Aspartate-27 and glutamate-473 are involved in catalysis by *Zymomonas mobilis* pyruvate decarboxylase

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Zymomonas mobilis pyruvate decarboxylase (EC 4.1.1.1) was subjected to site-directed mutagenesis at two acidic residues near the thiamin diphosphate cofactor in the active site. Asp-27 was changed to Glu or Asn, and Glu-473 was mutated to Asp (E473D) or Gln (E473Q). Each mutant protein was purified to near-homogeneity, and the kinetic and cofactor-binding properties were compared with those of the wild-type protein. Despite the very conservative nature of these alterations, all mutants had a very low, but measurable, specific activity ranging from 0.025 % (E473Q) to 0.173 % (E473D) of the wild type. With the exception of E473Q, the mutants showed small decreases in the affinity for thiamin diphosphate, and binding of the second cofactor (Mg^{2+}) was also weakened somewhat. With E473Q, both cofactors seemed to be very tightly bound so that they were not removed by the treatment that was effective for the wild-type enzyme and other mutant forms. All mutants showed minor changes in the K_m for substrate, but these alterations did not account for the low activities. These low specific activities, accompanied by little change in the K_m for pyruvate, are consistent with a quantitative model of the catalytic cycle in which the main effect of the mutations is to slow the decarboxylation step with a minor change in the rate constant for pyruvate binding.

Key words: active site, catalytic mechanism, enzyme kinetics, site-directed mutagenesis, thiamin diphosphate.

INTRODUCTION

Pyruvate decarboxylase (PDC; EC 4.1.1.1) catalyses the penultimate step in ethanol fermentation in which pyruvate is converted to acetaldehyde. Given the historical reliance of many civilizations on yeast to perform this fermentation, PDC from this organism has received more attention than the enzyme from other sources.

The substrate saturation curve of yeast PDC does not follow simple Michaelis–Menten kinetics [1]. Rather, the curve is sigmoidal with a Hill coefficient of approx. 2 and this has been interpreted as indicating that there is a site, distinct from the active site, that binds pyruvate, resulting in activation of the enzyme. This activator site is not specific for pyruvate and can also bind oxomalonate [2] and 2-oxoacid amides such as pyruvamide [3]. A sigmoidal substrate curve is a general feature of all PDCs that have been studied (reviewed in [4]) with the single exception of the enzyme from the bacterium *Zymomonas mobilis*, which shows Michaelis–Menten kinetics. For this reason, over the past 8 years we have focused our studies (reviewed in [5]) of PDC on the enzyme from *Z. mobilis*.

The three-dimensional structures of several forms of PDC have been determined. These structures include the non-activated enzyme from the yeasts *Saccharomyces uvarum* [6] and *S. cerevisiae* [7], the *S. cerevisiae* enzyme crystallized in the presence of the activator pyruvamide [8], and, most recently, *Z. mobilis* PDC [9]. These structures have revealed many features that are involved in subunit interactions, the binding of the two cofactors [thiamin diphosphate (ThDP) and Mg^{2+}] and residues that might participate in catalysis. The roles of several of these amino acids have been investigated by site-directed mutagenesis (reviewed in [5,10]).

The active-site region contains at least four acidic residues of proven or potential importance (Figure 1). It is widely believed that the active form of ThDP is the carbanion formed by ionization at C-2 of the thiazole ring; Glu-50 (*Z. mobilis* numbering) is needed for catalysis [11], apparently by promoting the rate of this ionization [12]. Asp-440 binds the Mg^{2+} ion that anchors ThDP to the enzyme [13,14]. The two other acidic residues, Asp-27 and Glu-473, have been subjected to little experimental scrutiny, although several papers [5–7,9,15–19] have drawn attention to their proximity to ThDP (Figure 1).

