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Effects of substitution of aspartate-440 and tryptophan-487 in the thiamin diphosphate binding region of pyruvate decarboxylase from *Zymomonas mobilis*

Russell J. Diefenbach, Judith M. Candy, John S. Mattick and Ronald G. Duggleby

Department of Biochemistry, University of Queensland, Brisbane 4072, Australia

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A tryptophan residue at position 487 in *Zymomonas mobilis* pyruvate decarboxylase was altered to leucine by site-directed mutagenesis. This modified *Z. mobilis* pyruvate decarboxylase was active when expressed in *Escherichia coli* and had unchanged kinetics towards pyruvate. The enzyme showed a decreased affinity for the cofactors with the half-saturating concentrations increasing from 0.64 to 9.0 μM for thiamin diphosphate and from 4.21 to 45 μM for Mg^{2+}. Unlike the wild-type enzyme, there was little quenching of tryptophan fluorescence upon adding cofactors to this modified form. The data suggest that tryptophan-487 is close to the cofactor binding site but is not required absolutely for pyruvate decarboxylase activity. Substitution of asparagine, threonine or glycine for aspartate-440, a residue which is conserved between many thiamin diphosphate-dependent enzymes, completely abolishes enzyme activity.

Pyruvate decarboxylase; Site-directed mutagenesis; Thiamin diphosphate binding; *Zymomonas mobilis*

1. INTRODUCTION

Pyruvate decarboxylase (PDC; EC 4.1.1.1) catalyses the decarboxylation of pyruvate to produce acetaldehyde and CO_2. Enzyme activity is dependent on the cofactors thiamin diphosphate (ThDP) and Mg^{2+}.

On the basis of chemical modification studies on PDC from yeast [1] and *Zymomonas mobilis* (unpublished results obtained within this laboratory) it appears that one or more tryptophan residues interact directly with bound ThDP. This concept is also supported by model studies that show a direct interaction between the indole nucleus of tryptophan and ThDP [2,3], and cofactor binding experiments on PDC apoenzyme from yeast [4] and *Z. mobilis* [5] which demonstrate that tryptophan fluorescence is quenched by ThDP binding in the presence of Mg^{2+}.

Hawkins et al. [6] have identified a common sequence motif in a range of ThDP-dependent enzymes. This region of approximately 30 residues, which begins with the sequence glycine-aspartate-glycine and ends with a double asparagine, has been postulated to form at least part of the ThDP-binding site of these enzymes. However, none of the seven tryptophan residues of *Z. mobilis* PDC are found in this region [7]. A comparison of the protein sequences of PDC from *Z. mobilis* [7], yeast [8] and maize [9], and the related enzyme indolepyruvate decarboxylase from *Enterobacter cloacae* [10], identifies a conserved tryptophan residue which lies just downstream of this putative ThDP-binding motif (Fig. 1). This tryptophan residue is located at position 487 in the sequence of *Z. mobilis* PDC (numbering as in [7] but starting with the initiating methionine) and is the only tryptophan conserved across all four enzymes.

On the basis of this homology and the evidence for the role of tryptophan in cofactor binding it was decided to determine the importance of this tryptophan residue by altering it to a leucine residue in PDC from *Z. mobilis* using site-directed mutagenesis. The kinetic properties of mutant and wild-type *Z. mobilis* PDC expressed in *Escherichia coli* were then compared to establish the effects of this substitution. Three other mutants, in which the conserved aspartate at the N-terminal end of the ThDP-binding motif was changed to asparagine, threonine or glycine were also prepared and characterised.

Abbreviations: ADH, alcohol dehydrogenase; PDC, pyruvate decarboxylase; ThDP, thiamin diphosphate.

Correspondence address: R.G. Duggleby, Department of Biochemistry, University of Queensland, Brisbane 4072, Australia. Fax: (61)(7) 365 1499.

2. EXPERIMENTAL

All reagents, which were of analytical grade or better, and enzymes (except PDC) were obtained from commercial sources.

