The role of His113 and His114 in pyruvate decarboxylase from *Zymomonas mobilis*

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Pyruvate decarboxylase (PDC) is one of several enzymes that require thiamin diphosphate (ThDP) and a divalent cation as essential cofactors. Recently, the three-dimensional structures of the enzyme from two yeasts have been determined. While these structures shed light on the binding of the cofactors and the reaction mechanism, the interactions between the substrate pyruvate and the enzyme remain unclear. We have used PDC from *Zymomonas mobilis* as a model for these enzymes in order to study substrate binding. The recombinant enzyme was expressed in *Escherichia coli*. High yield, simplicity of purification, high stability and simple kinetics make this model well suited for these studies. Activity measurements in the pH range between 5.8 and 8.5 indicated that a His residue may be involved in substrate binding. Analysis of an alignment of all known PDC protein sequences showed two invariant His residues (His113 and His114) which, according to the crystal structure of yeast PDC, are in the vicinity of the active site. Here we demonstrate that replacement of His114 by Gln does not have a great effect on cofactor and substrate binding. However, the k_{cat} is decreased indicating that His114 may assist in catalysis. In contrast, substitution of His113 by Gln renders the enzyme completely inactive. This mutant has decreased affinity for both cofactors, as revealed by measurements of tryptophan fluorescence quenching. However, this decreased affinity is insufficient to account for the complete loss of activity. Despite its inability to support overall catalysis, this [Gln113]PDC mutant is capable of releasing acetaldehyde from 2-(1-hydroxyethyl)thiamin diphosphate supplied exogenously. It is proposed that upon substrate binding, His113 is placed close to C2 of the thiazole ring. Subsequent deprotonation of this atom leads to a conformational change that allows a flexible loop (residues 105−112) that precedes His113 to close over the active site. Hence, replacement of His113 by another residue interferes with this closure of the active site and thus disrupts the catalytic process.

Keywords: pyruvate decarboxylase; *Zymomonas mobilis*; site-directed mutagenesis; substrate binding; catalysis.

Pyruvate decarboxylase (PDC), a cytosolic enzyme found in fungi, plants and some bacteria, is an enzyme that uses thiamin diphosphate (ThDP) as a cofactor. All ThDP-dependent enzymes are believed to follow a similar reaction mechanism. The initial step is the formation of a carbanion at C2 of the thiazole ring of the enzyme-bound cofactor (Kern et al., 1997). The negative charge on this carbon atom enables the nucleophilic attack on the carbonyl carbon of the substrate (Breslow, 1958, 1962; Breslow and McNelis, 1959; Kuo and Jordan, 1983). Cleavage of an adjacent carbon-carbon bond results in the release of the first product (CO_2 in PDC) and formation of a second carbanion that is then protonated to give 2-(1-hydroxyethyl)thiamin diphosphate (he-ThDP). In the final step ThDP is regenerated and the second product (acetaldehyde) is released.

PDC has a sequence motif that is common to all ThDP-binding enzymes (Hawkins et al., 1989). The motif usually begins with the sequence Gly-Asp-Gly at the N-terminal and ends with Asn-Asn at the C-terminal. Various residues are conserved to a lesser extent within the motif. The three-dimensional structure of the ThDP-dependent enzymes *Saccharomyces cerevisiae* transketolase (Lindqvist et al., 1992; Nikkola et al., 1994), *Lactobacillus plantarum* pyruvate oxidase (POX) (Müller and Schulz, 1993), *S. uvarum* PDC (Dyda et al., 1993), *S. cerevisiae* PDC (Arjunan et al., 1996) and *Pseudomonas putida* benzoylformate decarboxylase (BDC) (Hasson et al., 1996) have been determined. These structures are composed of three distinct domains. In PDC, BDC and POX, the ThDP motif is located in the C-terminal domain while in transketolase it is in the N-terminal domain. Interestingly, the three-dimensional structures suggest that while PDC, BDC and POX are collinear, transketolase seems to be a circular permutation of the others where the first and second domain of transketolase correspond to the third and first domains of the other two enzymes (Müller et al., 1993).

Organism	Sequence
Z. mobilis	^{103}NNDHAAGHVLHHALGKTDYHYQ^{124}
Common fungal	ISSQAKQLLLHHTLGNGDFTVF
S. cerevisiae 6	ISAQAKQLLLHHTLGNGDFTVF
N. crassa	TNDPSQYHILHHTLGHPDYTYQ
A. parasiticus	RASQESRALIHHTFNDGDYQRF
K. lactis	VSSQAKQLLLHHTLGNGDFTVF
H. uvarum	LASQAKQLLLHHTLGNGDFDVF
Common plant	SNDYGTNRILHHTIGLPDFSQE
A. thaliana 2	SNDYGTNRILHHTIGLPDFTQE
N. tabacum 1	SNDYGTNRILHHTIGLQDFSQE
N. tabacum 2	TNDYGTNRILHHTIGLPDFSQE

Fig. 1. Alignment of the region around the conserved His residues in 18 PDC protein sequences. The *Z. mobilis* sequence was taken from Genbank (Accession code X59558) and corresponds to residues 103–124. The underlined segment coincides with a region that is invisible in the X-ray structure of yeast PDC (Dyda et al., 1993; Arjunan et al., 1996). The common fungal sequence is identical over this region in *S. cerevisiae* 1 (X77316), *S. cerevisiae* 5 (X15668) and *Kluyveromyces marxianus* (L09727). Other fungal sequences are *S. cerevisiae* 6 (X66843), *Neurospora crassa* (L09125), *Aspergillus parasiticus* (U00967), *K. lactis* (X85968) and *Hanseniaspora uvarum* (U13635). The common plant sequence is identical over this region in *Oryza sativa* 1 (U26660), *O. sativa* 2 (U27350), *O. sativa* 3 (U07338), *Zea mays* (X59546), *Arabidopsis thaliana* 1 (U71121) and *Pisum sativum* (Z66543). Other plant sequences are *A. thaliana* 2 (U71122), *Nicotiana tabacum* 1 (X81854) and *N. tabacum* 2 (X81855). The conserved His are shown in bold type.

Robinson and Chun (1993) came to similar conclusions using a sequence alignment of PDC, transketolase, and the E1 component of the pyruvate dehydrogenase complexes of *Homo sapiens*, *Bacillus stearothermophilus* and *Escherichia coli*. However, this alignment failed to identify the conserved Glu residues that are now known to be required for catalysis in transketolase (Wikner et al., 1994) and PDC (Candy et al., 1996a).