In yeast PDC, each of these two residues has been subjected to mutagenesis. Initially Jordan et al. [20] described the mutant in

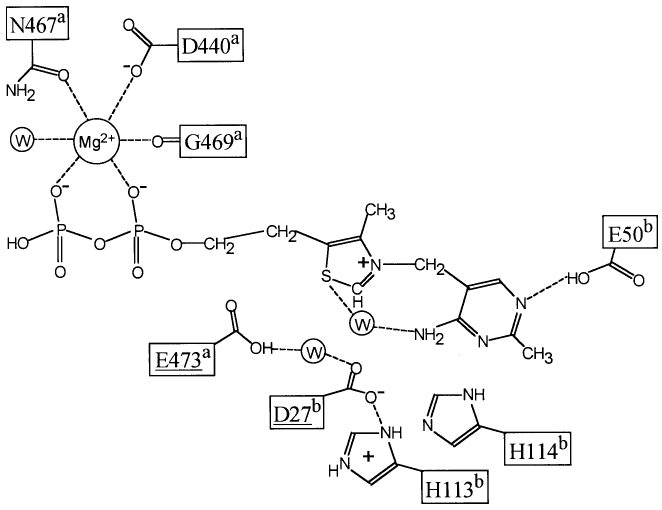

Figure 1 Schematic representation of the active site of *Z. mobilis* PDC

The structure illustrated in Figure 6 of Dobritzch et al. [9] has been redrawn and simplified to eliminate most residues that are not germane to the present study. The superscripts a and b indicate residues from adjacent subunits; Asp-27 (D27) and Glu-473 (E473) are underlined.

Abbreviations used: PDC, pyruvate decarboxylase; ThDP, thiamin diphosphate.

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which Asp-28 was changed to alanine (here abbreviated D28A, equivalent to D27A in *Z. mobilis* PDC) as having an activity of approx. one-seventh of the wild type, although this value was later reported as 1.5 % of wild type [21]. Another mutation (yeast D28N), as well as several (to Gln, Asp and Asn residues) at Glu-473 (yeast Glu-477), all have activities on the order of 1 % of the wild type [21]. Little other information has been published on these mutants apart from the Hill coefficient, which varies between 1.3 and 2.1, again with some discrepancies between the two publications (thus for E477D, Jordan et al. [20] give a Hill coefficient of 1.3, whereas Jordan et al. [21] report a value of 2.0). There is no published information on the affinities for substrate and cofactors of these mutants.

In the present study we have mutated Asp-27 (to Glu and Asn) and Glu-473 (to Gln and Asp) of *Z. mobilis* PDC. In all cases, the activity was very low, pointing to an important role for these residues. The effect was primarily on catalysis, with little effect on the Michaelis constant for pyruvate. Some decreases are observed in the affinity for the Mg^{2+} and for ThDP, with one exception: E473Q bound the cofactors much more tightly than the wild-type enzyme and they could not be removed by the procedure that was effective for wild-type *Z. mobilis* PDC and for every other mutant of this enzyme that we have so far tested.

MATERIALS AND METHODS

Restriction endonucleases and molecular biology products

Restriction enzymes, T4 DNA ligase and T4 DNA polymerase were purchased from New England Biolabs (Beverly, MA, U.S.A.) and deoxyribonucleotides from Perkin–Elmer (Norwalk, CT, U.S.A.). For sequencing we used the Prism Ready Dye Deoxy Terminator Cycle Sequencing Kit from Perkin–Elmer Applied Biosystems.

Bacterial strains and plasmids

Bacterial culture, plasmid DNA preparations, restriction endonuclease digestions, ligations and transformations were performed in accordance with standard protocols [22]. *Escherichia coli* strain JM109 was obtained from Promega (Madison, WI, U.S.A.). The pPLZM plasmid for heat-inducible PDC expression has been described previously [14]. All *E. coli* cultures containing the wild-type enzyme and the mutant constructs were maintained on Luria broth plates with 100 μg/ml ampicillin. For long-term storage, liquid cultures were kept in 15 % (v/v) glycerol and stored at -70 °C.

Mutagenesis

All mutants were constructed by using the 'Altered Sites' mutagenesis system *in vitro* (Promega, Sydney, Australia), as described previously [13]. Mutagenic primers were 21–30 bases in length with a G+C content of 50 % or higher. Silent mutations were introduced where possible to add a restriction endonuclease site to facilitate the screening of transformants. An *Eco*RI/*Nco*I (Asp-27 mutants) or *Nco*I/*Sph*I (Glu-473 mutants) fragment of the PDC gene with the desired mutations was subcloned into appropriately digested pPLZM to construct the full-length mutated gene for expression. The introduction of the desired base changes was confirmed by DNA sequencing.

