# Investigation of the cofactor-binding site of *Zymomonas mobilis* pyruvate decarboxylase by site-directed mutagenesis

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Several enzymes require thiamin 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 and its simple kinetic properties. A sequence motif of approx. 30 residues, beginning with a glycine-aspartate-glycine (-GDG-) triplet and ending with a double asparagine (-NN-) sequence, has been identified in many of these enzymes [Hawkins, Borges and Perham (1989) FEBS Lett. **255**, 77–82]. Other residues within this putative ThDP-binding motif are conserved, but to a lesser extent, including a glutamate and a proline residue. The role of the elements of this motif has been clarified by the determination of the three-dimensional structure of three of these enzymes [Muller, Lindqvist, Furey, Schulz, Jordan and Schneider (1993) Structure **1**, 95–103]. Four of the residues within this motif were modified by site-directed mutagenesis of the cloned PDC gene to evaluate their role in cofactor binding. The mutant proteins were expressed in *Escherichia coli* and found to purify normally, indicating that the tertiary structure of these enzymes had not been grossly

perturbed by the amino acid substitutions. We have shown previously [Diefenbach, Candy, Mattick and Duggleby (1992) FEBS Lett. **296**, 95–98] that changing the aspartate in the -GDG- sequence to glycine, threonine or asparagine yields an inactive enzyme that is unable to bind ThDP, therefore verifying the role of the ThDP-binding motif. Here we demonstrate that substitution with glutamate yields an active enzyme with a greatly reduced affinity for both ThDP and Mg$^{2+}$, but with normal kinetics for pyruvate. Unlike the wild-type tetrameric enzyme, this mutant protein usually exists as a dimer. Replacement of the second asparagine of the -NN- sequence by glutamine also yields an inactive enzyme which is unable to bind ThDP, whereas replacement with an aspartate residue results in an active enzyme with a reduced affinity for ThDP but which displays normal kinetics for both Mg$^{2+}$ and pyruvate. Replacing the conserved glutamate with aspartate did not alter the properties of the enzyme, while the conserved proline, thought to be required for structural reasons, could be substituted with glycine or alanine without inactivating the enzyme, but these changes did reduce its stability.

## INTRODUCTION

Thiamin diphosphate (ThDP) is the cofactor for a number of enzyme-catalysed reactions. In all cases there is an initial reaction of a carbonyl group in the substrate with C-2 of the thiazole ring of the enzyme-bound cofactor. The resulting adduct then undergoes cleavage of a carbon–carbon bond, releasing part of the original substrate molecule. In most cases [pyruvate decarboxylase (PDC), pyruvate dehydrogenase, pyruvate oxidase, indolepyruvate decarboxylase, benzoylformate decarboxylase, acetolactate synthase, glyoxylate carboligase, 2-oxoglutarate decarboxylase, 2-oxoglutarate dehydrogenase and the branched-chain-2-oxoacid dehydrogenase], the released fragment is $CO_2$. The enzyme-bound aldehyde may then be released (PDC, indolepyruvate decarboxylase, benzoylformate decarboxylase, 2-oxoglutarate decarboxylase and benzaldehyde lyase), oxidized (pyruvate oxidase, pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and the branched-chain-2-oxoacid dehydrogenase) or transferred to a second substrate (acetolactate synthase, glyoxylate carboligase, transketolase and dihydroxyacetone synthase).

In 1989, Hawkins et al. identified a sequence motif common to all the ThDP-dependent enzymes that had been sequenced at the time. This motif has since been found in other PDC sequences from *Saccharomyces cerevisiae* (Hohmann and Cederberg, 1990; Hohmann, 1991), *Zea mays* (Kelley et al., 1991), *Sarcina ventriculi*

(Lowe and Zeikus, 1992) and *Neurospora crassa* (Alvarez et al., 1993), indolepyruvate decarboxylase from *Enterobacter cloacae* (Koga et al., 1991), acetolactate synthase from *Brassica napus*, *Arabidopsis thaliana* (Bekkaoui et al., 1991), *Zea mays* (Fang et al., 1992), *Spirulina platensis* (Milano et al., 1992), *Synechococcus* PCC7942 (Friedberg and Seijffers, 1990), *Klebsiella pneumoniae* (Peng et al., 1992), *Porphyra umbilicalis* (Reith and Munholland, 1993), transketolase from humans (McCool et al., 1993), *S. cerevisiae* (Fletcher et al., 1992) and *R. sphaeroides* (Chen et al., 1991), glyoxylate carboligase from *Escherichia coli* (Chang et al., 1993), pyruvate dehydrogenase from *Bacillus subtilis* (Hemilä et al., 1990) and pyruvate synthase from *Halobacterium halobium* (Plaga et al., 1992).