While the crystal structures (Dyda et al., 1993; Arjunan et al., 1996) and mutagenesis studies (Diefenbach et al., 1992; Candy and Duggleby, 1994; Candy et al., 1996a) of PDC have shed light on the binding of the cofactors and the reaction mechanism, the interaction between substrate and enzyme remains unclear. In contrast, the three-dimensional structure of yeast transketolase (Nikkola et al., 1994) shows an identifiable substrate channel located at the subunit interface. This funnel-shaped channel is lined by various invariant or conserved residues; one of them (His481) has been implicated with both substrate binding and catalysis (Lindqvist et al., 1992; Nikkola et al., 1994). However, it is now known that in mammalian transketolases this particular His residue is replaced by Gln (Schenk et al., 1997) rendering a possible function in catalysis less likely.

We have used the recombinant *Zymomonas mobilis* enzyme expressed in *E. coli* for our studies of the structure and function of PDC due to the high yield, simplicity of purification, stability and simple kinetics of this enzyme. Alignment of known PDC protein sequences from bacteria, fungi and plants reveals that all are collinear, so studies on any one PDC are of relevance to all these enzymes. The sequence alignment shows a pair of His (His113 and His114 in *Z. mobilis* PDC) that are conserved absolutely; this region of the aligned PDC sequences is shown in Fig. 1. The crystal structure of yeast PDC (Arjunan et al., 1996), shows that both His are close to the active site (7.9 Å and 6.2 Å, respectively; Fig. 2) and form part of a cleft leading to the re-
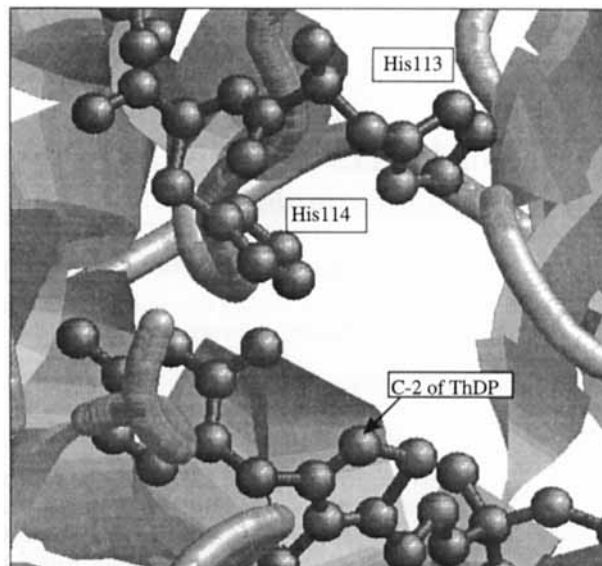

Fig. 2. Structure of the active site of PDC. The structure shown was drawn using the coordinates of *S. cerevisiae* PDC (Arjunan et al. 1996). The positions of His113 and His114 in *Z. mobilis* PDC are assumed to be equivalent to the corresponding residues (His114 and His115) in *S. cerevisiae* PDC.

active centre. Given that these residues and most others surrounding the ThDP are conserved between yeast and *Z. mobilis* PDC, we believe that the active site of both enzymes would be similar. A molecular modeling study (Lobell and Crout, 1996a) suggested that both His are protonated and His114, together with the 4'-amino group of the cofactor, has been implicated in interactions with the alcoholate ions of lactyl-ThDP and he-ThDP (Harris and Washabaugh, 1995). Interestingly, these two residues are preceded by a flexible loop (Dyda et al., 1993; Arjunan et al., 1996) of yet unknown function. The positions of the atoms in this loop are partially resolved in the structure of yeast PDC crystallized in the presence of the activator, pyruvamide (Lu et al., 1997). It is conceivable that this region of the protein might form a mobile flap that closes over the active site upon substrate binding which might then result in the movement of the His residues closer to the active site. This closure of the active site is in agreement with recent studies on the catalysis and regulation of PDC activity, which suggest a sequestration of the active site during catalysis (Alvarez et al., 1995; Harris and Washabaugh, 1995; Lobell and Crout, 1996b). Several possible roles for His113 and/or His114 are conceivable: (a) action as a base to assist ionization of ThDP; (b) provision of a cationic group to favour binding of the anionic substrate; (c) action as a source of the proton that is necessary for the overall reaction; (d) participation in cofactor binding.

In order to investigate the function of these residues we replaced both His113 and His114 by Gln. In this paper the effects of these mutations on substrate binding, cofactor binding and catalysis are discussed. A conference report of part of this work has appeared elsewhere (Candy et al., 1996b)

MATERIALS AND METHODS

Restriction endonucleases and molecular biology products. Restriction enzymes were purchased from New England Biolabs, T4 DNA ligase from Boehringer Mannheim, deoxyribonucleotides from Perkin Elmer and *Taq* polymerase from Dynazyme. For sequencing we used the Prism Ready Dye Deoxy

Table 1. Primers used for mutagenesis of His113 and His114. Base differences between wild type and the two Gln113-containing oligonucleotides are shown in lower case. These include a silent mutation that is introduced into the His114 codon to create an *Sph*I site (underlined). The lower case bases in Gln114/Rev indicate differences from the [Gln113]PDC template DNA; these repair the [Gln113]PDC mutation, introduce the [Gln114]PDC mutation, and eliminate the *Sph*I site.

Oligonucleotide	Sequence
pPL450/For	5′-GGGCAGCATTCAAAGCAGAAGGC-3′
pPL450/Rev	5′-CGTTGTAAAACGACGGCCAGT-3′
	ValLeuHisHisAlaLeuGly
Wild-type	5′-CGTGTTGCATCACGCTCTTGGC-3′
Gln113/For	5′-CGTGTTGCAgCAtGCTCTTGGC-3′
Gln113/Rev	3′-GCACAACGTcGTaCGGAGAACCG-5′
Gln114/Rev	3′-GCACAACGTaGTcCGAGAACCG-5′

Terminator cycle sequencing kit from Applied Biosystems. PCR reactions were performed with a Perkin Elmer DNA thermal cycler (Model 480).