2.1. *Mutagenesis procedures*

Mutation of aspartate-440 to asparagine was performed by overlap extension using the polymerase chain reaction [11]. However, this method was found to introduce additional random mutations which necessitated complete sequencing of the entire gene of several isolates to find one with only the desired mutation. The remaining site-specific

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                ↓                                                    ↓
ZmPDC   RRNILMVGDGSFQLTAQEVAQMVRLKLPVIIFLINNYGYTIEVMIHDGP...YNNIKNWDYAGL
YPDC    KRVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIEKLIH.GPKAQYNEIQGWDHLSL
MPDC    KRVIACIGDGSFQVTAQDVSTMLRCGQKSIIFLINNGGYTIEVEIHDGP...YNVIKNWDYTGL
IPDC    RRVIVLTGDGAAQLTIQELGSMLRDKQHPIILVLNNEGYTVERAIH.GAEQRYNDIALWNWTHI
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putative ThDP-binding domain

Fig. 1. Alignment of a portion of the protein sequences of PDC from *Z. mobilis* (ZmPDC), yeast (YPDC) and maize (MPDC) with indolepyruvate decarboxylase from *E. cloacae* (IPDC). The conserved aspartate and tryptophan residues are indicated with arrows. The underlined sequence beginning with GDG and ending with NN is common to most ThDP-dependent enzymes [6].

alterations of the *Z. mobilis pdc* gene were performed according to the Altered Sites in vitro mutagenesis procedure (Promega Corp.). The procedure was based on the use of a single-stranded DNA template and two primers, one of which was designed to introduce the desired mutation into the PDC coding sequence, while the other corrects a defective ampicillin resistance gene for selection purposes [12–14]. The 1.8 kb DNA fragment used for mutagenesis which contains the *Z. mobilis pdc* gene was identical to that obtained from a *PstI*/*Eco*RI double digest of pZAN3A [15]. The codon changes for alteration of tryptophan (TGG) to leucine (TTG) and aspartate (GAT) to asparagine (AAT), threonine (ACT) or glycine (GGT) were confirmed by dideoxy DNA sequencing [16]. The mutant and wild-type *Z. mobilis pdc* genes were then cloned into the *PstI*/*Eco*RI site of pUC18, where expression was under control of the *lac* promoter, before transformation into *E. coli* strain DH5α.

2.2. *Purification of enzyme*

Recombinant *E. coli* cells were grown at 37°C with agitation in a medium containing 1.6% (w/v) bacto-tryptone, 1% (w/v) bacto-yeast extract and 0.5% (w/v) NaCl. Ampicillin (50 μg/ml) was used to select transformants of *E. coli* and PDC expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside and *E. coli* cells were harvested and lysed as described previously [5]. PDC was purified using a one-step hydroxylapatite batch procedure [5]. Prior to storage at −20°C in 50% (v/v) glycerol, phosphate was removed by dialysis against 50 mM Mes/KOH, pH 6.5 which also contained 0.1 mM ThDP, 5 mM Mg^{2+} and 1 mM dithiothreitol.

2.3. *Protein, ThDP and enzyme assay*

All protein determinations were performed according to the method of Smith et al. [17] using a bicinchoninic acid protein determination kit (Sigma Chem. Co.). The ThDP content of purified PDC was measured as described previously [18]. PDC activity was assayed by measuring the rate of production of acetaldehyde, determined by measuring the oxidation of NADH in the presence of alcohol dehydrogenase (ADH). The rate of change of absorbance at 340 nm and 30°C, in 50 mM Mes-KOH, pH 6.5, was recorded. Unless otherwise stated, final 2 ml assay mixtures contained 5 mM $MgCl_2$, 0.1 mM ThDP, 0.15 mM NADH, 10 units/ml of ADH and 10 mM pyruvate. One unit of activity is defined as the quantity of enzyme that catalyses the formation of 1 μmol of product/min.