Expression, protein purification and preparation of apoenzyme

For expression of PDC on a 2-litre scale, the cells were grown in 2YT medium containing 100 μg/ml ampicillin at 30 °C. When the cell culture reached a D_{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 ***g***; the cell pellet was stored at -20 °C. The purification of PDC was based on the protocol described previously [23] except that an additional Sephacryl S-300 (AMRAD Pharmacia Biotech, Melbourne, Australia) gel-filtration step on a 2 cm × 90 cm column was introduced as the final stage of purification. The purified enzyme was mixed with an equal volume of glycerol and stored at -20 °C. Removal of cofactors to yield the apoenzyme was performed by using our published procedure [23,24].

Activity assay for PDC

PDC activity was measured in a coupled enzyme assay at 30 °C. The rate of production of acetaldehyde from 5 mM pyruvate was determined by following the oxidation of NADH at 340 nm in the presence of alcohol dehydrogenase [24]. Reactions were started by the addition of PDC; the amount of enzyme was chosen so that a steady decrease in NADH concentration could be monitored over 5 min. During purification, assays were performed with and without the addition of alcohol dehydrogenase. The latter was taken as a measure of the lactate dehydrogenase activity that is present in cell extracts but is separated during purification [23]. The K_m for pyruvate was determined in the standard assay mixture with various concentrations of substrate.

Measurement of cofactor activation, cofactor binding and ThDP content

Cofactor activation studies were performed by measuring the activity of the reconstituted holoenzyme. The apoenzyme was preincubated for 15 min at 30 °C with a saturating concentration of one cofactor while the concentration of the other was varied. The reaction was started by adding a pyruvate/NADH/alcohol dehydrogenase mixture and the data obtained were analysed as described below.

Cofactor binding was also measured by monitoring the quenching of tryptophan fluorescence of PDC [24] as a function of time with a Jasco Model FB-770 spectrofluorimeter. Excitation was at 300 nm (bandwidth 5 nm) and emission was measured at 340 nm (bandwidth 5 nm).

The ThDP content of enzyme samples was measured by oxidation to the fluorescent derivative thiochrome diphosphate, as described previously [24].

Data analysis

Kinetic parameters were determined by fitting the appropriate equation to the data by non-linear regression with INPLOT (GraphPad Software, San Diego, CA, U.S.A.), GraFit (Erithacus Software, Staines, U.K.) or an adaptation of DNRP53 [25]. The best-fit and S.E.M. values obtained from this analysis are reported. Substrate saturation curves were fitted to the Michaelis–Menten equation to obtain K_m values, and cofactor saturation curves were fitted to eqn. (1) to obtain the cofactor activation constant K_c:

$$v = V_{max}[C]/(K_c + [C]) \quad (1)$$

where [C] is the cofactor concentration. Results of tryptophan fluorescence quenching were analysed by the method of Diefenbach and Duggleby [24]. In brief, time courses were fitted to an exponential decay curve to obtain an apparent first-order rate constant at a given cofactor concentration. The slope of the

linear relationship between this rate constant and the cofactor concentration represents the rate constant for association (k_{on}), whereas the rate constant for cofactor dissociation (k_{off}) was obtained from the intercept on the ordinate.

Protein analysis

SDS/PAGE was performed as described by Laemmli [26] and proteins were detected by staining with 0.1% (w/v) Coomassie Blue. For routine measurements of protein concentrations, the dye-binding method of Sedmac and Grossberg [27] was used. More precise measurements were made by using the A_{280} value in 0.1 M NaOH; the expected molar absorption coefficient at this wavelength was calculated from the known tryptophan and tyrosine content [28] and the spectral data of Mihalyi [29].