Bacterial strains and plasmids. *E. coli* strain DH5α was obtained from Gibco BRL. The plasmid pPLZM was constructed by subcloning a 1.8-kb *Eco*RI−*Sph*I fragment of pIDT1A (Candy and Duggleby, 1994), containing the entire PDC gene from *Z. mobilis*, into the expression vector pPL450 (Elvin et al., 1990) (a gift from Dr N. E. Dixon, Research School of Chemistry, Australian National University). All *E. coli* cultures containing the wild-type and the mutant constructs were maintained on Luria broth plates (Sambrook et al., 1989) with 100 μg/ml ampicillin. For long-term storage, liquid cultures were kept in 15% glycerol and stored at −20°C. Plasmid DNA preparations, restriction endonuclease digestions, ligations and transformations were carried out according to standard protocols (Sambrook et al., 1989).

In vitro **mutagenesis by symmetric overlap extension PCR.** The mutation of His113 to Gln (designated [Gln113]PDC) was introduced by symmetric overlap extension PCR. Two complementary mutagenic oligonucleotide primers (Gln113/For and Gln113/Rev; Table 1) and two flanking primers (pPL450/For and pPL450/Rev; Table 1) were designed. The mutagenic primers introduce a silent change in the codon for His114 and thereby create an additional *Sph*I site that was used to screen for the [Gln113]PDC mutation. The first step involved amplification of two DNA fragments using one mutagenic and one flanking primer. Due to the complementarity of the mutagenic primers, these two fragments (0.5 kb and 1.4 kb) were overlapping (Ho et al., 1989). In the second step the two fragments and the two flanking primers were mixed and amplified by PCR. An *Asp*718−*Nco*I 709-bp fragment with the desired mutation was subcloned into pPLZM. The mutation was confirmed by DNA sequencing and restriction digestion.

The following protocol proved to be successful for the first-round amplification. In 500 μl PCR tubes (Perkin Elmer), 50 ng template DNA (pPLZM linearized with the restriction enzyme *Sca*I) was mixed with 250 μM dNTPs, 1 μM of each sequence-specific primer (pPL450/For plus Gln113/Rev for one mutagenic fragment and pPL450/Rev plus Gln113/For for the other, respectively) and reaction buffer [10 mM Tris/HCl pH 8.8, 50 mM KCl, 0.01% (by vol.) Tween 20, 0.01% (by vol.) Nonidet P40]. Mg^{2+} concentrations of 2.0 mM or 2.5 mM were equally suitable for amplification of the desired fragments. Water was added to a final volume of 49 μl. The reaction mix was heated to 96°C for 2 min to denature the double-stranded template DNA and destroy any contaminating nucleases before the *Taq* polymerase was added. The tube was spun down to collect any solution from

the top of the tube and placed immediately in the thermal cycler at 96°C. *Taq* polymerase (1 U) was added and the mixture was overlaid with 40 μl paraffin oil to reduce evaporation at high temperatures (Mezei, 1990) and to facilitate efficient transfer between cycles. Subsequently, the samples were subjected to 25 cycles of denaturation (96°C, 1 min), annealing (51°C, 1 min) and extension (72°C, 2 min). A final extension period of 7 min at 72°C was added to make sure that the production of fully extended double-stranded molecules from all nascent strands was complete. In the second round of amplification 50 ng of both mutagenic fragments were mixed with 250 μM dNTPs, 1 μM of each flanking primer (pPL450/For and pPL450/Rev) and reaction buffer. Mg^{2+} concentrations between 2.0−3.0 mM were suitable for amplification. Water was added to a final volume of 49 μl and the reactions were started as described above; the annealing temperature was 55°C.

Mutagenesis by one-sided overlap extension PCR. The mutation of His114 to Gln was introduced by one-sided overlap extension PCR (Landt et al., 1990). One mutagenic oligonucleotide primer (Gln114/Rev; Table 1) and two flanking primers (pPL450/For plus pPL450/Rev; Table 1) were used. The template used contained the [Gln113]PDC mutation and the mutagenic primer repaired this change, thereby removing the *Sph*I site; restriction digestion with *Sph*I allowed initial screening for the [Gln114]PDC mutation. The first step involved amplification of one mutagenic DNA fragment (0.5 kb) using the mutagenic primer and pPL450/For. In the second round of PCR this fragment was used as a megaprimer (Landt et al., 1990) together with pPL450/Rev. An *Asp*718−*Nco*I fragment containing the desired mutation was subcloned into pPLZM and the mutation was confirmed by DNA sequencing and restriction digestion.

For the first round of PCR, the conditions were the same as described for the first-round amplification of the [Gln113]PDC mutation (see above). In the second round 50 ng template DNA (pPLZM containing the [Gln113]PDC mutation, linearized with *Sca*I), 250 μM dNTPs, 1 μM pPL450/Rev (flanking primer), 2.5 mM Mg^{2+} and reaction buffer were mixed. Amplifications using 10 ng, 20 ng, 30 ng, 40 ng and 50 ng of the megaprimer from the first round produced fragments of the expected size. The 25 PCR cycles were started as described above; the annealing temperature was 55°C.

Expression and protein purification. For large-scale expression of PDC the cells were grown in 10 l 2YT medium (Sambrook et al., 1989) containing 100 μg/ml ampicillin at 30°C in a 20-l Chemap fermenter with aeration and stirring (320 rpm). When the cell culture reached an A_{600} of 0.5 the temperature was rapidly increased to 42°C and the induction was maintained for 3 h. The cells were harvested by centrifugation at 4°C for 15 min at $2500 \times g$. The cell pellet was stored at −20°C.

The purification of PDC was based on the protocol described by Diefenbach and Duggleby (1991) with some modifications. The cells were resuspended in lysis buffer (6 ml/g cell paste) consisting of 100 mM potassium phosphate pH 6.8, 0.1 mM ThDP, 5 mM $MgCl_2$, 0.6 mg/ml lysozyme, 0.01 μg/ml DNase I, 18.5 mM 2-mercaptoethanol, 2 μl/ml Nonidet P-40, 20 μg/ml leupeptin, 0.02 U/ml aprotinin and 0.3 μg/ml bestatin; 1 g glass beads/g cell paste was added and the cells were stirred at room temperature (≈22°C) for 2 h. The enzyme was partially purified using a one-step hydroxyapatite batch procedure (1 g hydroxyapatite/6 ml lysate) (Diefenbach and Duggleby, 1991). The eluate was concentrated to 15 mg/ml and dialyzed overnight against three changes of 50 mM Mes/KOH pH 6.5, containing 2 mM $MgCl_2$, 0.1 mM ThDP and 1 mM dithiothreitol. PDC was further purified by ion-exchange chromatography on a DEAE-Sephacel column. The enzyme was eluted by a linear 2−50 mM $MgCl_2$

gradient. Peak fractions were pooled and stored at $-20\,^{\circ}$C after mixing with an equal volume of glycerol.

