The role of residues glutamate-50 and phenylalanine-496 in *Zymomonas mobilis* pyruvate decarboxylase

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Several enzymes require thiamine diphosphate (ThDP) as an essential cofactor, and we have used one of these, pyruvate decarboxylase (PDC; EC 4.1.1.1) from *Zymomonas mobilis*, as a model for this group of enzymes. It is well suited for this purpose because of its stability, ease of purification, homotetrameric subunit structure and simple kinetic properties. Crystallographic analyses of three ThDP-dependent enzymes [Müller, Lindqvist, Furey, Schulz, Jordan and Schneider (1993) Structure **1**, 95–103] have suggested that an invariant glutamate participates in catalysis. In order to evaluate the role of this residue, identified in PDC from *Zymomonas mobilis* as Glu-50, it has been altered to glutamine and aspartate by site-directed mutagenesis of the cloned gene. The mutant proteins were expressed in *Escherichia coli*. Here we demonstrate that substitution with aspartate yields an enzyme with 3 % of the activity of the wild-type, but with normal kinetics for pyruvate. Replacement of Glu-50 with glutamine yields an enzyme with only 0.5 % of the catalytic activity of the wild-type enzyme. Each of these mutant enzymes has a decreased affinity for both ThDP and Mg^{2+}. It has been reported that the binding of cofactors to apoPDC quenches the intrinsic tryptophan fluorescence [Diefenbach and Duggleby (1991) Biochem. J. **276**, 439–445] and we have identified the residue responsible as Trp-487 [Diefenbach, Candy, Mattick and Duggleby (1992) FEBS Lett. **296**, 95–98]. Although this residue is some distance from the cofactor binding site, it lies in the dimer interface, and the proposal has been put forward [Dyda, Furey, Swaminathan, Sax, Farrenkopf and Jordan (1993) Biochemistry **32**, 6165–6170] that alteration of ring stacking with Phe-496 of the adjacent subunit is the mechanism of fluorescence quenching when cofactors bind. The closely related enzyme indolepyruvate decarboxylase (from *Enterobacter cloacae*) has a leucine residue at the position corresponding to Phe-496 but shows fluorescence quenching properties that are similar to those of PDC. This suggests that the fluorescence quenching is due to some perturbation of the local environment of Trp-487 rather than to a specific interaction with Phe-496. This latter hypothesis is supported by our data: mutation of this phenylalanine to leucine, isoleucine or histidine in PDC does not eliminate the fluorescence quenching upon addition of cofactors.

INTRODUCTION

Thiamine diphosphate (ThDP) is an essential cofactor for a number of enzyme-catalysed reactions. In all cases, catalysis involves the initial formation of a carbanion at C-2 of the thiazole ring followed by nucleophilic attack at the carbonyl carbon of the substrate. Then there is cleavage of an adjacent carbon–carbon bond to release the first product with formation of a second 2-α-carbanion. Until recently, the base responsible for the abstraction of the thiazole C-2 proton was unknown, although studies with analogues of ThDP [1] suggested that the 4′-NH_2 might be involved. Removal of the 4′-NH_2 group abolishes catalytic activity, as does replacement of the N-1′, but not the N-3′, nitrogen.

The determination of the three-dimensional structures of yeast transketolase by Lindqvist et al. [2] rationalized these findings and highlighted a critical role for a glutamate residue (Glu-418) that is very close to N-1′. Thus it was proposed [3] that the neutral form of Glu-418 donates a proton to N-1′ while a base (His-481) abstracts a proton from the 4′-NH_2 to yield an imine. This process is then reversed, with C-2 acting as the proton donor and Glu-418 as the base, resulting in the formation of the carbanion at C-2. This is an attractive hypothesis, although the proposed role of His-481 is questionable since mammalian transketolases contain a glutamine at the equivalent position (G. Schenk, R. Layfield, J. M. Candy, R. G. Duggleby and Nixon, P. F., unpublished work).

The determination of the three-dimensional structures of two other ThDP-dependent enzymes {*Lactobacillus plantarum* pyruvate oxidase by Müller and Schulz [5] and yeast pyruvate decarboxylase (PDC; EC 4.1.1.1) by Dyda et al. [6]} is consistent with the proposition that a glutamate residue promotes the ionization at the thiazole C-2. Comparison of the three structures [7] reveals that each folds into three domains of approximately equal size but that the middle domain of transketolase (which contains Glu-418) corresponds to the N-terminal domain of the other two proteins. Nevertheless, there is a glutamate in both PDC (Glu-51) and pyruvate oxidase (Glu-59) contained within this so-called 'Pyr domain' [7] that is close to the N-1′ of ThDP in a position that is equivalent to that of Glu-418 in transketolase. The conservation of a glutamate in ThDP-dependent enzymes (Figure 1) supports the hypothesis of a catalytic role for this residue, as does the observation that mutation of Glu-418 in yeast transketolase to glutamine or alanine markedly reduces enzymic activity [9]. Curiously, neither of these mutants is totally inactive, suggesting that the formation of a hydrogen bond