The principal features of this motif are a glycine-aspartate-glycine (GDG) sequence separated by approx. 25 residues from an asparagine doublet (NN). Within the motif are other residues that are conserved to a lesser extent: for example, an acidic amino acid eight residues from the GDG and a proline that is seven residues before the NN. As shown below, all of these features are found in *Zymomonas mobilis* PDC, the enzyme that is the subject of the present paper:

[439]<u>GDG</u>SFQLTAQ<u>E</u>VAQMVRLKL<u>P</u>VIIFL<u>INN</u>[467]

Until recently, the role of these conserved residues was not known, and there was limited experimental evidence for the involvement of this motif in ThDP binding. We showed that

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substitution of aspartate-440 in *Z. mobilis* PDC with glycine, threonine or asparagine yields an inactive enzyme that is unable to bind ThDP, therefore verifying the role of the ThDP-binding motif (Diefenbach et al., 1992). Russell et al. (1992) showed that replacement of the second glycine with alanine, serine or methionine greatly reduced the activity of the pyruvate dehydrogenase multienzyme complex, but they did not investigate whether ThDP binding was affected.

The determination of the three-dimensional structures of three ThDP-dependent enzymes (yeast transketolase by Lindqvist et al., 1992; *Lactobacillus plantarum* pyruvate oxidase by Muller and Schulz, 1993; and yeast PDC by Dyda et al., 1993) has gone a long way to explaining the functions of some elements of this motif. In all three enzymes, the side chains of both the aspartate and the second asparagine co-ordinate to the essential bivalent-metal ion, which is itself co-ordinated to an oxygen on each of the phosphate groups. Curiously, this motif does not interact at all with the thiamin portion of the cofactor; rather, it interacts with several residues dispersed along the sequence and forms a different domain. Thus it appears that the presence of this common motif in these enzymes is more a reflection of a common evolutionary heritage. It is noteworthy in this context that, while the sequence of pyruvate oxidase and PDC are co-linear with each other, but not with transketolase, the three-dimensional structures suggest that transketolase is a circular permutation of the others, with the first and second domains of transketolase corresponding to the third and first domains respectively of PDC (Muller et al., 1993). A similar conclusion, but including pyruvate dehydrogenase, had earlier been reached by Robinson and Chun (1993) on the basis of sequence alignment.

Several questions remain unanswered by the three-dimensional structures. For example, the fact that the PDC mutant Asp[440] → Asn (Asp440Asn) cannot bind ThDP suggests that the negative charge is important, something that could be examined further by making the mutant Asp440Glu. The structures suggest that asparagine-467 might be replaceable by another residue with a side chain capable of co-ordinating to the metal. The roles of glutamate-449 and proline-459 are not clear, despite being conserved in many ThDP-dependent enzymes. Here we report some properties of several mutant forms of PDC with alterations in the four residues mentioned above. The mutant PDC genes were expressed in *E. coli* and the resulting enzymes characterized to determine the effect of the substitution on cofactor and substrate binding.

## MATERIALS AND METHODS

### Restriction endonucleases and molecular-biology products

Restriction enzymes and ligases were purchased from New England Biolabs, Boehringer Mannheim or Toyobo Corp. Radionucleotides for sequencing were purchased from Amersham Corp. *Taq* DNA polymerase was purchased from Biotech (Australia). Pharmacia Ultrapure dNTPs were used in all PCR reactions. Lysozyme and DNAase I were purchased from Boehringer Mannheim. The 'Altered Sites' *In Vitro* Mutagenesis System was purchased from Promega.

### Bacterial strains and plasmids

*E. coli* strains were obtained from BRL or Promega. Plasmid pIDT1A, constructed from a 1.8 kb *Eco*RI/*Pst*I fragment of pZAN2 (Neale et al., 1988), subcloned into pUC18 (I. Tonks of this laboratory), contains the entire PDC gene from *Z. mobilis*.