Preparation of apoenzyme. All glassware, plastic cuvettes and tips were soaked in 20% (by vol.) HNO_3 and rinsed several times with metal-free water (18.2 mΩ/cm, MilliQ water purification system, Millipore). PDC preparations were diluted 20-fold with 50 mM Tris/HCl pH 8.5 containing 1 mM EDTA and 1 mM dithiothreitol. The solution was stirred at room temperature for 30 min and concentrated to 1 mg/ml by ultrafiltration. After passage through a BioGel P-6DG (BioRad) column (30 cm$\times$ 2.5 cm^2) equilibrated with Tris/EDTA/dithiothreitol buffer the sample was concentrated and passed through another BioGel P-6DG column (25 cm$\times$2.5 cm^2) equilibrated with metal-free 50 mM Mes/KOH pH 6.5 containing 1 mM dithiothreitol. The peak fraction was collected and stored at 4°C. Metal-free Mes buffer and ThDP stock solutions used for cofactor binding studies were passed through a Chelex 100 (BioRad) column to remove divalent metal ions.

Activity assay for PDC. PDC activity was assayed in a coupled enzyme assay at 30°C. The rate of acetaldehyde production from 10 mM pyruvate was determined by measuring the oxidation of NADH in the presence of alcohol dehydrogenase (ADH) (Diefenbach and Duggleby, 1991). In general, unless otherwise mentioned, reactions were started by addition of PDC. The amount of enzyme was chosen so that a steady decrease of NADH absorption could be monitored over 5 min. During purification, assays were performed with and without addition of ADH. The latter was taken as a measure for lactate dehydrogenase (LDH) activity that is present in cell extracts but separated during purification. The activity unit is defined as the amount of enzyme that catalyses the formation of 1 μmol product/min. The K_m for pyruvate was determined in the standard assay mixture with varying concentrations of substrate.

pH studies. The effect of pH on the kinetic properties of PDC was determined by conducting assays as described above, varying the pyruvate concentration and pH. All assays were performed in duplicate. The buffer contained 50 mM Mes, 100 mM Tris and 50 mM acetic acid adjusted to the appropriate pH with NaOH or HCl; this mixture allows the ionic strength to remain constant over a wide range of pH values (Ellis and Morrison, 1982). Approximate K_m values for each pH were estimated from preliminary studies at pH 5.5, 6.5, 7.5, 8.5 and 9.5. Subsequently, assays were performed over a range of eight pyruvate concentrations (0.1$-$5 K_m, and 12 pH values (5.8$-$8.5).

Analytical methods. The concentration of stock pyruvate was determined by NADH oxidation in the presence of LDH as described previously (Candy and Duggleby, 1994). ThDP stock solutions were assayed spectrophotometrically using the ε_{267} of 8520 M^{-1} cm^{-1} (Diefenbach and Duggleby, 1991). Mg^{2+} concentrations in stock solutions was measured by atomic absorption spectrophotometry at 285.5 nm. The bicinchoninic acid protein determination kit (Sigma Chemical Company) was used to measure protein concentrations (Smith et al., 1985).

Measurement of cofactor binding. Cofactor binding was studied by measuring the activity of the reconstituted holoenzyme. The apoenzyme was incubated for 15 min at 30°C with saturating concentration of one cofactor while varying the concentration of the other. The reaction was started by adding a pyruvate/NADH/ADH mixture and the data obtained were analyzed as described elsewhere (Candy and Duggleby, 1994).

Cofactor binding was also measured by monitoring the quenching of tryptophan fluorescence of PDC (Diefenbach and Duggleby, 1991) using a Jasco model FB-770 spectrofluorimeter. Excitation was at 300 nm (bandwidth 5 nm) and emission was measured at 340 nm (bandwidth 5 nm). Results were analyzed according to Diefenbach and Duggleby (1991).
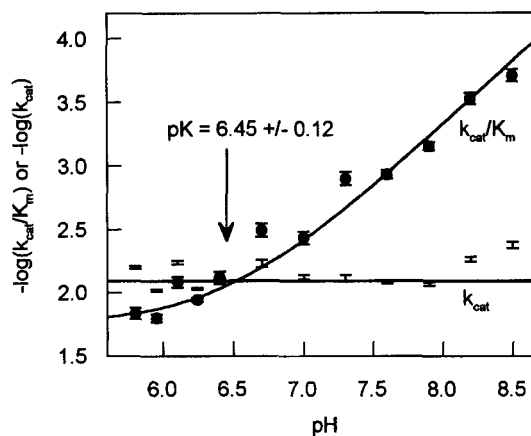

Fig. 3. Effect of pH on the kinetic properties of wild-type PDC. Values for k_{cat} and k_{cat}/K_m were determined over a range of pH values and the negative log (base 10) of these values plotted against pH. The lines are the best fit for a constant k_{cat} and k_{cat}/K_m following the simple titration curve $(k_{cat}/K_m)_{limit} \cdot (10^{-pH}/10^{-pK_a})/[1+(10^{-pH}/10^{-pK_a})]$ with pK_a = 6.45$\pm$0.12.

ThDP analogues. Thiochrome diphosphate was prepared according to the method of Nishimune et al. (1988) while oxy-ThDP (in which the 4'-amino group is replaced by a hydroxyl) was a gift from Dr Stefan König (Martin-Luther-Universität Halle-Wittenberg). he-ThDP was prepared by the method of Krampitz and Votaw (1966), separated from unreacted ThDP by ion-exchange chromatography (Deus et al., 1970), and freeze-dried. The product was completely free of ThDP, as shown by ^{1}H-NMR; he-ThDP concentrations were determined by absorbance measurements at 272.5 nm (Schellenberger and Hübner, 1965).