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ZmPDC     EQVYCC-NELNCGFSAEGYARAKG
ScPDC1    RWAGNA-NELNAAYAADGYARIKG
ScPDC5    RWAGNA-NELNAAYAADGYARIKG
ScPDC6    RWAGNA-NELNAAYAADGYARIKG
HuPDC     RWAASL-NELNAAYAADGYSRIKG
KmPDC     RWAGNA-NELNAAYAADGYARLKG
ApPDC     KWVGNC-NELNAGYAADGYSRIKD
NcPDC     KEVGCA-NELNCSLAAEGYARANG
NtPDC     RWAGNA-NELNAAYAADGYARLKG
OsPDC1    KLIACC-NELNAGYAADGYARALV
OsPDC3    SVVGCC-NELNAGYAADGYARARG
MaPDC     TLVGCC-NELNAGYAADGYARSRG
EcIPDC    CWVGCA-NELNASYAADGYARCKG
AbIPDC    PLHTLS-HEPAVGFAADAAARYSS
OfOCDC    RFYSFR-HEQHAGYAASIAGYIEG
PpBFDC    RYILAL-QEACVVGIADGYAQASR
AtALS     RNVLPR-HEQGGVFAAEGYARSSG
MaALS     ANHLFR-HEQGEAFAASGYARSSG
ScALS     NFVLPK-HEQGAGHMAEGYARASG
EcILVG    EHLLCR-HEQGAAMAAIGYARATG
LpPOX     HYIQVR-HEEVGAMAAAADAKLTG
PfBZL     PIIDTR-HEAAAGHAAEGYARAGA

ScTK      RYIRYGIREHAMGAIMNGISAFGA
HsTK      RFIECYIAEQNMVSIAVGCATRNR
HpTK      RYIEYGIREHAMCAIANGLAAYNK
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Figure 1   Amino acid sequence alignment of the region near the catalytic glutamate residue in a series of ThDP-dependent enzymes

The enzymes are PDC from *Z. mobilis* (ZmPDC, where the glutamate is Glu-50 and is shown in bold), *Saccharomyces cerevisiae* (ScPDC1, ScPDC5, ScPDC6; Glu-51), *Hanseniaspora uvarum* (HuPDC; Glu-51), *Kluyveromyces marxianus* (KmPDC; Glu-51), *Aspergillus parasiticus* (ApPDC; Glu-52), *Neurospora crassa* (NcPDC; Glu-56), *Nicotiana tabacum* (NtPDC; Glu-73), *Oryza sativa* (OsPDC1, Glu-90; OsPDC3, Glu-71) and *Zea mays* (MaPDC; Glu-95); IPDC from *Enterobacter cloacae* (EcIPDC; Glu-52) and *Azospirillum brasilense* (AbIPDC; Glu-48); oxalyl-CoA decarboxylase from *Oxalobacter formigenes* (OfOCDC; Glu-56); benzoylformate decarboxylase from *Pseudomonas putida* (PpBFDC; Glu-47); acetolactate synthase from *Arabidopsis thaliana* (AtALS; Glu-144), *Z. mays* (MaALS; Glu-112), *S. cerevisiae* (ScALS; Glu-139) and *E. coli* (EcILVG, Glu-47); pyruvate oxidase from *L. plantarum* (LpPOX; Glu-59); benzaldehyde lyase from *Pseudomonas fluorescens* (PsBL; E50); and transketolase from *S. cerevisiae* (ScTK; Glu-418), *Homo sapiens* (HsTK; Glu-366) and *Hansenula polymorpha* (HpTK; Glu-433). Sequences were obtained from GenBank or PIR. Transketolases, and the other sequences, were separately aligned using ClustalW computer program [8], then the two groups were aligned on the basis of the equivalent residues in the three-dimensional structures [7].

between Glu-418 and N-1′ is not absolutely essential for catalysis. This is not entirely consistent with the failure of the ThDP analogue that lacks N-1′ to support catalysis, despite the demonstration that this compound binds to transketolase in a manner that is structurally equivalent to ThDP binding [10]. Clearly it is very important to investigate the extent to which this glutamate is essential in other ThDP-dependent enzymes. Here we report the results of mutating Glu-50 of *Z. mobilis* PDC to glutamine or aspartate.

