutant enzyme is open for sterically more demanding 2-keto acids, the substrate spectrum of PDC*L112A* was studied. The data (Table 3) show that the substrate range of the mutant does not differ from that of the wt enzyme, and therefore a direct participation of L112 in the active site can be excluded.

Mutation of I472. According to the X-ray structure of yeast PDC, the side chain of I476 (corresponding to I472 in PDC*Z.m.*) has a distance of only 0.5 nm to C2-ThDP. Thus, an interaction of this side chain with ThDP-bound intermediates during catalysis is likely. This idea has recently been supported by modelling studies [11, 13]. These authors suggest that this isoleucine residue is responsible for the strict stereo control during carboligation of acetaldehyde with aromatic aldehydes (e.g. PAC), in such a way that one site of the carbanion-enamine is protected by the isoleucine side-chain during the ligation step.

Table 3. Comparison of the substrate ranges of the decarboxylation reaction of PDC*I472A*, PDC*L112A* and wt PDC*Z.m.* n.d., not determined.

	PDC*I472A* activity	PDC*L112A* activity	wt-PDC activity
	%		
Linear 2-keto acids			
2-Keto-propanoic acid (pyruvate)	100	100	100
2-Keto-butanoic acid	120	77	70
2-Keto-pentanoic acid	108	15	11
2-Keto-hexanoic acid	80	3.4	< 1
C3-branched 2-keto acids			
2-Keto-3-methylbutanoic acid	< 0.1	n.d.	< 0.1
2-Keto-3-methylpentanoic acid	0.3	n.d.	0
2-Keto-3,3-dimethylpentanoic acid	0	n.d.	0
C4-branched 2-keto acids			
2-Keto-4-methylpentanoic acid	1.8	n.d.	0.3
2-Keto-4,4-dimethylpentanoic acid	0	n.d.	0
2-Keto-4-methylhexanoic acid	3	n.d.	0
Cyclic and aromatic 2-keto acid			
2-Keto-3-cyclohexylpropanoic acid	0	n.d.	0
2-Keto-3-phenylpropanoic acid (phenylpyruvate)	0.4	n.d.	0
2-Keto-phenylpropanoic acid (benzoylformate)	3	0	0

We exchanged I472 for F, L, A, S and C. Only the mutant PDC*I472A* could be obtained in sufficient amounts for further characterisation. The mutation reduced the decarboxylase activity with respect to pyruvate to about 30 % of the wt activity; this was associated with an eightfold increase in K_m for pyruvate. The substrate range of the decarboxylation reaction shows that the C_4 and C_5 2-keto acids are preferable over pyruvate (C_3) as substrates (Table 3, Fig. 3 A, B). The relative activities increase in the order C4 > C5 > C3 > C6 and the K_m values decrease in the order: $C_3 > C_4 > C_5 > C_6$ (Fig. 3 A, B).

The specific carboligase activity with respect to formation of PAC was reduced to 60 % relative to wt PDC*Z.m.*. The result-

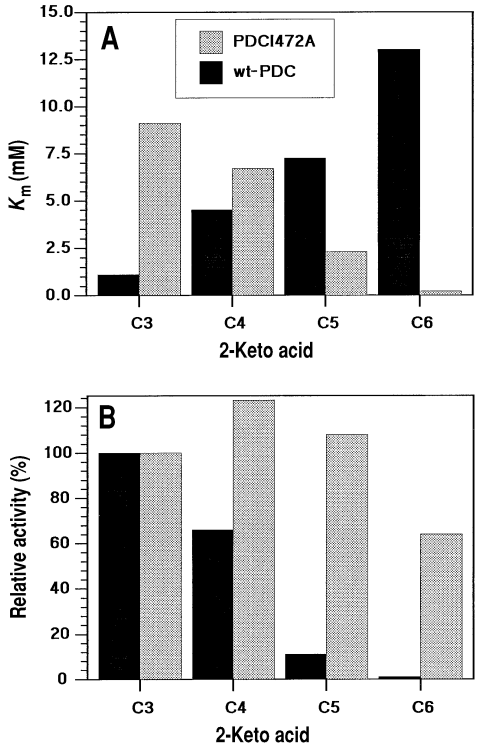
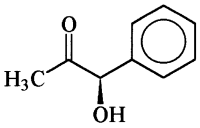
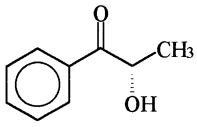
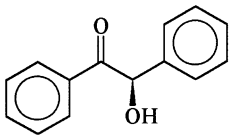
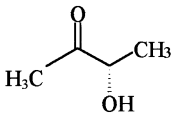


Fig. 3. (A) K_m values for the decarboxylation of various 2-keto acids of PDC*I472A* and wt PDC*Z.m.* (B) Relative decarboxylase activities of PDC*I472A* compared with wt PDC*Z.m.* with respect to various 2-keto acids.