2.4. *Preparation of apoenzyme*

For preparation of cofactor-free apoenzyme, PDC obtained directly from purification (2 mg/ml; 10 ml vol.) was diluted 10-fold with 50 mM Tris-HCl, pH 8.5, which also contained 1 mM EDTA. After 1 h incubation at room temperature the solution was reconcentrated by ultrafiltration to a 10 ml volume. This was then passed through a column of Sephadex G-25 (dimensions 5.3 cm^2 × 28 cm) equilibrated with the Tris/EDTA buffer and then through a similar column equilibrated with 50 mM Mes-KOH, pH 6.5; the apoenzyme preparation was stored at 4°C. This pH 6.5 Mes buffer, as well as the ThDP solutions used for assay and binding studies of apoenzyme, were passed through a column of Chelex 100 cation exchange resin (Na^+ form) to remove divalent metals.

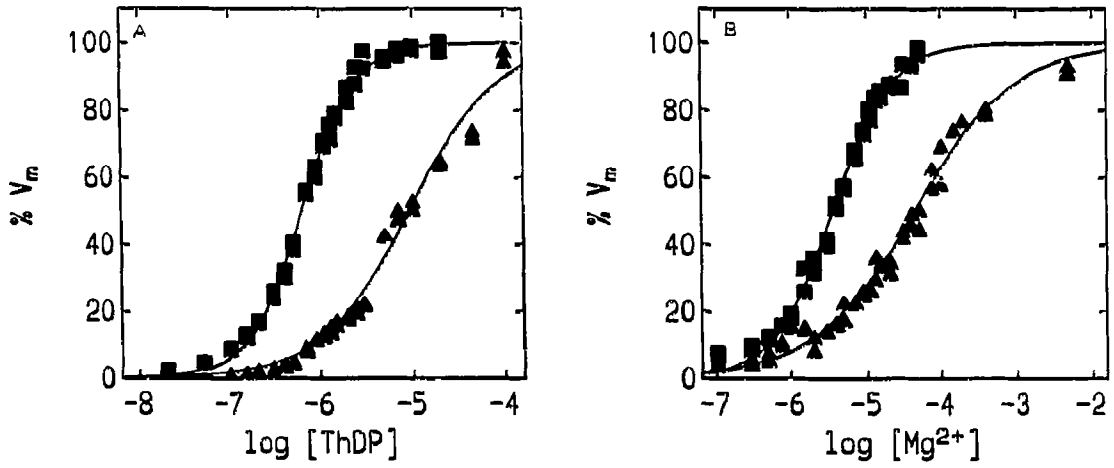

Fig. 2. Cofactor saturation curves for wild-type (W487L; squares) and mutant (triangles) *Z. mobilis* PDC apoenzyme. Rates (as % V_m) are plotted against log{[cofactor] (mM)} to accommodate the wide range of cofactor concentrations. (A) ThDP binding. Apoenzyme was preincubated with ThDP as indicated and 5 mM Mg^{2+} prior to assay. (B) Mg^{2+} binding. Apoenzyme was preincubated with Mg^{2+} as indicated and 0.1 mM ThDP prior to assay.

Table I

Comparison of the kinetic properties of PDC W487L and wild-type *Z. mobilis* PDC

Ligand	PDC enzyme	
	Wild-type	W487L
ThDP		
$K_{0.5}$(μM)	0.64 ± 0.02	9.0 ± 1.7
n	1.46 ± 0.05	0.92 ± 0.08
Mg^{2+}		
$K_{0.5}$(μM)	4.21 ± 0.16	45.0 ± 7.3
n	1.30 ± 0.08	0.63 ± 0.04
Pyruvate		
K_m(mM)	1.08 ± 0.05	0.86 ± 0.05
n	1.03 ± 0.04	0.99 ± 0.07

2.5. *Measurement of cofactor binding*

The kinetics of activation by cofactors were determined by preincubating apoenzyme with various cofactor combinations for 15 min at 30°C prior to initiation of assay by addition of a pyruvate/NADH/ADH mixture.