Acetaldehyde release from he-ThDP. Apoenzyme was added to a reaction mixture, yielding a final volume of 210 μl containing 95 μM he-ThDP, 3.97 mM MgCl$_2$, 119 μM NADH, 6.33 U/ml ADH and 0.23$-$1.14 mg/ml apoenzyme in 50 mM Mes/KOH pH 6.5. After incubation at 30°C for timed intervals, during which any acetaldehyde released is reduced to ethanol with the concomitant formation of NAD$^+$, the reaction was stopped by addition of 20 μl 1 M HCl. After 10$-$20 min at room temperature, 2 ml 6 M NaOH containing 10 mM imidazole was added, mixed immediately, incubated at 60°C for 20 min, and cooled to room temperature. The HCl destroys unreacted NADH while the strong alkali converts NAD$^+$ into an intensely fluorescent product (Passoneau and Lowry, 1993). The fluorescence was determined (excitation at 373.5 nm, emission at 454 nm) and the amount of acetaldehyde formed was estimated from a standard curve prepared by using known amounts of acetaldehyde in similar reaction mixtures. Fluorescence intensities were corrected against identical control reactions in which ThDP replaced he-ThDP. There was no release of acetaldehyde from he-ThDP in the absence of PDC.

Circular dichroism studies. Near-ultraviolet CD spectra were measured at 25°C with a Jasco J-710 spectropolarimeter using a path length of 0.1 cm. Protein concentrations were 2.45 mg/ml and 5 mg/ml for measurements with wild-type PDC and the [Gln113]PDC mutant, respectively.

RESULTS

Effect of pH on kinetic properties of wild-type PDC. Any effects on kinetic constants by changes in pH indicate the involvement of ionisable side chains in the kinetic mechanism. The present study shows that the ratio k_{cat}/K_m only, but not k_{cat},

Table 2. Purification of the [Gln114]PDC mutant.

Step	Activity	Protein	Specific activity	Yield	Purifi- cation
	U	mg	U/mg	%	fold
Soluble extract	3474	520	6.7	(100)	(1.0)
Hydroxapatite eluate	2500	150	16.7	72	2.5
DEAE-Sephacel eluate	1085	44	24.7	31	3.7
Best DEAE-Sephacel fraction	83	2.8	29.6	2.4	4.4

is affected by changes in pH (Fig. 3), suggesting that an ionisable group of the enzyme is involved in substrate binding but not catalysis. The shape of the k_{cat}/K_m profile suggests that this ionisable group must be protonated for effective substrate binding.

The pK_a for this group is 6.45 ± 0.12, which is similar to that for free His (6.1). It does not represent ionization of the substrate which has a pK_a of 2.49 (Dawson et al., 1969). The next nearest pK_a values of free amino acid side chains are around 4 for glutamate or Asp and 8.3 for cysteine; while the data would be consistent with such a side-chain if it had an unusual pK_a, the most plausible hypothesis is that a His residue is involved in substrate binding. Thus it is suggested that the carboxyl group of pyruvate forms an ion pair with a protonated His residue in the active site. Sequence alignment (Fig. 1) and structural data (Fig. 2) suggest that either of two highly conserved His, His113 and His114, may provide the ionisable group involved.

Mutagenesis, expression and purification of mutant PDC. The mutations [Gln113]PDC and [Gln114]PDC were introduced into separate constructs using PCR; each mutation was confirmed by DNA sequencing and restriction digestion. No unintended base changes were detected.

The synthesis of recombinant PDC in *E. coli* DH5α cells was induced by a rapid temperature shift from 30°C–42°C (inactivation of the thermolabile λ repressor results in a derepression of the strong P_R and P_L promoters). Analysis of samples, collected at 30-min intervals from the moment of induction, by Coomassie-blue-stained SDS/PAGE gels, showed the production of large amounts of recombinant enzyme with an estimated molecular mass of 62 kDa (data not shown). Only a minor fraction of the overexpressed protein was insoluble. The [Gln114]PDC mutant has measurable activity while the [Gln113]PDC mutant is completely inactive. Under the assay conditions used, we would have easily detected a specific activity that is 1/15000 of that of wild-type PDC, placing a value of 6 mU/mg as the upper limit of its activity. Table 2 summarizes the purification of the [Gln114]PDC mutant. The fraction with the highest purity ($\geq 90\%$) after DEAE-Sephacel chromatography had a specific activity of 30 U/mg. Extrapolation to a pure preparation suggested a maximum value of 32–33 U/mg. Based on this maximum specific activity the recombinant protein in the induced extract (6.7 U/mg; Table 2) makes up 20% of the total soluble protein.

Characterization of the mutants. The affinity of the [Gln113]PDC mutant for its substrate pyruvate could not be determined because it is completely inactive even at a substrate concentration of 100 mM; the K_m value of recombinant wild-type PDC is approximately 0.5 mM (Candy and Duggleby, 1994). However, the cofactors will bind to this [Gln113]PDC mutant. The progress of cofactor binding to apoenzyme can be monitored by following the decrease in tryptophan fluorescence
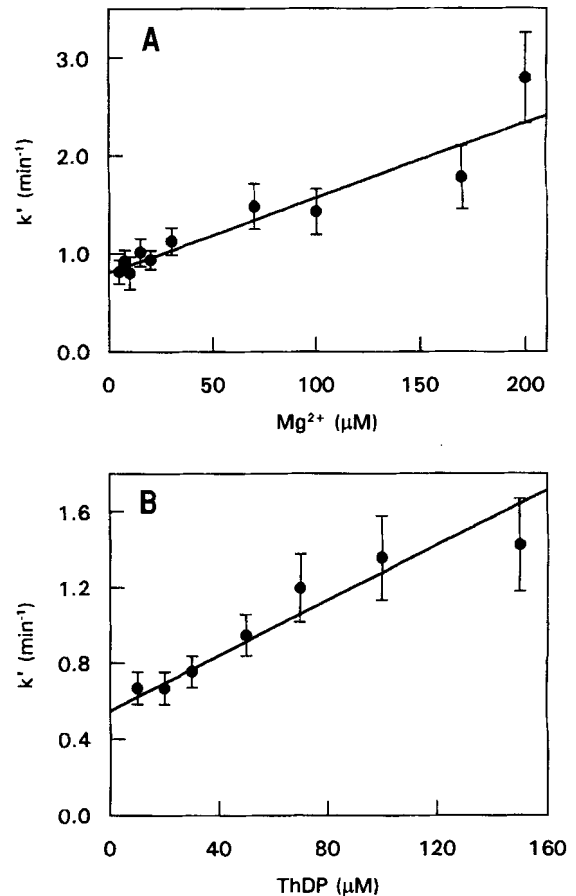

Fig. 4. Cofactor binding of Mg^{2+} and ThDP to [Gln113]PDC. Tryptophan fluorescence quenching of apoenzyme was followed with time after preincubation for 15 min with a fixed concentration of one cofactor followed by addition of the other cofactor. These time courses yielded an apparent first-order rate constant, k'. From the dependence of k' on $[Mg^{2+}]$ (A, incubated with 0.1 mM ThDP) or [ThDP] (B, incubated with 5 mM Mg^{2+}) the off and on rate constants were determined from the y intercept and slope, respectively, of the best-fit lines that are shown. The ratio of these rate constants is equal to the dissociation constant for the cofactor.