This plasmid was used as the DNA template for mutagenesis by PCR of the proposed ThDP-binding region. *E. coli* cultures containing the pIDT1A construct or mutant constructs were maintained on Luria-broth plates (Sambrook et al., 1989) with 100 $\mu$g/ml ampicillin. Plasmid pSEL-PDC, used as the DNA template for *in vitro* mutagenesis, was constructed by subcloning the same 1.8 kb *Eco*RI/*Pst*I fragment into the phagemid vector pSELECT-1. *E. coli* cultures containing pSEL-PDC or its derivatives were maintained on Luria-broth plates, which contained tetracycline (15 $\mu$g/ml) or ampicillin (125 $\mu$g/ml) as appropriate. Plasmid DNA preparations, restriction-endonuclease digests, isolation of DNA fragments, ligations and transformations were carried out under standard conditions (Sambrook et al., 1989).

### Expression and purification of mutant PDC from *E. coli*

For large-scale production of PDC (wild-type and mutant) in *E. coli*, cells were grown at 37 °C with shaking in 2YT medium (Sambrook et al., 1989) containing 100 $\mu$g/ml ampicillin. Expression of the PDC gene was induced by addition of 1 mM isopropyl $\beta$-D-thiogalactopyranoside (Progen) to cells in the early exponential growth phase. The purification procedure was based on that described by Diefenbach and Duggleby (1991) in which cells are lysed and PDC purified using a one-step hydroxyapatite batch procedure. The enzyme was concentrated to a protein concentration of 15 mg/ml and dialysed against 50 mM Mes/KOH, pH 6.5, which 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. The mutant Pro459Ala was further purified for analysis of ThDP content by chromatography on DEAE-Sephacel (Diefenbach and Duggleby, 1991). Wild-type and mutant PDC apoenzymes were prepared from glycerol stocks as described previously (Diefenbach et al., 1992), except that all buffers were supplemented with 1 mM DTT.

### 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 (Diefenbach and Duggleby, 1991). Unless stated otherwise, reactions were initiated by the addition of PDC obtained directly from purification or from glycerol stocks diluted in 50 mM Mes/KOH, pH 6.5. 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 (LDH) activity. One unit of activity is defined as the quantity of enzyme that catalyses the formation of 1 $\mu$mol of product/min. For each of the active PDC mutants, the $K_m$ for the substrate pyruvate was determined in the standard assay mixture containing various concentrations of the pyruvate.

### Analytical methods

The concentration of stock pyruvate was determined by NADH oxidation in the presence of LDH, modified from the assay of Bucher et al. (1965). Solutions of ThDP were assayed spectrophotometrically by using a $\epsilon_{267}$ of 8520 M$^{-1}\cdot$cm$^{-1}$ determined in this laboratory by Diefenbach (1991). The concentration of Mg$^{2+}$ in stock solutions was determined by atomic-absorption spectrophotometry at 285.2 nm. All protein determinations were performed according to the method of Smith et al. (1985) using a

**Table 1    Sequence of oligonucleotide primers used in mutagenesis reactions**

Mismatches from the *Z. mobilis* PDC sequence are indicated in **bold**. pUCRP* and T7/T3α* are 5' and 3' PCR flanking primers respectively. R = A,G; S = G,C; N = A,C,G,T; D = G,A,T; M = A,C.

| Primer | Sequence | | | Method of mutagenesis |
|---|---|---|---|---|
| pUCRP* | 5' | CGA-CGT-TGT-AAA-ACG-ACG-GCC-AGT | 3' | |
| T7/T3α* | 5' | CAC-AGG-AAA-CAG-CTA-TGA-CCA-TGA | 3' | |
| D4404 | 5' | C-ATG-GTT-GGT-**SAD**-G**S**T-TCC-TTC-C | 3' | *In vitro* mutagenesis |
| E4491 | 5' | ACG-GCT-CAG-GA**C**-GTC-GCT-CAG | 3' | *In vitro* mutagenesis |
| P4591A | 5' | CTG-AAA-CTG-**GGT**-GTT-ATC-ATC-TTC-TTG | 3' | Symmetric overlap extension |
| P4591B | 3' | GCG-GAC-TTT-GAC-**CCA**-CAA-TAG-TAG | 5' | |
| P4592 | 5' | CTG-AAA-CTG-**RNG**-GTT-ATC-ATC | 3' | *In vitro* mutagenesis |
| N4671 | 5' | TTG-ATC-AAT-**RMM**-TAT-GGT-TAC-ACC | 3' | *In vitro* mutagenesis |
| N4672 | 5' | TTG-ATC-AAT-**CAG**-TAT-GGT-TAC-ACC | 3' | One-sided overlap extension |

BCA (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 the addition of a pyruvate/ADH/NADH mixture. Data from the cofactor-binding experiments were analysed by non-linear regression using the DNRP53 computer program (Duggleby, 1984) or a development of this program (DNRPEASY).