The time course for cofactor binding to PDC apoenzyme was determined by following the quenching of tryptophan fluorescence. Measurements were done on an Aminco SPF 500 spectrofluorimeter with an excitation wavelength of 300 nm (bandwidth 5 nm) and an emission wavelength of 340 nm (bandwidth 5 nm). This unusually high excitation wavelength was chosen so as to avoid excessive absorption of the incident light by ThDP. Reactions were performed at 30°C and initiated by addition of apoenzyme (0.09 mg/ml) to a mixture of 0.1 mM ThDP and 5 mM Mg^{2+} in 50 mM Mes-KOH, pH 6.5.

2.6. *Data analysis*

For cofactor and pyruvate saturation curves the rate of reaction (v, change in absorbance per s) as a function of ligand concentration ([L]) was described by the equation:

$$v = V_m[L]^n/\{(K_{0.5})^n + [L]^n\}$$

where the parameters are V_m (the rate at saturating ligand), $K_{0.5}$ (the half-saturating concentration of ligand) and n (the Hill coefficient). In the case of pyruvate $K_{0.5}$ was equivalent to the Michaelis constant (K_m). This equation was fitted to the data using the DNRP53 computer program [19].

3. RESULTS AND DISCUSSION

Substitution of leucine for tryptophan-487 yields a mutant PDC which is active when expressed in *E. coli*. Comparison of the kinetic properties of this mutant (PDC W487L) and wild-type *Z. mobilis* PDC expressed in *E. coli* shows that this substitution affects cofactor binding (Fig. 2; Table I). The affinity of PDC W487L apoenzyme for both cofactors is decreased significantly as seen by a 10–15-fold increase in the half-saturating concentrations ($K_{0.5}$) of either cofactor (Table I). In addition, the Hill coefficient for each cofactor is decreased. For ThDP the value decreases from 1.46 to 0.92 which indicates that binding is no longer cooperative while for Mg^{2+} the value decreases from 1.30 to 0.63, a change from positive to negative cooperativity (Table I). In contrast, there is little or no effect on the binding of pyruvate (Fig. 3; Table I) which indicates that alteration of tryptophan-487 has not grossly disturbed the active site.

Addition of saturating cofactors (0.1 mM ThDP and 5 mM Mg^{2+}) to PDC W487L apoenzyme was found to have little effect on tryptophan fluorescence (Fig. 4). In contrast, wild-type PDC apoenzyme showed an appreciable decrease in tryptophan fluorescence upon addition of saturating cofactors (Fig. 4). Tryptophan-487 must therefore be close to or part of the cofactor binding site since cofactor quenching of tryptophan fluorescence observed with wild-type PDC is not apparent in PDC W487L. This conserved tryptophan residue must therefore be the tryptophan whose fluorescence has been shown previously to be quenched by cofactor binding to PDC apoenzyme from *Z. mobilis* [5] and, by homology, from yeast [4].

The mutation of aspartate-440 to asparagine, threonine or glycine yielded proteins (PDC D440N, PDC D440T or PDC D440G, respectively) which were de-