Table 3. Kinetic properties of wild-type and mutants of PDC. The K_m values for pyruvate were determined from activity measurements on holoenzyme, while the K_d values for cofactors were estimated from activity (wild-type and [Gln114]PDC) or tryptophan fluorescence quenching ([Gln113]PDC) measurements on apoenzyme. Results for wild-type are taken from Candy et al. (1996a). n.d., not determined.

Parameter	Value for		
	wild-type PDC	[Gln113]PDC	[Gln114]PDC
Activity (U/mg)	89	<0.006	33
K_m for pyruvate (mM)	0.52 ± 0.05	n.d.	0.71 ± 0.04
K_d for ThDP (µM)	2.53 ± 0.16	75 ± 9	1.25 ± 0.12
K_d for Mg^{2+} (µM)	5.87 ± 0.42	105 ± 15	14.9 ± 2.8

upon cofactor binding (Wittorf and Gubler, 1970; Diefenbach and Duggleby, 1991). Binding affinities for cofactors were determined by measuring the fluorescence quenching under conditions where the concentration of one cofactor was varied while the other was present at a fixed concentrations. At cofactor concentrations in excess of the PDC subunit concentration, these
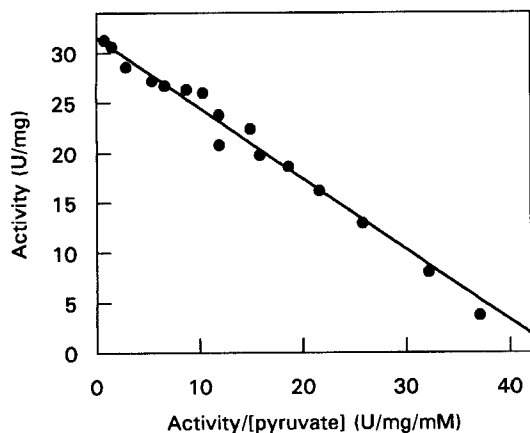
Fig. 5. Pyruvate saturation curve of [Gln114]PDC. Rates were determined over a range of substrate concentrations and are shown here as an Eadie-Hofstee transformation. The line represents the best fit of the Michaelis-Menten equation to the untransformed data.
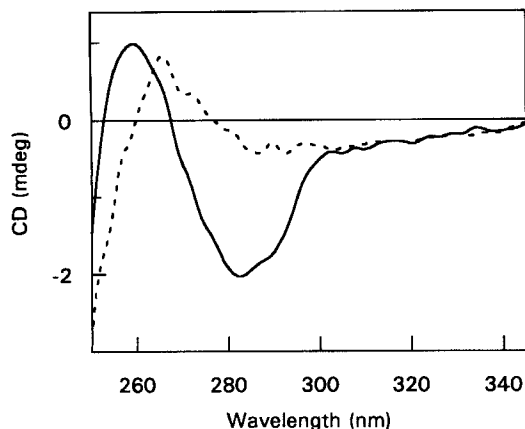

Fig. 6. CD spectra of wild-type PDC and [Gln113]PDC. Protein concentrations were 2.45 mg/ml for wild-type (——) and 5.0 mg/ml for [Gln113]PDC (– – –).
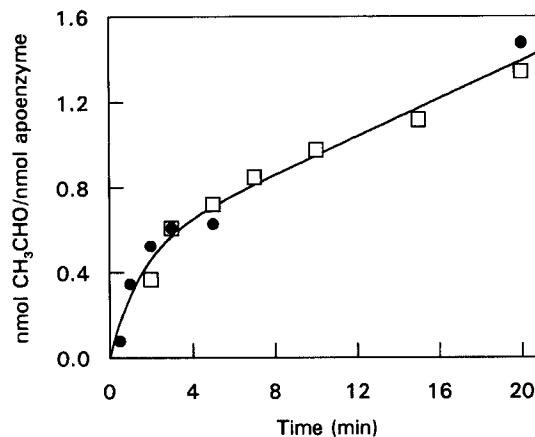

Fig. 7. Acetaldehyde release from he-ThDP, catalysed by the apoenzyme of [Gln113]PDC. Enzyme was mixed with he-ThDP and the released acetaldehyde was trapped and quantified as described in the text. The different symbols are from experiments with 0.83 nmol (□) or 1.67 nmol (●) subunit. The line is the best fit to the data of the equation $y = \alpha \cdot \text{time} + \beta \cdot [1 - \exp(-\gamma \cdot t)]$.

curves follow apparent first-order kinetics (Diefenbach and Duggleby, 1991). The apparent first-order rate constant was determined and plotted against the concentration of the limiting cofactor (Fig. 4). The slope and the intercept on the ordinate correspond to the association (k_1) and dissociation (k_2) rate constants, respectively. At equilibrium the rate of association equals the rate of dissociation and the dissociation constant K_d corresponds to the ratio of k_2/k_1. These results are summarized in Table 3.

The affinity of the [Gln114]PDC mutant for pyruvate was determined by activity measurements at different substrate concentrations. This mutant PDC displays normal Michaelis-Menten kinetics (Fig. 5) with a K_m for pyruvate of 0.716 ± 0.035 mM. This value is similar to that reported for the recombinant wild-type enzyme of 0.52 ± 0.05 mM (Candy and Duggleby, 1994). Dissociation constants for the two cofactors were derived from activity assays; apoenzyme was incubated in the presence of a limiting amount of one cofactor and saturating concentration of the other. The activity of apoenzyme in the absence either cofactor was 0.15% of that of the holoenzyme. The saturation curves are rectangular hyperbolas (data not shown). The results are listed in Table 3.