RESULTS

Expression and purification

SDS/PAGE of cell-free extracts (results not shown) exhibited a prominent band of approx. 60 kDa, indicating that each mutant PDC was expressed well. However, enzyme assays revealed very little PDC activity. These assays were complicated by the fact that *E. coli* extracts contain lactate dehydrogenase activity that obscured small amounts of PDC. To determine whether the mutations had completely abolished PDC activity, each of the mutant proteins was purified.

By using the same procedure as for wild-type PDC, each of the mutant enzymes was purified successfully. All eluted at approximately the same position during gel-filtration chromatography, indicating that association to form tetramers was not affected by the mutations. The purity of the final products was assessed by SDS/PAGE (Figure 2); only traces of impurities are visible. Lactate dehydrogenase was separated during the purification [23]; this permitted the demonstration that each of the mutants exhibits a small amount of PDC activity.

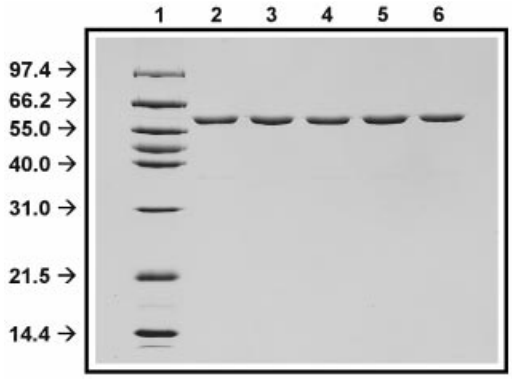

Figure 2 SDS/PAGE of purified wild-type *Z. mobilis* PDC and mutants

Lane 1, molecular mass standards (molecular mass in kDa are indicated at the left); lane 2, wild-type PDC; lane 3, D27E; lane 4, D27N, lane 5, E473D; lane 6, E473Q.

Activity and substrate saturation curves

Measured at a pyruvate concentration of 5 mM, the specific activity of each of the mutants was quite low, ranging from 0.025% (E473Q) to 0.173% (E473D) of the wild-type enzyme (Table 1). A possible explanation for these low activities would be a massive increase in the K_m for pyruvate so that in assays at 5 mM the mutant enzymes were well below saturation. We therefore measured substrate saturation curves. We illustrate our results in Figure 3(A) and below with D27E as an example, but results of similar quality were obtained for the other mutants and for wild-type PDC. Some variations in K_m from the wild-type value are seen (Table 1) but these are relatively small and in three of the mutants there is a decrease in K_m. In all cases, measurements at 5 mM pyruvate would give a good approximation of the specific activity.

Cofactor activation, cofactor binding and ThDP content

As noted earlier, Asp-27 and Glu-473 are so close to the active site (Figure 1) that mutation could affect the affinity for ThDP and, indirectly, that for Mg^{2+}. We therefore attempted to prepare the apoenzymes to measure K_c for each of the cofactors. For the wild-type protein and all mutants except for E473Q, apoenzyme

Table 1 Activity and kinetic properties of wild-type and mutant *Z. mobilis* PDCs

Where more than one value is shown, they are the result of several independent experiments. For the various kinetic constants, the values shown represent the best-fit value ± S.E. obtained from fitting the appropriate equation to the data. Abbreviations: S.A., specific activity expressed as a percentage of the wild-type value of 70.0 units/mg; n.d., not determined.