Table 4. Product range obtained using wt PDC and PDC*I472A* (50 µg/ml) as catalysts and pyruvate (35 mM) and benzaldehyde (50 mM) as educts. The yields of 2-hydroxy ketones were measured by HPLC (PAC, 2-HPP, benzoin) and GC (acetoin) after incubation for 16 h at 30°C.

Product	Enantiomeric excess		Yield	
	wt-PDC	PDC*I472A*	wt-PDC	PDC*I472A*
	%		mM	
 (*R*)-PAC	98	70	2.4	1.6
 (*S*)-2-HPP	–	70	0	0.4
 (*R*)-benzoin	–	> 98	0	0.1
 (*S*)-acetoin	20	50	4.0	4.0

ing PAC consisted of significant amounts of the *(S)*-enantiomer, reducing the enantiomeric excess of *(R)*-PAC to 70 % (Table 4). Additionally, the formation of PAC was connected with the synthesis of significant amounts of the tautomeric product (*S*)-2-hydroxypropiophenon ((*S*)-2-HPP) as well as (*R*)-benzoin and acetoin (Table 4). The carboligase activity of PDC*I472A* with respect to the different 2-hydroxy ketones decreased in the order: acetoin > PAC > 2-HPP > benzoin. These results show that the selectivity but not the total carboligase activity of PDC*I472A* is reduced compared with wt PDC.

The conformational stability of PDC*I472A* is only slightly reduced compared with the wt enzyme; this can be deduced from the midpoints of the urea-denaturation curves (Table 2). The mutant enzyme reached its full activity in the presence of cofactor concentrations of 0.1 mM ThDP and 5 mM $MgSO_4$, like the wt enzyme, and retained the cofactors during the purification procedure. Thus, the mutation did not influence cofactor binding significantly.

Mutation of I476. I476 (corresponding to I480 in yeast PDC) is another conserved residue among pyruvate decarboxylases (Fig. 2) and is located about 0.8 nm from C2-ThDP. According to the X-ray structure of yeast PDC, the side chains of I476 and I480 (I472 and I476 in PDC*Z.m.*) are arranged in the cofactor binding cleft, one upon the other. The possibility that I476 may also influence transition states at C2-ThDP was probed by site-directed mutagenesis to L, V, A and E. All mutants showed normal expression rates and purification behaviour.

The decarboxylase activity of the mutant enzymes decreased in the order: I ≫ L > V > A ≫ E (Table 2). The K_m values for pyruvate increased by a factor of 4 (PDC*I476L*) up to a factor of 8 (PDC*I476V*). While the substitution of I476 with L or V has only little effect on the carboligase activity and the enantiomeric excess (ee) of (*R*)-PAC; PDC*I476A* exhibited only 4 % of the wt carboligase activity and produced (*R*)-PAC with an ee of only 90 % (Table 2). The situation changed completely when a glutamate residue was introduced at position 476. Apart from the generally low catalytic activity of this mutant, the resulting PAC was produced with an excess of the (*S*)-enantiomer (Table 2). In contrast to PDC*I472A*, no 2-HPP or benzoin was detected.

Mutations of I476 influenced the enzyme stability significantly. All mutant holoenzymes lost the cofactors rapidly in cofactor-free buffer (Table 2). The thermostability and the conformational stability, as deduced from urea-unfolding curves, decrease in the order I ≫ L > V > A ≫ E.

DISCUSSION

In the present study the function of conserved amino acids in the cofactor binding cleft of PDC have been investigated with respect to their influence on the decarboxylase and carboligase activities, on the kinetic constants and on enzyme stability. E473 was shown to be essential for both the catalytic activities and the structural integrity of the enzyme. Thus, substitution by D or N resulted in inactive mutant enzymes, whereas the exchange for hydrophobic residues such as L, I or V obviously impaired the structural integrity of the enzyme. Expression of these mutants was not detectable, probably due to rapid degradation of misfolded proteins.