The crystal structures of these ThDP-dependent enzymes [2,5,6] have clarified the way in which cofactors bind. In each of these enzymes, the side-chains of both an aspartate and an asparagine co-ordinate to the essential bivalent metal ion that is itself co-ordinated to oxygen atoms of the phosphate groups. These residues correspond to the aspartate and the second asparagine in a GDGX$^{24-27}$NN sequence motif that is common to all ThDP-dependent enzymes [11]. This motif is contained in a 'PP domain' [7] that constitutes the N-terminal domain of transketolase, but the C-terminal domain of the other two enzymes. The thiazole and pyrimidine portions of the cofactor
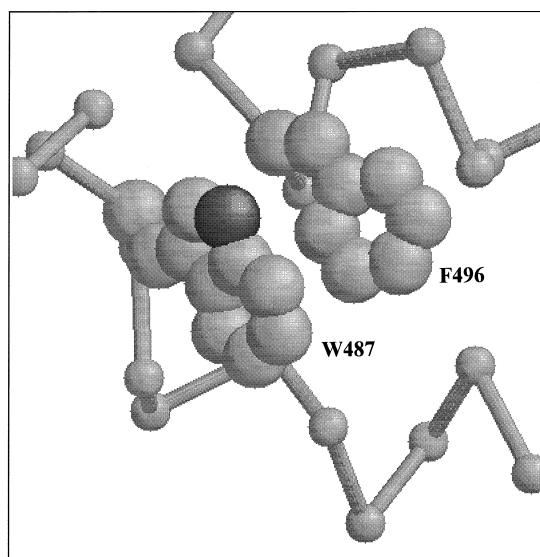

Figure 2   Structure of the Trp-487/Phe-496 region of PDC

The structure of yeast PDC was taken from Dyda et al. [6] and renumbered according to the *Z. mobilis* PDC sequence. The locations of the α-carbon atoms of residues 484–495 (lower strand from left to right) and residues 491–497 (upper strand from right to left) are shown, together with the side-chains of Trp-487 and Phe-496.

do not interact at all with this motif; rather, they interact with several residues dispersed along the sequence and located at the subunit interface. Most of these residues are in an adjacent subunit and form the Pyr domain mentioned earlier.

The fact that ThDP binds across the subunit interface emphasizes the importance of subunit interactions in these oligomeric enzymes. Thus it has been proposed [12] that increasing pH leads to the dissociation of yeast PDC which coincides with loss of bound ThDP. Lowering the pH allows tetramers to reform, but these are not active until cofactors are added. We have observed [13] that adding cofactors to the *Z. mobilis* PDC apoenzyme at pH 6.5 results in a structural change that is accompanied by a marked decrease in tryptophan fluorescence. Structural changes in yeast PDC on adding cofactors have also been adduced from tryptophan fluorescence [14], cysteine reactivity [15], solution X-ray scattering [16] and CD [17] studies. The residue responsible for the tryptophan fluorescence changes in *Z. mobilis* PDC was later identified as Trp-487 by mutating it to leucine [18]. At the time we suggested that there was a direct interaction between Trp-487 and ThDP, but the crystal structure of yeast PDC [6] later proved this hypothesis to be incorrect. Trp-487 is at the subunit interface and is close to Phe-496 on an adjacent monomer (Figure 2); thus Dyda et al. [6] suggested that cofactor binding causes a perturbation of ring stacking between Trp-487 and Phe-496 and it is this that results in the change in tryptophan fluorescence. This proposal is not entirely convincing as the rings do not appear to be parallel to one another (Figure 2).

Trp-487 is conserved across all known PDCs as well as in the closely related enzyme indolepyruvate decarboxylase (IPDC) (Figure 3). However, Phe-496 is not absolutely conserved and may be substituted by leucine, isoleucine or methionine. Thus it was of interest to determine the fluorescence quenching properties that result from such substitutions. Here we report the results of

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ZmPDC      NNYGYTIEVMIH--DGPYNNIKNWDYAGLMEVFNG
ScPDC1     NNDGYTIEKLIHGPKAQYNEIQGWDHLSLLPTFGA
ScPDC5     NNNGYTIEKLIHGPHAEYNEIQGWDHLALLPTFGA
ScPDC6     NNDGYTIEKLIHGPHAEYNEIQTWDHLALLPAFGA
HuPDC      NNNGYTIEKLIHGPTAQYNMIQNWKQLRYLTNFGA
KmPDC      NNDGYTIERLIHGETAQYNCIQSWKHLDLLPTFGA
ApPDC      NNDGYTIERCIHGRNQAYNDVAPWRYLKAAEFFGA
NcPDC      NNRGYTIEVEIH--DGSYNKIKNWDYAMLVEAFNS
NtPDC      NNGGYTIEVEIH--DGPYNVIKNWDYTGLVDAIHN
OsPDC1     NNGGYTIEVEIH--DGPYNVIKNWDYTGLIDAIHN
OsPDC3     NNGGYTIEVEIH--DGPYNVIKNWDYVGLVNAIHN
MaPDC1     NNGGYTIEVEIH--DGPYNVIKNWDYTGLVNAIHN
MaPDC2     NNGGYTIEVEIH--DGPYNVIKNWNYTGLVEAFHN
MaPDC3     NNGGYTIEVEIH--DGPYNVIKNWNYTGFVDAIHN
EcIPDC     NNEGYTVERAIHGAEQRYNDIALWNWTHIPQALSL
AbIPDC     NNASWEMLRTFQ-PESAFNDLDDWRFADMAAGMGG
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Figure 3   Amino acid sequence alignment of the region near Trp-487 (numbering according to Z. mobilis PDC) in PDC and related enzymes