The enzyme shows co-operative kinetics for the activation by each cofactor (Diefenbach and Duggleby, 1991). For $Mg^{2+}$ cofactor saturation curves, the rate of the reaction ($v$) as a function of the cofactor concentration ([C]) was described by the equation:

$$v = V_m([C]+[M]_0)^h/[K^h+([C]+[M]_0)^h]$$

where $V_m$ is the rate at saturating cofactor, $K$ is the half-saturating concentration (abbreviated later as $s_{0.5}$) of the cofactor, and $h$ is the Hill coefficient. The additional term, $[M]_0$, is only required when the $Mg^{2+}$ concentration has to be corrected for background metal levels. The equation for ThDP cofactor saturation was as above, but without the background term.

Cofactor binding was determined by monitoring the quenching of the tryptophan fluorescence of PDC on an Aminco SPF-500 spectrofluorimeter with excitation and emission wavelengths of 300 nm (bandwidth 5 nm) and 340 nm (bandwidth 5 nm) respectively.

For stoichiometry determinations, ThDP was measured by oxidation to its fluorescent derivative thiochrome diphosphate, as described by Diefenbach and Duggleby (1991).

## Sephacryl 200 chromatography

The molecular masses of proteins were determined by gel filtration through a column of Sephacryl 200 (Pharmacia). The column, equilibrated with 50 mM Mes, pH 6.5, containing 1 mM DTT, at a flow rate of 5 ml·min⁻¹, was calibrated using

ovalbumin (molecular mass 45 kDa), BSA (66 kDa) and LDH (140 kDa). Blue Dextran (approx. 2 MDa) was used to determine the void volume. Fractions were collected and assayed for PDC activity using the standard activity assay conditions.

## Mutagenesis strategies

During the course of this work, a variety of methods for mutagenesis was tried. Each involves the use of one or more synthetic oligonucleotides. Mutagenic primers for PCR and *in vitro* mutagenesis (Table 1) were designed to be 24–33 bases in length, having a 50 % G+C content or higher where possible, and incorporating single or two to three consecutive base mismatches more than six residues from the 3' end of the primer (Kwok et al., 1990). All mutagenic primers were designed taking into consideration the codon usage for *Z. mobilis* PDC (Neale et al., 1987). The introduction of the base changes in these mutant genes was confirmed by the dideoxynucleotide-chain-termination method of DNA sequencing (Sanger et al., 1977) using a T7 DNA Polymerase Sequencing Kit (Phamacia–LKB Biotechnology).

## Mutagenesis by symmetric overlap extension PCR

Construction of mutant Pro459Gly was by symmetric-overlap-extension PCR. This is a three-reaction procedure requiring two complementary mutagenic oligonucleotide primers (P4591A and P4591B; Table 1) and two flanking oligonucleotide primers (pUCRP* and T7/T3α*; Table 1) and involving the generation of two DNA fragments that, by virtue of having complementary oligonucleotide primers in independent PCR reactions, can be joined at the ends (Ho et al., 1989). A 0.4 kb fragment and a 1.5 kb fragment of the gene containing the desired mutation were amplified in separate reactions using one mutagenic and one flanking primer. These products were mixed with each other and the flanking primers and amplified in a third PCR reaction. The entire PDC gene with the desired mutations was subcloned into pUC18 as a 1.8 kb *Eco*RI/*Pst*I fragment for expression.

The DNA sequence of the entire gene was determined to ensure that only the desired mutation was present. This is necessary, as we have shown previously (Diefenbach et al., 1992) the presence of additional random base changes when making

Asp440 mutants by PCR. Two such mutants were chosen for further study: the mutated regions of Thr119Ile (ACC → ATC) and Thr132Ala (ACG → GCG) were subcloned into wild-type pIDT1A using EcoRI/NcoI and EcoRI/PstI respectively.