Although this glutamate residue is conserved among all PDC sequences known so far as well as in indole-3-pyruvate decarboxylase from *Enterobacter cloacae* [26] (Fig. 2), it is not con-

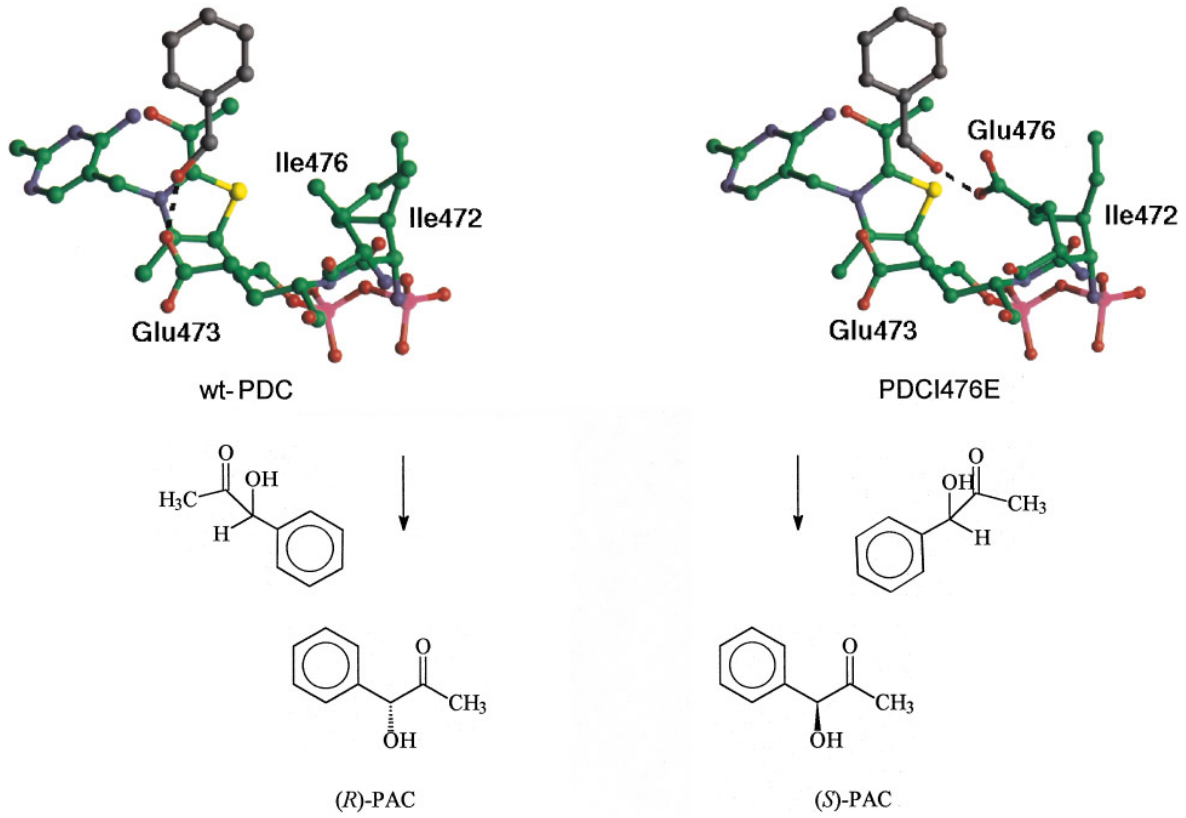


Fig. 4. Structural models to explain the synthesis of (*R*)-PAC by wt PDC and predominantly (*S*)-PAC by the mutant enzyme PDC*I476E*. In PDCI476E both glutamate side chains (E473 and E476) may coordinate benzaldehyde prior to C−C bond formation.

served in benzoylformate decarboxylase (BFD) from *Ps. putida* [27], where a leucine is found at the equivalent position (Fig. 2). Since the carboxylic group of this glutamate residue in PDCs is predicted to play a crucial role in the protonation of the carbanion-enamine (species 3 in Fig. 1) and the coordination of the aldehyde cosubstrate during carboligation [11, 13], alternative ways to perform these reaction steps must exist for BFD. The situation will probably be elucidated from the crystal structure of BFD [28] which will soon be available.