The enzymes are PDC from *Z. mobilis* (ZmPDC; Trp-487, Phe-496), *S. cerevisiae* (ScPDC1, ScPDC5, ScPDC6; Trp-493, Phe-502), *H. uvarum* (HuPDC; Trp-493, Phe-502), *K. marxianus* (KmPDC; Trp-493, Phe-502), *A. parasiticus* (ApPDC; Trp-499, Phe-508); *N. crassa* (NcPDC; Trp-491, Phe-500), *N. tabacum* (NtPDC; Trp-538, Phe-547), *O. sativa* (OsPDC1, Trp-526, Phe-535; OsPDC3, Trp-509, Phe-518) and *Z. mays* (MaPDC1; Trp-534, Phe-543; MaPDC2 and MaPDC3 are partial sequences); and IPDC from *E. cloacae* (EcIPDC; Trp-484, Phe-493) and *A. brasilence* (AbIPDC; Trp-477, Phe-486). Sequences were obtained from GenBank or PIR. Alignment was performed with the aid of the ClustalW computer program [8]. The first two residues in each sequence represent the end of the ThDP binding motif [11].

such studies on *Enterobacter cloacae* IPDC (which naturally contains a leucine residue), as well as mutagenesis of *Z. mobilis* PDC to change this residue to leucine, isoleucine or histidine.

MATERIALS AND METHODS

Restriction endonucleases and molecular biology products

Restriction enzymes and ligases were purchased from New England Biolabs, Boehringer Mannheim or Progen. The Prism Ready DyeDeoxy Terminator Cycle Sequencing Kit for sequencing was purchased from Applied Biosystems. All PCR reactions were carried out using a Perkin-Elmer DNA Thermal Cycler (Model 480). The 'Altered Sites' *in vitro* Mutagenesis System was purchased from Promega. Lysozyme and DNase I were purchased from Boehringer Mannheim.

Bacterial strains and plasmids

Escherichia coli strains XL-1 Blue and DH5α were obtained from Stratagene or BRL. Plasmid pPL450 (a gift from Dr. N. E. Dixon, Research School of Chemistry, Australian National University, Canberra, Australia) contains the λ cI857 gene and both strong promoters P_R and P_L arranged in tandem [19]. The plasmid pPLZM was constructed from a 1.8 kb *Eco*RI/*Sph*I fragment of pIDT1A [20] containing the entire PDC gene from *Z. mobilis*, subcloned into pPL450. *E. coli* cultures containing the pPLZM construct or mutant constructs were maintained on Luria broth plates [21] with 100 μg/ml ampicillin. Plasmid pSEL-PDC, used as the DNA template for *in vitro* mutagenesis, was constructed by subcloning the *Eco*RI/*Sph*I fragment of pIDT1A into the phagemid vector pSELECT-1 (Promega). *E. coli* cultures containing pSEL-PDC or its derivatives were maintained on Luria broth plates which contained tetracycline (15 μg/ml) or ampicillin (125 μg/ml), as appropriate. Plasmid DNA preparations, restriction endonuclease digestions, isolation of DNA fragments, ligations and transformations were carried out under standard conditions [21].

In vitro mutagenesis

All mutants were constructed using the 'Altered Sites' *in vitro* mutagenesis system as described previously [18]. Mutagenic primers were 24–38 bases in length with a G+C content of 50 % or higher. Silent mutations were introduced where possible to create additional restriction endonuclease sites to facilitate in the screening of transformants. All mutagenic primers were designed taking into consideration the codon usage for *Z. mobilis* PDC [22]. The entire PDC genes with the desired mutations were subcloned into pPLZM as a 1.8 kb *Eco*RI/*Sph*I fragment for expression. The introduction of the base changes in these mutant genes was confirmed by DNA sequencing.

Expression and purification of enzymes

For large-scale production of PDC (wild-type and mutant) in *E. coli*, cells were grown at 30 °C in a 20 litre Chemap Fermenter with stirring (400 rev./min) and aeration. The cells were grown in 2YT medium [21] supplemented with 0.2 % (v/v) glycerol and containing 100 μg/ml ampicillin. Expression of the PDC gene was accomplished by a rapid temperature shift to 42 °C after the cells had reached an A_{590} of 0.5. Growth of the cells was maintained at 42 °C for 3–4 h.