## Mutagenesis by one-sided-overlap-extension PCR

The mutant Asn467Gln was constructed by one-sided-overlap-extension PCR. This two-reaction process requires a single mutagenic primer (N4672) and the two flanking primers (Landt et al., 1990). The 1 kb NcoI/PstI fragment of the PCR product containing the mutated 3' end of the PDC gene was subcloned into the 3.5 kb NcoI/PstI fragment of pIDT1A for expression; DNA sequencing confirmed the mutation.

## In vitro mutagenesis using a phagemid

Mutants Asp440Glu, Glu449Asp, Pro459Ala and Asn467Asp were constructed using the 'Altered Sites' In Vitro Mutagenesis System as described previously (Diefenbach et al., 1992). Mutants were screened by DNA sequencing, and the DNA with the desired mutation was then subcloned into pUC18 as a 1.8 kb EcoRI/PstI fragment. In the case of Glu449Asp, the base change introduced a unique AatII site, and this was used to screen for the desired mutation.

## RESULTS

### The Asp440Glu mutant

We have shown previously (Diefenbach et al., 1992) that substitution of Asp440 in PDC from Z. mobilis with Asn, Thr or Gly yields an inactive protein. In order to further characterize the role of this residue, it was replaced with glutamate. Expression

of the mutant Asp440Glu in E. coli yielded an active enzyme after purification; a final specific activity of 1.37 units/mg was obtained, and the protein appeared close to purity as judged by SDS/PAGE. This is very low when compared with the wild-type enzyme (usually around 70 units/mg) but, as will be discussed below, assaying this enzyme at very high ThDP concentrations gave a higher specific activity.

In contrast with the linear change in absorbance over time found in the activity assay of native Z. mobilis PDC and recombinant wild-type PDC, the Asp440Glu mutant exhibited a distinct lag phase in product formation when assayed without prior incubation with cofactors (Figure 1a). The presence of this lag phase made it very difficult to determine the activity of Asp440Glu. Clearly the initial rate does not represent its full catalytic potential; however, no true steady state was reached by the time NADH was exhausted from the assay. To test whether this lag phase was due to slow binding of the cofactors, the Asp440Glu holoenzyme was preincubated in reaction mix for a period of 1–20 min before adding pyruvate to initiate the reaction. These experiments showed that preincubation of Asp440Glu with cofactors neither increased the rate of the reaction nor eliminated the lag phase.

Varying the concentration of ThDP in the reaction mix over the range of 0.05–5 mM had a dramatic effect on the length of the lag phase (Figure 1a). However, even at extremely high concentrations of ThDP, the lag phase in product formation was not eliminated entirely for the Asp440Glu mutant PDC enzyme.

The affinity of both the Asp440Glu holoenzyme and apoenzyme for ThDP was determined by activity assay. The pH of the reaction mixtures was monitored to ensure that the pH was not altered by the high ThDP content. The resulting ThDP saturation curve for the holoenzyme is shown in Figure 1(b). Reaction rates were determined from measurements taken along the most linear part of the time-course curves, although there is clearly some uncertainty in the precision of these determinations. The $s_{0.5}$ obtained from ThDP was $1.47 \pm 0.11$ mM for the holoenzyme and $1.49 \pm 0.37$ mM for the apoenzyme. Because of the lower affinity of Asp440Glu for ThDP, a modified reaction mix containing a final ThDP concentration of 2 mM was used to evaluate the kinetics towards the metal-ion cofactor and substrate. The half-saturating $Mg^{2+}$ concentration was $346 \pm 58$ $\mu$M for the apoenzyme (results not shown), while the $K_m$ for pyruvate was $0.95 \pm 0.03$ mM. Further, the decreased affinity of Asp440Glu for ThDP causes the standard assay to greatly underestimate the specific activity of this mutant enzyme. By using a reaction mixture with 2 mM ThDP, the specific activity obtained for this mutant after purification increased from 1.37 to 14.2 units/mg of protein.