Enzyme	S.A. (%)	$K_m^{pyruvate}$ (mM)	K_c^{ThDP} (μM)	k_{on}^{ThDP} ($\mu M^{-1} \cdot h^{-1}$)	k_{off}^{ThDP} (h^{-1})	$K_c^{Mg^{2+}}$ (μM)	$k_{on}^{Mg^{2+}}$ ($\mu M^{-1} \cdot h^{-1}$)	$k_{off}^{Mg^{2+}}$ (h^{-1})
Wild-type	(100)	0.68 ± 0.02 0.66 ± 0.03	1.97 ± 0.15 1.81 ± 0.12 2.12 ± 0.12	1.11 ± 0.03	3.53 ± 0.75	8.19 ± 0.61 8.87 ± 0.86	0.132 ± 0.003	2.39 ± 0.53
D27E	0.072	0.25 ± 0.01 0.25 ± 0.01	7.11 ± 0.19 6.74 ± 0.22	1.49 ± 0.02	2.93 ± 0.75	17.0 ± 0.75 18.0 ± 0.89	0.155 ± 0.006	3.89 ± 0.52
D27N	0.049	0.48 ± 0.04 0.43 ± 0.03	3.30 ± 0.28	0.54 ± 0.04	25.86 ± 1.98	64.5 ± 4.65	0.125 ± 0.026	43.32 ± 3.00
E473D	0.173	0.19 ± 0.01 0.20 ± 0.02 0.18 ± 0.01	3.88 ± 0.45 4.29 ± 0.44 3.73 ± 0.47	1.36 ± 0.03	1.11 ± 0.03	16.2 ± 1.04 15.4 ± 0.81	0.141 ± 0.017	3.91 ± 0.96
E473Q	0.025	1.17 ± 0.15 1.14 ± 0.08 1.04 ± 0.08	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

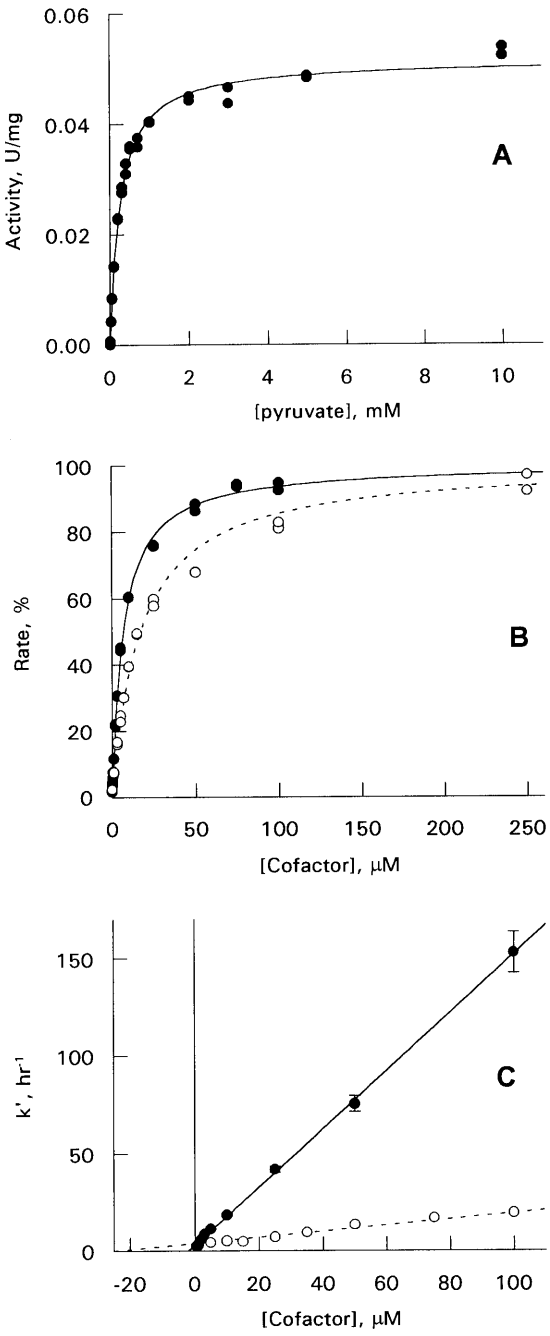

Figure 3 Kinetic and binding properties of the D27E mutant of *Z. mobilis* PDC

(**A**) Pyruvate saturation curve. (**B**) ThDP (●, solid line) and Mg^{2+} (○, dotted line) saturation curves. Rates are expressed as percentages of the activity with a saturating concentration of both cofactors. (**C**). Binding of ThDP (●, solid line) and of Mg^{2+} (○, dotted line); the apparent first-order rate constant (k') of tryptophan fluorescence quenching was determined and is plotted against the cofactor concentration. Where error bars are not shown they are contained within the dimensions of the data points.

preparation was successful, as judged by the low residual activity on adding back one cofactor only (Table 2).