We attempted to measure substrate binding to the inactive [Gln113]PDC mutant by monitoring changes in tryptophan fluorescence. Although some quenching was observed, this appeared to be due to light absorption by pyruvate as similar quenching

was observed when BSA was substituted for PDC (data not shown). In a second approach, the apoforms of wild-type PDC and [Gln113]PDC were reconstituted with the fluorescent cofactor analogue thiochrome diphosphate which is a competitive inhibitor with respect to ThDP, with a K_i of 4 µM (Diefenbach, 1991). However, the results obtained while monitoring thiochrome diphosphate fluorescence spectra as a function of pyruvate concentration resulted in no change that could be used to measure substrate binding (data not shown).

Circular dichroism has been widely used to characterize properties of yeast PDC (e.g. Hopmann, 1980; Ullrich, 1982). Recently, our laboratory reported the CD spectrum of wild-type and the [Glu50]PDC mutant of *Z. mobilis* PDC (Candy et al., 1996a). Here we attempted to follow the binding of pyruvate to the [Gln113]PDC mutant and wild-type PDC (reconstituted with the inactive analogue oxy-ThDP) by monitoring the CD spectrum between 240–340 nm. Although we could not show any significant changes with increasing substrate concentrations, a substantial difference between the spectra of wild-type and mutant was observed (Fig. 6). The wild-type showed characteristic maxima at 263 nm and 283 nm. However, this mutant protein displays only one maximum at 268 nm. The implications of these observations are discussed below.

Acetaldehyde release. The ability of [Gln113]PDC to promote the release of acetaldehyde from he-ThDP was investigated. After mixing apoenzyme with he-ThDP there was a burst of acetaldehyde production followed by a slower linear release (Fig. 7). Quantitatively similar results were obtained for wild-type PDC. The rate constants governing these two phases (0.657 ± 0.202 and 0.044 ± 0.005 min⁻¹, respectively) are considerably slower than the value of 4.5 · 10⁴ min⁻¹ estimated for product release from wild-type PDC during normal turnover (Sun et al., 1995). Unless the he-ThDP-PDC complex formed by addition of he-ThDP to apoenzyme is different from that formed by decarboxylation of pyruvate by holoenzyme, these results suggest that acetaldehyde release is not rate-limiting in these experiments. Estimation of the rate constants of [Gln113]PDC for he-ThDP binding and ThDP release (from experiments similar to that illustrated in Fig. 4; data not shown) gave values of 0.68 and 0.049 min⁻¹, respectively. Thus, in the first phase, the slow step is binding of he-ThDP to apoenzyme while the second phase is

dominated by the rate at which ThDP is released to be replaced by another molecule of he-ThDP.

DISCUSSION

In recent years the binding of the cofactors ThDP and the divalent metal ion (e.g. Mg^{2+}) and the mechanism of catalysis have been investigated thoroughly for various ThDP-dependent enzymes. Site-directed mutagenesis studies on PDC from *S. cerevisiae* and *Z. mobilis* revealed the importance of various residues in cofactor binding (Diefenbach et al., 1992; Candy and Duggleby, 1994), decarboxylase/carboligase activity and stability (Bruhn et al., 1995), and catalysis (Candy et al., 1996a). Unlike *Z. mobilis* PDC the homologous enzyme in brewer's yeast is subject to substrate activation (Boiteux and Hess, 1970; Hübner et al., 1978); a Cys residue (Cys221 in PDC1 from *S. cerevisiae*) has been found to mediate this regulatory function (Zeng et al., 1993; Baburina et al., 1994, 1996). Interestingly, none of the reported active mutants with changes in cofactor binding, specificity, stability, catalysis or regulation has a drastically altered K_m value for pyruvate indicating that substrate binding has not been strongly affected by any of these mutations.

The availability of the three-dimensional structures of PDC from *S. uvarum* at 2.4 Å (Dyda et al., 1993) and *S. cerevisiae* at 2.3 Å (Arjunan et al., 1996) has not clarified the residues in the enzyme that interact with the substrate. An alignment of all known PDC protein sequences (Fig. 1) shows two absolutely conserved His suggesting an important role for these residues. Measurements of the pH dependence of the enzyme activity revealed that an ionisable group with a pK_a of 6.45 may be involved in substrate binding (Fig. 3). Even though pK_a values of side chains in proteins may change considerably depending on their microenvironment, we suggest that one of the two conserved His may be involved in substrate binding. Inspection of the crystal structure of *S. cerevisiae* PDC (Arjunan et al., 1996) shows that both His are part of a wall of the cavity leading to the active site and both are involved in subunit interactions. According to a molecular modeling study of PDC (Lobell and Crout, 1996a) both these His are positively charged. Thus, we decided to replace both His and chose Glns for these studies because nearly isosteric substitutions will minimize structural changes (Dayhoff et al., 1982).

Mutations were introduced by overlap extension PCR despite the potential for misincorporation of bases by *Taq* polymerase. In order to verify that only the desired mutation was introduced, the whole PCR-amplified fragment was sequenced; no unintentional base changes were detected.

Both mutants were expressed in *E. coli* using the expression vector pPL450 resulting in a very high level of recombinant protein. The synthesis of the recombinant enzyme could be maintained for several hours indicating that its overexpression is not detrimental for the host. While most of the recombinant protein was soluble, a minor amount was found in the insoluble fraction, most likely accumulated in inclusion bodies. For our purposes it was not necessary to recover PDC from the insoluble fraction.

The mutant proteins could be purified without modification of the protocol optimized for the wild-type enzyme (Diefenbach and Duggleby, 1991), consistent with the structures of the mutants being similar to that of the wild-type (Plapp, 1995). It is reasonable to assume that both mutants have folded in the correct conformation and this is borne out by the finding that each mutant will bind the cofactors. Kinetic constants for the substrate pyruvate and the cofactors were determined and the results summarized in Table 3. These data suggest that residue His114

is involved in binding neither the substrate nor the cofactors. However, the k_{cat} of the [Gln114]PDC mutant is decreased to about 35% compared to that of the wild-type enzyme. This is consistent with this residue assisting in catalysis and supports the recent suggestion that the corresponding His in PDC from *S. carlsbergensis* (His115), together with the aminopyrimidinyl group of ThDP, interacts with the alcoholate anion of enzyme-bound α-lactyl-ThDP and he-ThDP (Harris and Washabaugh, 1995). Additionally, it was proposed that this particular His residue in PDC from *S. cerevisiae* forms a hydrogen bond with an Asp (Asp28 in *S. cerevisiae*) which is proposed to be involved in the abstraction of the proton from C2 of the thiazolium ring of the cofactor ThDP (Harris and Washabaugh, 1995). It appears that the substitution of His by Gln weakens the hydrogen bonding pattern but does not completely disrupt it. A plausible role for His114 is that it orients the intermediates formed in the catalytic pathway.