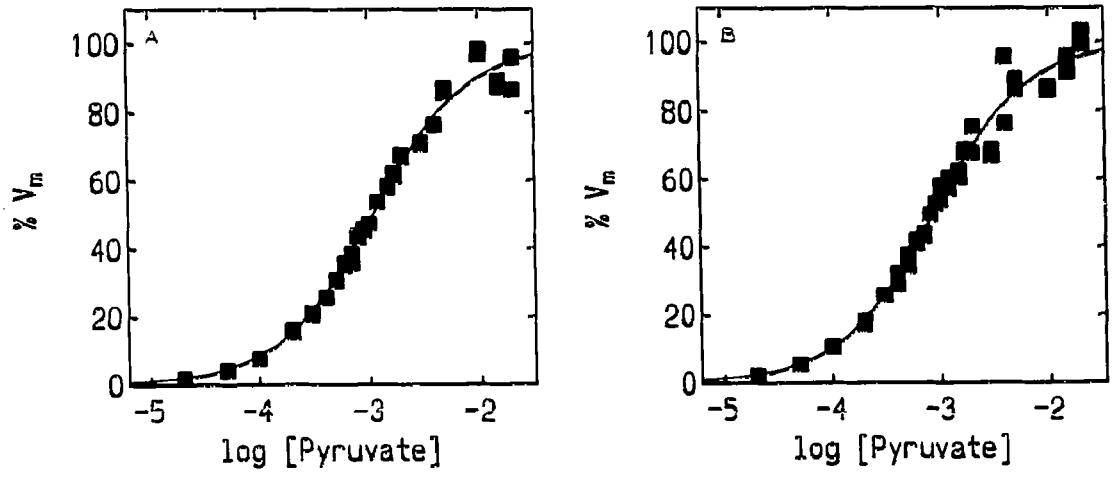

Fig. 3. Pyruvate saturation curve for *Z. mobilis* PDC. (A) Wild-type. (B) Mutant W487L.

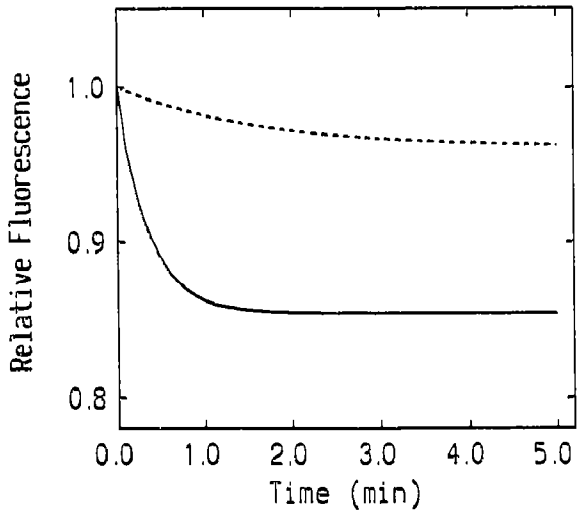

Fig. 4. Fluorescence time course for the binding of cofactors to wild-type (solid line) and mutant (W487L; broken line) *Z. mobilis* PDC apoenzyme. Both fluorescence time course curves were corrected for the slow decrease in fluorescence of apoenzyme alone over the same time period. The initial fluorescence was greater for mutant apoenzyme due to a higher background of non-PDC protein and the curves have also been normalised to an initial fluorescence value of 1.0.

void of activity. However, the structure of none of these appeared to be grossly perturbed as they purified normally. None of these proteins was capable of binding ThDP, as judged by the fact that none contained any ThDP when isolated and none showed the characteristic quenching of tryptophan fluorescence upon addition of cofactors. These results confirm the importance of the conserved aspartate identified by Hawkins et al. [6] and are consistent with their proposal that this residue forms part of the ThDP-binding site.

The fact that the binding of both ThDP and Mg^{2+} has been affected in PDC W487L is most likely due to the expected close interaction of the cofactors at the cofactor binding site of PDC. This is supported by previous observations on cofactor binding to *Z. mobilis* PDC apoenzyme [5] which shows that the quenching of tryptophan fluorescence occurs both with limiting ThDP and Mg^{2+} when the other cofactor is saturating. Therefore alteration of tryptophan-487 would be expected to affect both ThDP and Mg^{2+} binding.

Clearly tryptophan-487 plays some role in cofactor binding to PDC but since PDC W487L apoenzyme is still capable of binding cofactors, though with a reduced affinity, then substitution with leucine is not sufficient to destroy the cofactor binding site of *Z. mobilis* PDC. ThDP must still be accommodated into the hydrophobic binding site and, if the proposed interaction of ThDP with tryptophan has been removed, then it is still likely that ThDP is bound to PDC by other groups on the enzyme. These residues may interact directly with the pyrophosphate group of ThDP or indirectly through Mg^{2+}.

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