An indirect proof of the central function of E473 was obtained by influencing relevant side chain interactions of this residue with N482. The X-ray structure of yeast PDC, which served as a model for PDC*Z.m.*, revealed that the carbonyl groups of E477 (E473 in PDC*Z.m.*) and N488 (N482 in PDC*Z.m.*) are in contact. The repulsion of the carbonyl groups of both side chains in yeast PDC is probably limited by hydrogen bonds formed between the amide group of N488 with main-chain carbonyl groups of H481 and G482. These amino acids correspond to H477 and probably D478 in PDC*Z.m.* The sequence alignment in Fig. 2 shows an insertion of two amino acids in the sequence of PDC*S.c.* relative to PDC*Z.m.* in this region. Thus, a direct comparison of the interacting amino acids is not possible before the X-ray structure of PDC*Z.m.* becomes available. In yeast PDC this 'complex' is shielded against water by the side chains of L31 and I480. Both hydrophobic residues are conserved among PDCs and correspond to L30 and I476 in PDC*Z.m.* An alteration of the side chain polarity of N482 by exchanging for D is expected to result in an alteration of the hydrogen bonds and thus influence the surrounding amino acid residues. As a consequence, the function of E473 is probably impaired, which may explain the reduced decarboxylase activity of the mutant enzyme (about 10% wt activity) and the disturbed carboligase activity of PDC*N482D*.

Since N482 does not contribute to monomer−monomer interactions, this residue is not directly involved in the stabilisation of the dimer or cofactor binding. Thus, the thermostability and the stability of the cofactor binding of the mutant enzyme are comparable to that of the wt enzyme.

Mutation of I472 and I476 demonstrate the sterical influence of these side chains on the transition states during catalysis. I472 has a distance of about 0.5 nm to C2-ThDP whereas I476 is about 0.8 nm from the active centre. A sequence comparison of ThDP-dependent decarboxylases revealed that both isoleucine residues are conserved among PDCs and indole-3-pyruvate decarboxylase, whereas in BFD there is an alanine residue at position 472 and a phenylalanine residue at position 476 (Fig. 2). It was possible to exchange I472 for A, but not for L, V, S, C or F, which probably impair the structural integrity of the protein. By contrast, substitution of I476 for L, V, A and E gave mutant enzymes of sufficient stability for purification and characterisation.

The reduction of the side chain size at position 472 resulted in an enlarged substrate binding site. As a consequence, the affinity of PDC*I472A* for unbranched aliphatic 2-keto acids larger than pyruvate increased, as deduced from the K_m values (Fig. 3A). A further consequence of the enlarged substrate binding site is the reduced selectivity observed during carboligation. PDC*I472A* catalyses the formation of (*S*)-2-HPP and (*R*)-benzoin in addition to (*R*)-PAC, by-products which are not observed during biotransformations using the wt enzyme or other mutant enzymes (Table 4). The formation of both by-products requires the initial binding of benzaldehyde to C2-ThDP similar to BFD

from *Pseudomonas putida*, which catalyses the synthesis of (*S*)-2-HPP and (*R*)-benzoin [29]. The enantioselectivity of the carboligation was significantly affected by the mutation of I472, since the ee of (*R*)-PAC was reduced to 70 %. Furthermore, (*S*)-2-HPP was obtained with 70 % ee, whereas (*R*)-benzoin was obtained nearly enantiomerically pure with > 98 % ee (Table 4).

These results unambiguously demonstrate that I472 is significant for both substrate specificity and the selectivity of the decarboxylation and carboligation reactions. Although I472 is located within a direct-interaction distance to C2-ThDP, the capacity of the mutant enzyme to bind the cofactors is not significantly affected. This is in contrast to mutations at position 476 which have a more pronounced effect on enzyme stability (Table 2), although I476 is further away from the active centre. As mentioned above, I476 probably shields the 'E473/N482 complex' from water and, thus, is important for the correct function of E473. This interaction may explain why replacement of I476 by smaller aliphatic residues impair the protein stability. Mutation of I476 to E results in a very unstable mutant enzyme, which catalyses predominantly the synthesis of (*S*)-PAC (60 % ee). This switch of the ee can be explained from the 3D structure, assuming that two glutamate residues in the mutant enzymes (at position 473 and 476) may compete for the coordination of benzaldehyde during carboligation and thereby account for two different enantiomers (Fig. 4).

CONCLUSIONS

The present study elucidates molecular factors accounting for the selectivity of the decarboxylation and carboligation reactions of PDC relative to other decarboxylases, especially BFD. Important amino acids influencing the size of the substrate-binding site and the enantioselectivity of the carboligation are I472 and I476. Exchange of I472 for alanine generates a sequence similar to BFD in this position, and PDC*I472 A* has a catalytic activity similar to BFD. An essential residue for enzymatic activity is E473. The correct functioning of E473 is influenced by mutations of neighbouring residues N482 and I476.

This work was supported by the *Deutsche Forschungsgemeinschaft* grant no. Po558/1-1. We thank Dr S. Bringer-Meyer for providing polyclonal rabbit anti-PDC antibodies and S. Münch for excellent technical assistance.

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