With E473Q, apoenzyme preparation was not successful because the activity of the enzyme in the absence of either cofactor was similar to that when both cofactors were added. To confirm that this effect resulted from a failure to release the

Table 2 Cofactor requirements and ThDP content of wild-type and mutant *Z. mobilis* PDCs

Apoenzyme was prepared and assayed for ThDP content and for residual activity in the absence of one cofactor at a saturating concentration (0.1 mM ThDP or 5 mM Mg^{2+}) of the other. Activity measurements are expressed as a percentage of the activity restored by the addition of a saturating concentration of both cofactors. Values for the wild-type enzyme are taken from [24]. Abbreviation: n.d., not determined.

	Activity (%)		
Enzyme	Without added ThDP	Without added Mg^{2+}	ThDP (mol per subunit)
Wild-type	0	2	0.01
D27E	2	1	0.02
D27N	7	3	n.d.
E473D	6	6	0.04
E473Q	90	84	0.82

cofactors, the ThDP content of E473Q and other mutants was determined. In all cases there was a good correlation between the activity in the absence of ThDP and the ThDP content. In particular, the activity of the preparation of E473Q in the absence of added ThDP was clearly due to the fact that it contained nearly 1 mol of ThDP per subunit, despite its being treated in a manner that removes cofactors from wild-type PDC.

The K_e values for each cofactor of the remaining three mutants were measured. Activation curves were hyperbolic in each case (examples are shown in Figure 3B) and the cofactor activation constants (Table 1) were all elevated as compared with the wild-type values. Surprisingly, the effects on Mg^{2+} activation were more pronounced than those on ThDP activation. The latter were elevated by no more than 3.5-fold (D27E), while an increase approaching 8-fold was observed in the K_e for Mg^{2+} of D27N. Nevertheless these results show that the low specific activities (measured at 0.1 mM ThDP/5 mM Mg^{2+}) observed for the mutants could not have resulted from difficulty in binding the cofactors.

It could be argued that the low specific activities of the mutants resulted from some difficulty in attaining the active conformation and that the enzymes that we have purified consisted of a mixture containing a very small fraction of fully active enzyme together with large amounts of inactive protein. This is difficult to rule out, although the fact that the mutants behaved normally during purification, including gel filtration chromatography, indicates that no massive structural perturbation had occurred. As a further check, we measured cofactor binding to apoenzyme by its ability to quench the fluorescence of Trp-487 [13,24]. Because this is a property of the bulk protein we would expect no fluorescence quenching if most of the protein had lost the ability to bind cofactors and was therefore inactive. This did not happen: the extent of tryptophan fluorescence quenching was similar to that of wild-type (results not shown). Similar experiments were not possible for E473Q for the reason mentioned above. However, measurement of the ThDP content of this protein suggested that most or all of the protein was in the active conformation.

The quenching of tryptophan fluorescence that occurs when cofactors bind is a slow process [24] and, by following the time course, allows the determination of k_{on} and k_{off} values (Table 1). The values of k_{on} for each cofactor with the mutants were similar to those of wild-type PDC, except for a small decrease in ThDP

binding to D27N. The values of k_{off} for the mutants were also similar to those of wild-type PDC, again with the exception of D27N. With that mutant there were substantial increases in the rates of dissociation of both ThDP (7-fold) and Mg^{2+} (18-fold).

The ratio k_{off}/k_{on} (i.e. the negative intercept on the abscissa in Figure 3C) is a cofactor dissociation constant and the values of this ratio are generally similar to those obtained by cofactor activation measurements. However, the values do not exactly coincide. The conversion of apoenzyme to the active holoenzyme is a multistep process [24,30] and measurements of cofactor affinity based on the restoration of activity and those determined by tryptophan fluorescence quenching might not, and usually do not, yield identical values because the two types of measurement do not probe the same subset of equilibria.