The enzyme extraction and purification procedure was based on that described by Diefenbach and Duggleby [13] but with some modifications. Cells were lysed for 1 h at room temperature with lysozyme (0.3 μg/ml) in 100 mM KH_2PO_4/KOH buffer (pH 6.8) containing ThDP (0.1 mM), $MgCl_2$ (5 mM), DNaseI (0.01 μg/ml), 2-mercaptoethanol (18.5 mM), Nonidet P-40 (2 μl/ml), leupeptin (20 μg/ml), aprotinin (0.02 unit/ml), bestatin (0.3 μg/ml) and toluene (0.1 %, v/v). Glass beads of equal volume to that of the packed cells were added to aid in cell disruption.

PDC was purified using a one-step hydroxyapatite batch procedure [13]. The enzyme was concentrated to 15 mg/ml protein and dialysed against 50 mM Mes/KOH, pH 6.5, that also contained 2 mM $MgCl_2$, 0.1 mM ThDP and 1 mM dithiothreitol (DTT). An equal volume of glycerol was added after dialysis and the enzyme preparation was stored at −20 °C until required. These enzymes were further purified for analysis of ThDP content by chromatography on either DEAE-Sephacel or HighTrapQ (Pharmacia). IPDC was isolated from *E. coli* strain DH5α harbouring plasmid pIP27, which contains the *E. cloacae* IPDC gene inserted into the *Sma*I site of pKK223-3 [23]. The enzyme used for these studies was purified to apparent homogeneity by $(NH_4)_2SO_4$ fractionation, followed by chromatography on Q-Sepharose and phenyl-Sepharose [24].

Preparation of apoenzyme

In order to minimize contamination by metal ions, all glassware, plastic cuvettes and tips were soaked in 20 % (v/v) HNO_3 after cleaning, and rinsed several times with metal-free water (18 MΩ·cm⁻¹; MilliQ water purification system, Millipore). Wild-type and mutant PDC apoenzymes were prepared from the enzyme stored in 50 % glycerol by 20-fold dilution with 50 mM Tris/HCl, pH 8.5, which contained 1 mM EDTA and 1 mM DTT. After 30 min of stirring at room temperature the solution was concentrated by ultrafiltration. The solution was then passed through a BioGel P-6DG (Bio-Rad) column (30 cm × 2.5 cm²) equilibrated with Tris/EDTA/DTT buffer, followed by passage through a similar column (25 cm × 2.5 cm²) equilibrated with metal-free Mes/KOH buffer (50 mM, pH 6.5) containing 1 mM DTT. This buffer and the ThDP solutions used for binding

studies of the apoenzymes had been passed through a column of Chelex 100 (Bio-Rad) to remove bivalent metal ions.

Activity assay for PDC

PDC activity was assayed at 30 °C by measuring the rate of production of acetaldehyde, determined by measuring the oxidation of NADH in the presence of alcohol dehydrogenase (ADH), as described previously [13]. Unless stated otherwise, reactions were initiated by the addition of PDC obtained directly from purification, or by dilution in 50 mM Mes/KOH (pH 6.5) of enzyme stored in 50 % glycerol. During purification of recombinant PDC isolated from *E. coli* clones, assays were performed with and without ADH, the latter taken as a measure of background lactate dehydrogenase activity. One unit of activity is defined as the quantity of enzyme that catalyses the formation of 1 μmol of product per min. Unless stated otherwise, the K_m for the substrate was determined in the standard assay mixture containing various concentrations of pyruvate.

Analytical methods

The concentration of stock pyruvate was determined by NADH oxidation in the presence of lactate dehydrogenase, modified from the assay of Bucher et al. [25]. Solutions of ThDP were assayed spectrophotometrically using an ϵ_{267} of 8520 $M^{-1}\cdot cm^{-1}$ determined in this laboratory by Diefenbach [26]. The concentration of Mg^{2+} in stock solutions was determined by atomic absorption spectrophotometry at 285.2 nm. Protein determinations were performed according to the method of Smith et al. [27] using a bicinchoninic acid protein determination kit (Sigma Chemical Co.).

Measurement of cofactor binding

In cofactor binding experiments, the wild-type and mutant PDC apoenzymes were preincubated in mixtures containing a saturating concentration of one cofactor and various concentrations of the other cofactor for 15 min at 30 °C prior to initiation of the assay by addition of a pyruvate/ADH/NADH mixture. Data from the cofactor binding experiments were analysed as described previously [20].

When cofactor binding could not be measured by activity assay, it was determined at 30 °C by monitoring the quenching of the tryptophan fluorescence of PDC as described previously [20] using a Jasco Model FP-770 spectrofluorimeter with an excitation wavelength of 300 nm (bandwidth 5 nm) and an emission wavelength of 340 nm (bandwidth 10 nm). These assays were conducted in a total volume of 3 ml.

Circular dichroism

Near-UV CD spectra were measured with a Jasco J-710 spectropolarimeter using a path length of 0.1 cm at 25 °C, with a protein concentration of 5.4 mg/ml (wild-type enzyme) or 2.40 mg/ml (E50Q mutant).