The failure of high cofactor concentrations to eliminate the lag phase implies the existence of a slow step in activation of the enzyme. Although Diefenbach and Duggleby (1991) have shown that both the apoenzyme and holoenzyme are tetrameric, Asp440Glu may undergo dissociation into monomers or dimers, and the lag phase may represent the time required for these other forms to be converted into the tetramer. The elution profiles of the Asp440Glu holoenzyme and apoenzyme were compared with those obtained for wild-type PDC purified from Z. mobilis. PDC holoenzyme from Z. mobilis was eluted as a single peak which corresponded to the void volume of the column (exclusion size 200 kDa) and at the elution position expected of the tetramer (Figure 2). By contrast, both Asp440Glu holoenzyme and Asp440Glu apoenzyme were eluted as a molecular mass of 126 kDa (Figure 2). This suggests that the Asp440Glu mutant exists almost entirely in the dimeric form, at least under non-turnover conditions.
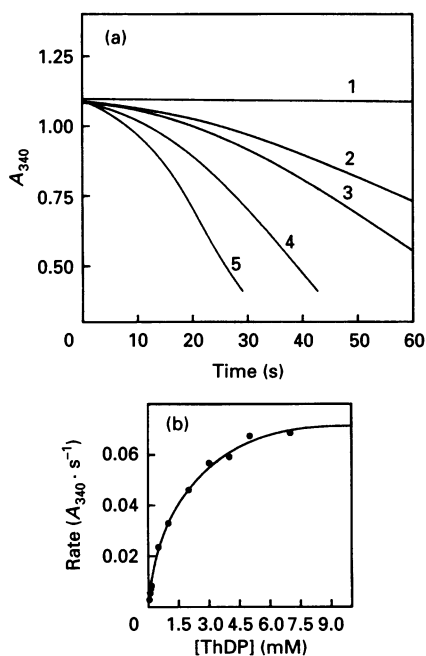


**Figure 1   Activation of Asp440Glu PDC by ThDP**

(a) Progress curves in the presence of 5 mM $Mg^{2+}$ and ThDP concentrations of 0 mM (1), 0.143 mM (2), 0.25 mM (3), 0.5 mM (4) and 1 mM (5). (b) ThDP saturation curve.
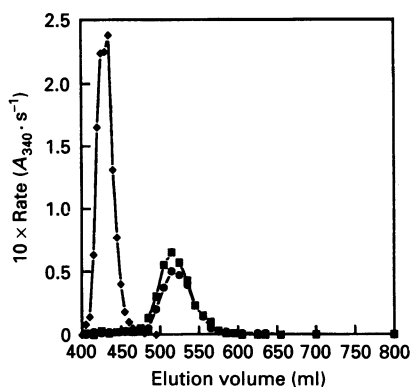
**Figure 2   Activity profiles of Z. mobilis PDC and Asp440Glu PDC after elution from Sephacryl 200**

PDC activity profiles of *Z. mobilis* PDC (◆), Asp440Glu holoenzyme (●) and Asp440Glu apoenzyme (■).

**Table 2   Affinity of PDC for substrate and cofactors**

| Enzyme | $K_m$ for pyruvate (mM) | $s_{0.5}$ ($\mu$M) for: ThDP | $s_{0.5}$ ($\mu$M) for: Mg$^{2+}$ |
|---|---|---|---|
| Wild-type PDC | 0.52 ± 0.05 | 2.53 ± 0.16 | 5.87 ± 0.43 |
| Thr119Ile | 0.74 ± 0.05 | * | * |
| Thr132Ala | 0.53 ± 0.04 | 0.60 ± 0.03 | 15.5 ± 1.5 |
| Asp440Glu (holoenzyme) | 0.95 ± 0.03 | 1472 ± 109 | ND |
| Asp440Glu (apoenzyme) | ND‡ | 1485 ± 365 | 346 ± 58 |
| Glu449Asp | 0.52 ± 0.04 | 2.11 ± 0.16 | 7.26 ± 0.31 |
| Pro459Gly | 0.87 ± 0.03 | 4.71 ± 0.61 | 11.1 ± 0.1 |
| Pro459Ala | 0.67 ± 0.03 | 1.81 ± 0.17 | 4.34 ± 0.28 |
| Asn467Gln | † | * | * |
| Asn467Asp | 0.95 ± 0.02 | 1466 ± 141 | 4.84 ± 0.76 |

* No activation by added cofactor.
† Inactive.
‡ ND, not determined.

## The Glu449Asp mutant

Changing Glu449 to Asp yielded an active enzyme that had a specific activity after purification of 12.5 units/mg; despite this rather low value, the enzyme appeared to be close to purity by SDS/PAGE. The kinetics towards substrate and cofactor were found to be similar to those of wild-type PDC (Table 2).