Since the results above showed that His114 is not involved in substrate binding, we investigated whether His113 might be the residue that interacts with pyruvate. The mutation of His113 to Gln rendered the enzyme completely inactive making it impossible to measure a K_m value for the substrate pyruvate. Although cofactor binding was impaired by this mutation (Table 3), it is clear that the reduced affinity for the cofactors does not account for the complete loss of activity.

CD spectra have been used widely to study the interactions between transketolase and ThDP and its substrates (Kochetov et al., 1970; Heinrich et al., 1971). A strong negative band between 300−350 nm is possibly due (Kochetov and Usmanov, 1970) to the formation of a charge-transfer complex between ThDP and two aromatic residues (Phe445 and Tyr448) at the cofactor binding site of the enzyme (Lindqvist et al., 1992; Nikkola et al., 1994). The CD spectrum undergoes changes when substrates are added (Heinrich et al., 1971; Usmanov and Kochetov, 1983). These spectroscopic properties of transketolase have been used to study the effects of various mutations in *S. cerevisiae* transketolase on binding of cofactor and substrates and on catalysis in this enzyme (Wikner et al., 1994, 1995).

CD spectra for PDC have been reported previously for the yeast (Hopmann, 1980; Ullrich, 1982) and *Z. mobilis* (Candy et al., 1996a) enzymes. The CD spectra of wild-type PDC, reconstituted with oxy-ThDP, and [Gln113]PDC holoenzyme were monitored as a function of increasing pyruvate concentrations (0−100 mM). However, the substrate did not affect the spectra (data not shown). In contrast, there was a great difference between the spectra of wild-type and mutant protein (Fig. 6). Wild-type PDC has a characteristic two-signal dichroic band with a positive maximum at 263 nm, a negative maximum at 283 nm and two shoulders around 270 nm and 290 nm. These bands are thought to be due to the binding of ThDP to the asymmetric protein environment (Hopmann, 1980; Ullrich, 1982). The signal is weaker than that of transketolase because there are no aromatic residues present in PDC that may form a charge-transfer complex with the cofactor. The [Gln113]PDC mutant has a positive maximum at 268 nm and a negative diffuse band between 280−340 nm (Fig. 6). The difference from wild-type PDC implies that the replacement of His113 by a Gln may have changed the π-electron distribution in the pyrimidine ring of the cofactor, i.e. by weakening or removing a hydrogen bond. According to the crystal structure (Dyda et al., 1993; Arjunan et al., 1996), this hydrogen bond is likely to be between His113 and N3' of the cofactor. In agreement with a weakening of this hydrogen bond, the affinity of the mutant for ThDP is decreased (Table 3).

We found that upon adding he-ThDP to wild-type and mutant apoenzyme, acetaldehyde was formed at similar rates. These

rates are very much slower than the overall rate of catalysis by wild-type PDC. This is because the process is limited by the rate at which he-ThDP binds (in the initial exponential phase shown in Fig. 7) and the rate at which ThDP is released (linear phase). Nevertheless, it appears that [Gln113]PDC, while inactive for pyruvate decarboxylation, retains some ability to perform the last step of catalysis (release of the product). This implies that His113 is involved in abstraction of the proton from C2 of the thiazolium ring, in binding or reaction of pyruvate to form lactyl-ThDP, or in decarboxylation.

We are conscious that both His residues appear to be somewhat distant from the thiazole C2 (7.9 Å and 6.2 Å, respectively) in the crystal structure (Dyda et al., 1993; Arjunan et al., 1996). However, it is of interest that the eight residues immediately preceding His113 are not visible in the structure, implying that they form a mobile loop. Recently, Lu et al. (1997) have solved the structure of yeast PDC crystallized in the presence of the activator pyruvamide. In this structure, the mobility of this loop is restricted, making it visible in two of the subunits; one end of this loop is near to the channel leading to the thiazole C2. In this context it is relevant that it has been proposed that the active site during catalysis is closed (Alvarez et al., 1995; Lobell and Crout, 1996b). Further, it has been suggested (Alvarez et al., 1995) that, for the allosteric yeast PDC, opening and closing of the active site is controlled by the reaction of the activator pyruvamide, or a second substrate molecule with a sulfhydryl group. In *S. cerevisiae* PDC residue Cys221 has been identified as being responsible for this activation (Baburina et al., 1994). However, in *Z. mobilis* PDC no such regulation mechanism has been found (Sun et al., 1995); moreover, no Cys residues are conserved in regions corresponding to Cys221 of *S. cerevisiae* PDC. However, a model presented by Kluger and Smyth (1981) suggested that the exergonic addition of ThDP to pyruvate might be used to drive the sequestration (closing of the active site). It is likely that His113 interacts with pyruvate allowing Cα of the substrate to be located close to the active centre. Subsequent deprotonation of C2 of the cofactor and formation of lactyl-ThDP lead to conformational changes causing a sequestration of the active site through closure of the cleft by the residues of the mobile loop; upon formation of the product the active site is opened and acetaldehyde is released. This closure of the active site appears to be a necessary requirement for the catalytic mechanism (Alvarez et al., 1995; Lobell and Crout, 1996b). It is suggested that His113 mediates the opening and closing of the active site, possibly by ion-pairing with the carboxyl group of pyruvate, which would explain why [Gln113]PDC is completely inactive.

It is of interest that the two residues preceding His113 are invariably a pair of branched-chain amino acids (Fig. 1); we suggest that these may pack around the methyl group of pyruvate when the active site closes. Although the orientation of these residues in the structure of Lu et al. (1997) is not compatible with this proposition, the structure may undergo further movements of the loop when the substrate binds. If this loop then makes contact with the methyl group of pyruvate, changing Val111 and Leu112 could alter the substrate specificity of PDC. Mutations at these residues, as well as further changes to His113 and His114, are currently being prepared for expression and kinetic characterization.

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