RESULTS

E50Q mutant

Wikner et al. [9] have shown that substitution of a glutamate residue (Glu-418) with glutamine in yeast transketolase yields a protein with 2 % of the catalytic activity of the wild-type transketolase. Using PDC from *Z. mobilis* we have also replaced the equivalent glutamate (Glu-50) with a glutamine residue, resulting in an active enzyme. A final specific activity of 0.32 unit/mg was obtained when the enzyme was fully activated

Table 1   Parameters characterizing the interaction of PDC with substrate and cofactors

The results are reported as the best-fit value $\pm$ standard error. Values shown for E50Q were determined from fluorescence measurements. ND, not determined. $S_{0.5}$ is the half-saturating concentration.

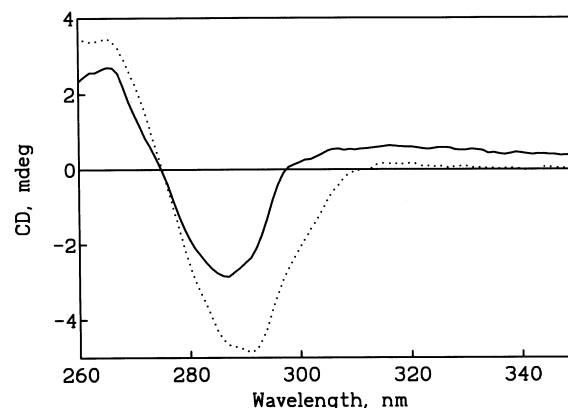| Enzyme	K_m for pyruvate (mM)	$S_{0.5}$ for ThDP (μM)	$S_{0.5}$ for Mg^{2+} (μM)
Wild-type	0.52 ± 0.05	2.53 ± 0.16	5.87 ± 0.43
E50Q	ND	10.7 ± 4.0	194 ± 65
E50D	0.50 ± 0.02	113 ± 5	84.1 ± 4.2
F496L	0.97 ± 0.02	0.92 ± 0.03	7.30 ± 0.31
F496I	1.11 ± 0.06	0.87 ± 0.03	8.12 ± 0.38
F496H	1.06 ± 0.05	1.60 ± 0.06	7.47 ± 0.50

Figure 4   CD spectrum of PDC

The solid line represents the spectrum of the wild-type *Z. mobilis* enzyme, while that of the E50Q mutant is shown by the broken line.

with cofactors (see below), and the protein appeared close to purity by SDS/PAGE. This specific activity is less than 0.5 % of that of the wild-type enzyme, which is usually close to 70 units/mg. Gel filtration indicated a molecular size indistinguishable from that of the wild-type.

In contrast to the linear change in absorbance over time found in the activity assay of native *Z. mobilis* PDC and recombinant wild-type PDC, the E50Q mutant exhibited a distinct lag phase in product formation when assayed without prior incubation with cofactors. This lag was still evident after 5 min with 10 mM $MgCl_2$ plus 0.2 mM ThDP; under these conditions, the half-time for activation of the wild-type apoenzyme is less than 6 s [13]. Prolonged storage of the enzyme in 10 mM $MgCl_2$ plus 0.2 mM ThDP did not eliminate this lag phase.

The presence of this lag phase, together with extremely low activity, made kinetic studies very difficult. Therefore the binding of ThDP and Mg^{2+} was determined by their effect on fluorescence quenching. The half-saturating concentration for ThDP was determined to be 10.7 μM while that for Mg^{2+} was 194 μM (Table 1). Each of these values is substantially higher than those found for the wild-type enzyme.

The CD spectra of E50Q and wild-type PDC are shown in Figure 4. The wild-type enzyme shows a positive peak at 265 nm, a negative peak at 286 nm and a weak diffuse band in the 300–340 nm region. For E50Q, the last band was absent while the negative peak was shifted 5 nm towards the visible region.
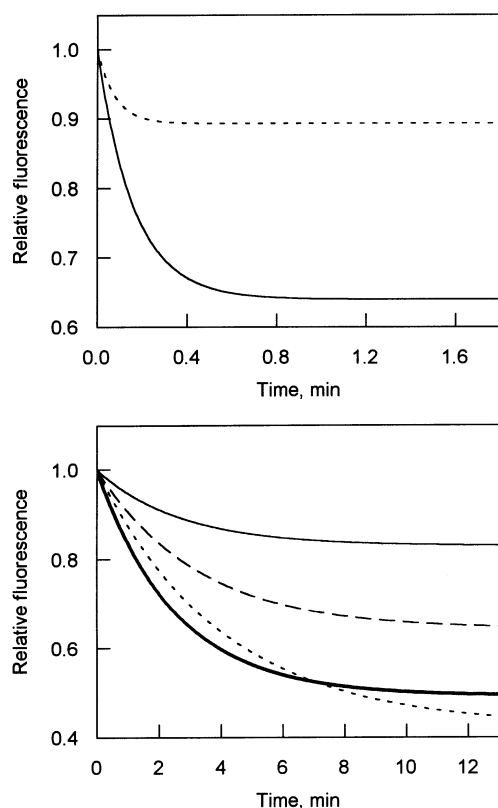
Figure 5 Fluorescence quenching of IPDC and PDC