## Pro459 mutants

The mutants Pro459Gly and Pro459Ala are both active, with final specific activities of 13 units/mg of protein for Pro459Gly and 3.6 units/mg of protein for Pro459Ala. Both enzymes exhibited kinetic properties towards pyruvate, ThDP and Mg$^{2+}$ that are similar to those of wild-type PDC (Table 2). The low specific activity of Pro459Ala was investigated further by chromatography on DEAE-Sephacel, since it appeared to be reasonably pure on SDS/PAGE. The pooled active fractions had a specific activity of 28.2 units/mg of protein, and the ThDP

content of this material was found to be 6.27 $\mu$mol/g. From these values, the activity may be expressed as 4.5 units/nmol of ThDP, a value similar to that for wild-type PDC (6.2 units/nmol of ThDP). Thus the apparently low specific activity of Pro459Ala seems to be due mainly to the presence of a substantial proportion of inactive PDC protein rather than an intrinsically low activity of the mutant enzyme.

## Asn467 mutants

Expression of the mutant Asn467Asp in *E. coli* yielded an active enzyme with a final specific activity of 8.4 units/mg of protein after purification. This enzyme has a $K_m$ for pyruvate of 0.95 ± 0.02 mM. The affinity of this mutant PDC for Mg$^{2+}$ was similar to that of *Z. mobilis* PDC and wild-type recombinant PDC, with a value for the half-saturating concentration of 4.84 ± 0.76 $\mu$M. However, the affinity of Asn467Asp for ThDP was significantly decreased, with an $s_{0.5}$ value of 1.47 ± 0.14 mM. In contrast with Asp440Glu, no lag phase in product formation was apparent in the time-course curves of Asn467Asp. The mutant Asn467Gln, although successfully purified, was inactive. This protein is unable to bind either ThDP or Mg$^{2+}$ in the presence of the other cofactor, as determined by fluorescence measurements.

## Thr119Ile and Thr132Ala

While there was no original intention to make these proteins, they provide a useful reference for the alterations in the properties of PDC that might be expected from arbitrary changes in the sequence. Each was purified to near homogeneity (as judged by SDS/PAGE) to yield a final product with a specific activity of 29 units/mg (Thr119Ile) and 6.4 units/mg (Thr132Ala). In the latter case this is well below the value observed for the wild-type enzyme and is probably due to extreme instability of this mutant, which lost 90 % of its activity during overnight dialysis which is the final step of the purification. Large losses were also observed when making the apoenzyme, making it difficult to determine the kinetics with respect to the cofactor. The half-saturating concentrations so determined appeared to be somewhat different from those of the wild-type, while the kinetics towards pyruvate were normal (Table 2). Thr119Ile also had unchanged kinetics towards the substrate, but was stable until cofactors were removed, whereupon it lost all activity and could not be reconstituted. Thus it was not possible to characterize the affinity for ThDP or Mg$^{2+}$.

## DISCUSSION

PDC protein from all mutants, including the inactive Asn467Gln mutant, was successfully purified using the hydroxyapatite batch procedure developed for native *Z. mobilis* PDC and recombinant wild-type PDC, indicating that the three-dimensional structure of these mutants has not been grossly altered.

Comparison of the kinetic properties of the mutant forms with the wild-type PDC expressed in *E. coli* showed that substitution of aspartate-440 or asparagine-467, residues at each end of the ThDP-binding motif, had large effects on cofactor binding without affecting the affinity for pyruvate (Table 2). In contrast, substitutions of other residues in this region had little effect. These findings may be interpreted by considering the three-dimensional structure of the enzyme's counterpart in yeast (Dyda et al., 1993).

Firstly, Glu449 does not seem to be particularly important, despite being highly conserved. However, the replacement tested
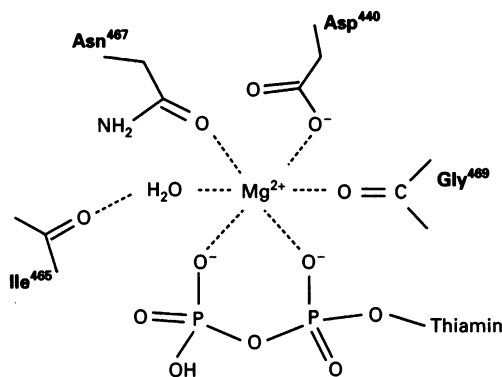
**Figure 3   Schematic representation of the Mg²⁺-binding region of PDC**

The structure is modified from that of the yeast enzyme (Dyda et al., 1993).