The upper panel shows the fluorescence of *E. cloacae* IPDC on adding 5 mM MgCl$_2$ and 0.1 mM ThDP to the apoenzyme (solid line) or holoenzyme (broken line). The lower panel shows the fluorescence of *Z. mobilis* PDC on adding 0.5 mM MgCl$_2$ and 0.01 mM ThDP to the wild-type enzyme (thick solid line), F496L (dotted line), F496I (dashed line) or F496H (thin solid line). In all cases, to facilitate comparison, the starting fluorescence has been normalized to an arbitrary value of 1.0.

E50D mutant

Changing Glu-50 to aspartate yielded an active enzyme that, when fully activated with cofactors (see below), had a specific activity after purification of 2.02 units/mg; despite this rather low value (less than 3 % of wild-type), the enzyme appeared to be close to purity by SDS/PAGE. Like E50Q, this mutant exhibited a distinct lag in product formation when assayed without prior incubation with cofactors. Preincubation, and varying the concentration of ThDP in the reaction mixture over the range 0.1–10 mM, had a dramatic effect on the length of this lag phase. Preincubation with concentrations at and above 1 mM eliminated the lag phase entirely. The K_m for the substrate pyruvate was determined by preincubating the enzyme for 15 min in 1 mM ThDP and 5 mM Mg^{2+} before addition to the standard assay mixture containing various concentrations of pyruvate. Under these conditions the K_m for pyruvate was indistinguishable from that of wild-type PDC (Table 1). In contrast, there was a marked decrease in the affinity of this mutant for cofactors: a 14-fold change for Mg^{2+} and a 45-fold change for ThDP.

Substitutions at Phe-496

In view of the suggested role [6] of ring stacking between Trp-487 and Phe-496 in the quenching of tryptophan fluorescence, it is of interest to examine the effects of alterations at position 496.

Table 2   Kinetics of tryptophan fluorescence quenching for wild-type and F496 mutants of PDC

The results are reported as the best-fit value $\pm$ standard error.

Enzyme	Amplitude	Rate constant (min^{-1})
Wild-type	0.508 $\pm$ 0.003	0.394 $\pm$ 0.008
F496L	0.587 $\pm$ 0.014	0.248 $\pm$ 0.015
F496I	0.358 $\pm$ 0.002	0.307 $\pm$ 0.005
F496H	0.171 $\pm$ 0.003	0.372 $\pm$ 0.017

Fortunately, Nature has already performed this experiment (Figure 3), and we investigated the fluorescence properties of *E. cloacae* IPDC (Figure 5, upper panel). We observed a substantial fluorescence quenching on adding cofactor to the apoenzyme, whereas there was little effect on the holoenzyme. This suggests that there is no specific requirement for Phe-496.

To investigate this further the *Z. mobilis* PDC mutants F496L, F496I and F496H were prepared; all were found to be active, with final specific activities of 52.3, 22.6 and 28.8 units/mg respectively. As with E50D, all these mutants exhibited a lag phase in product formation that was eliminated by preincubation with cofactors. The three mutants have a similar K_m for pyruvate that is approximately twice that of the wild-type (Table 1). Unexpectedly, each had an affinity for ThDP that was higher than that of the wild-type; in contrast, each showed a small but consistent decrease in affinity for Mg^{2+}.

The fluorescence quenching of each of these mutants was determined and compared with that of the wild-type enzyme. In each case (Figure 5, lower panel), there was a first-order decrease in fluorescence that was characterized with respect to the amplitude (relative to an arbitrary starting value of 1.0) and a rate constant (Table 2). For F496L, the amplitude was similar to that of the wild-type although the rate constant was somewhat lower. The amplitude for F496H was markedly decreased, although the rate constant was similar to that of the wild-type. F496I showed properties that were intermediate between the other mutants, with respect to both the amplitude and rate constant.

DISCUSSION

The crystal structures of three ThDP-dependent enzymes [7] are consistent with a glutamate residue being essential for catalysis, and it is therefore surprising that Wikner et al. [9] were able to mutate this residue in yeast transketolase to glutamine and retain 2 % activity. Here we have shown that the same substitution in *Z. mobilis* PDC also yields an active enzyme with 0.5 % of normal activity. Although this may give rise to some suspicion that this glutamate is not essential, it should be noted that this is not the first time that an 'essential' acidic residue has been substituted by the amide analogue with retention of some activity. For example, Corey and Craik [28] showed that the mutant D102N of trypsin can exhibit over 5 % of wild-type activity when assayed at an appropriate pH.