here, with aspartate, is a very conservative one, and more radical changes may result in significant changes to the properties of the enzyme. In much the same way, Pro459 is located at some distance from ThDP, and its replacement with other residues is easily accommodated in the structure without major activity changes. The only effect seems to be some difficulty in folding correctly, as the mutants purified together with substantial amounts of inactive PDC protein. This does not indicate any crucial role for Pro459, and similar effects have been observed in other mutants. For example, changing threonine-119 to iso-leucine results in an enzyme that has apparently normal properties until the cofactors are removed; it then undergoes rapid, ir-reversible, inactivation. This residue is not conserved across PDCs from different species and is located well away from the active site, so it is unlikely to be playing any special role. Similarly, changing threonine-132 to alanine gave an enzyme that is very unstable, even when both cofactors are present; again, this residue is nowhere near the active site.

The aspartate residue at position 440 in Z. mobilis PDC is highly conserved among ThDP-dependent enzymes. We have shown previously that substitution of aspartate-440 in PDC with glycine, threonine or asparagine results in the complete loss of enzyme activity (Diefenbach et al., 1992). These mutant enzymes contain no bound ThDP and are unable to bind ThDP even at levels far in excess of the half-saturating concentrations of the wild-type PDC. The requirement for an aspartate is not absolute, and we have now shown that replacement with a glutamate residue results in an enzyme that is still active, although with a substantial decrease in its affinity for cofactors.

The region around position 440 of the yeast PDC structure is illustrated in Figure 3. There is direct bonding to the Mg²⁺, and this may be why a negatively charged residue is required. Changing this residue to glutamate will cause the carboxy group to intrude further into the cofactor-binding region, making it more difficult for both cofactors to bind, as we observe.

An unusual feature of the Asp440Glu mutant is a prominent lag phase when the enzyme is assayed, and it may be significant that, in contrast with the tetrameric form of the Z. mobilis PDC (Diefenbach et al., 1992), both the Asp440Glu holoenzyme and apoenzyme exist in a dimeric form. We suggest that the lag phase in product formation exhibited by Asp440Glu PDC represents the time required for the dimeric form of this mutant to reassemble into active tetramers. This lag phase could be reduced by high levels of cofactors, but it could not be eliminated.

At the other end of the ThDP-binding motif, replacement of asparagine-467 with aspartate gave an active enzyme, while substitution with glutamine caused a loss of activity. This implies that size is crucial; changing Asn467 to glutamine would cause the carboxamido group to push further into the cofactor-binding region (Figure 3). Evidently there is little conformational flexi-bility in this part of the protein, as the result is a total loss of ThDP-binding capacity and, consequently, an inactive protein. It is noteworthy that this residue is very highly conserved in the ThDP-binding motif described by Hawkins et al. (1989). Indeed, with a slightly different alignment of the E1 component of the pyruvate dehydrogenase complex of E. coli it could be argued that this residue is conserved completely. Thus it is surprising that it can be replaced with aspartate to yield an active enzyme.

Perhaps the most puzzling result for Asn467Asp is that it has reduced affinity for ThDP, but a normal affinity for Mg²⁺. The fact that it is active is not too surprising, as the substitution involves no change in size, and the carboxy group is likely to co-ordinate to Mg²⁺ as well as, or better than, a carboxamide group. Why, then, should the affinity for ThDP be diminished? It may be that the additional negative charge totally neutralizes the charges on the Mg²⁺, thereby weakening the bonding to the negatively charged phosphate oxygens of ThDP (Figure 3). It is noteworthy that the 580-fold-weaker binding of ThDP observed in this mutant is very similar in magnitude to the enhancement of ThDP binding (530-fold) caused by the presence of Mg²⁺ in the wild-type enzyme (Diefenbach and Duggleby, 1991).

Some obvious new mutagenic targets beckon; the conserved glycine residues flanking aspartate-440 (Russell et al., 1992), as well as asparagine-466, clearly have important roles in the ThDP-binding motif. However, it is now evident from the structures that have been determined that this motif interacts only with the phosphates of ThDP. The thiazole and pyrimidine rings are involved in a host of other interactions with residues that are scattered through the sequence, mostly on an adjacent subunit. Of particular interest is glutamate-50. This conserved residue is close to N-1 of the pyrimidine ring and may participate in a proton relay that ultimately leads to the ionization of the thiazole C-2 (Muller et al., 1993; Schneider and Lindqvist, 1993). Experiments to test this hypothesis by mutating glutamate-50 are currently underway.

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