The E50Q mutant of *Z. mobilis* PDC is clearly a badly crippled enzyme; not only is it an inefficient catalyst, it is also impaired in its ability to bind cofactors (Table 1) and is slow to reach even its weak catalytic potential. The origin of these time lags is unclear. They are not due to slow cofactor binding, since storage of the enzyme in the presence of saturating concentrations of both MgCl$_2$ and ThDP had little effect. Neither are they due to alterations of the quaternary structure such as those we reported

previously [20] for the mutant D440E, which is dimeric, unlike the tetrameric wild-type enzyme. It is possible that the lags represent a slow conformational change that follows cofactor binding and requires the presence of substrate.

The CD spectrum of wild-type *Z. mobilis* PDC has not been reported previously but appears similar to that of the yeast enzyme [17,29,30] except for the presence of a weak diffuse band in the 300–340 nm region (Figure 4). Wikner et al. [9] reported a similar but more intense band in transketolase that they ascribe to a charge-transfer complex between ThDP and aromatic residues in the cofactor binding pocket. The weakness of this band in PDC, which has no aromatic residues in the corresponding region [6], is consistent with this interpretation.

The CD spectrum of the E418Q mutant of transketolase [9] is considerably different from that of the wild-type; this is interpreted by the authors to result from a changed electronic distribution in the pyrimidine ring. The corresponding PDC mutant (E50Q) shows much less marked changes; however, the CD spectrum of PDC is not expected to be as sensitive as that of transketolase to the electron distribution in the cofactor, since charge-transfer complexes do not appear to contribute.

Changing Glu-50 to aspartate, which retains the charge but alters the size, also gave an enzyme with markedly decreased activity, although it is six times more active than E50Q. This mutant enzyme clearly has some difficulty in binding ThDP in that such binding is slower (as reflected in the assay lags) and weaker (Table 1) than in the wild-type. However, once saturated with cofactors, the mutant enzyme has an unaltered K_m for pyruvate.

We considered the possibility that the residual activity observed in the E50Q and E50D mutants results from a low level of misreading of the mutant codon, thereby giving a small amount of wild-type enzyme that co-purifies with the inactive, mutant protein. Although the required error frequency would have to be much higher than is considered normal in *E. coli*, it has been shown that high rates of expression can increase the error frequency to values that would be consistent with the observed activity [31]. This explanation is effectively discounted, at least for E50D, by the observed alteration in affinity for cofactors which was determined from their effects on catalytic activity.

Thus the conservation of a glutamate in ThDP-dependent enzymes is not an indication that this residue is essential in the strictest sense. Rather, a glutamate is the most efficient means of achieving catalysis, and no effective way to use an alternative amino acid side-chain for this function has arisen during the evolution of this group of enzymes.

The binding of ThDP to this enzyme family is at a subunit interface, and it is expected that residues involved in contacts between subunits would be perturbed when the cofactor is added. One such residue is Trp-487, which is conserved in PDCs from all sources and in the closely related IPDC (Figure 3), and which has been shown to be the source of the fluorescence quenching that is observed upon adding cofactors to *Z. mobilis* PDC [18]. In the crystal structure of yeast PDC, this residue is close to a phenylalanine on the neighbouring subunit (Figure 2), although this phenylalanine is not conserved in related enzymes (Figure 3), in which it is replaced by other hydrophobic amino acids. However, it had not been demonstrated previously whether any of the enzymes that lack Phe-496 show characteristic fluorescence quenching; as shown in Figure 5 (upper panel), IPDC from *E. cloacae* has similar properties in this respect to *Z. mobilis* PDC, which suggests that ring-stacking, as proposed by Dyda et al. [6], may not be important for this property. To investigate this further, Phe-496 of *Z. mobilis* PDC was mutated to leucine, isoleucine or histidine. In each case, adding cofactors

to the apoenzyme resulted in a significant fluorescence quenching. Although the effect was smallest for F496H, it was clearly greater than that observed for the mutant W487L [18]. It is of interest that F496H, the only mutant for which ring-stacking might be conceivable, gave the smallest degree of fluorescence quenching. These results suggest that it is changes in the local environment of Trp-487, rather than any specific interaction with Phe-496, that is responsible for the fluorescence quenching.

Tryptophan fluorescence is expected to decrease with increases in the polarity of the local environment. Since cofactor binding results in a decrease in fluorescence, it appears that Trp-487 must be more exposed to solvent in the holoenzyme than in the apoenzyme This is consistent with the observation that the fluorescence change, and hence the alteration of the local environment, was smallest in F496H. Nevertheless, it is not consistent with the crystal structure of the PDC holoenzyme, which indicates that the indole ring of Trp-487 is largely inaccessible to solvent. There are two factors that are relevant in the context of this apparent inconsistency. First, the published structure is at 2.4 Å resolution and some caution is necessary when inferring fine details of solvent accessibility. Secondly, it is conceivable that crystal packing forces have compressed the subunit interface to the extent that it does not reflect the structure of this region in solution.

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