Quaternary structure

The removal of cofactors from PDC involves treatment under mildly alkaline conditions (pH 8.5); it has been shown that, under these conditions, yeast PDC dissociates from 240 kDa tetramers into dimers [31–33]. Presumably it is because cofactors bind across the subunit interface [6–8] that this dissociation promotes their release. It is known that wild-type *Z. mobilis* PDC holoenzyme and apoenzyme are both tetrameric at pH 6.5 [24,34], and X-ray solution scattering studies have suggested that the tetramers remain intact at higher pH values [35]. However, the latter experiments were performed at high protein concentrations (50–250 μM in subunits) that would suppress dissociation, leaving open the possibility that cofactor release from *Z. mobilis* PDC involves dimer formation. If this is the case, then the difficulty in removing cofactors from the E473Q mutant might have been due to an unusually high tetramer stability.

Gel-filtration chromatography showed that this mutant PDC (at a subunit concentration of less than 2.8 μM) was eluted at a position close to that expected for a 240 kDa globular protein. However, wild-type apoenzyme (at a very similar concentration) was also eluted at this position, demonstrating that, for *Z. mobilis* PDC, cofactor release does not depend on subunit dissociation. The higher tetramer stability of *Z. mobilis* PDC, as compared with the yeast enzyme, is consistent with the more extensive subunit interface in the former noted by Dobritzsch et al. [9]. Thus it seems that the failure of the E473Q PDC mutant to release cofactors at pH 8.5 was not caused by a higher stability of the tetramer.

DISCUSSION

We have prepared two mutants at each of two positions (Asp-27 and Glu-473; Figure 1) in *Z. mobilis* PDC. Each of the mutant proteins was expressed in *E. coli* and purified to near-homogeneity (Figure 2). The mutations were chosen to be quite conservative: converting each acidic residue to the corresponding amide or exchanging aspartate and glutamate at each position. Despite the conservative nature of these changes, all of the mutants had very low activity, ranging from 580-fold to 4000-fold lower than that of the wild-type enzyme. Recently, Pohl et al. [36] reported that the E473D mutant of *Z. mobilis* PDC has no activity. However, the first author (M. Pohl, personal communication) concedes that an activity of less than 1 % of wild type might not have been detected, a limit well above the activity that we have observed here (Table 1). Even measuring these low activities is quite challenging and is made possible only by the high efficiency of the expression and purification systems that we have used, which enable tens of milligrams of protein to be purified.

These same four mutants have also been prepared for yeast PDC [20,21]. Although only limited data have been reported on their properties, the specific activities are at least an order of magnitude higher than those that we observe. In this context, Sun et al. [37] have suggested that the regulation of yeast PDC by substrate activation is achieved at the cost of catalytic efficiency. Thus the mutation of residues involved in catalysis might be expected to have less effect on the yeast enzyme than on *Z. mobilis* PDC. Consistent with this hypothesis is the observation that mutation of the catalytic Glu-50 (Figure 1) in *Z. mobilis* PDC [11] results in 0.46 % (E50Q) or 2.9 % (E50D) of wild-type activity, whereas the same mutations of the corresponding yeast PDC Glu-51 [21] are each approx. 4-fold more active.

It is conceivable that the specific activities that we have measured are underestimates resulting from a mixture of active and inactive forms. Although such a possibility is difficult to disprove, it would require that the two forms co-purified during hydroxyapatite absorption and desorption, ion-exchange chromatography and gel-filtration chromatography. Moreover, if there is an inactive form present, it seems to be fully competent in cofactor binding. Although neither of these arguments is totally compelling, taken together they favour the simpler hypothesis that for each of the mutants there is only one form of the enzyme, and it has a very low specific activity.

Wild-type *Z. mobilis* PDC has a hyperbolic substrate saturation curve, unlike the PDC that has been characterized from other sources [4]. All of the mutants also show Michaelis–Menten kinetics with respect to pyruvate, although some differences from the wild type were noted in the K_m values (Table 1). Generally, the mutations at Glu-473 had a greater effect than those at Asp-27, but it would be superficial to explain this simply on the basis (Figure 1) that Glu-473 is closer (4.05 Å) to the C-2 atom of ThDP than is Asp-27 (6.38 Å).

The effects described above can be interpreted in terms of the quantitative model for the catalytic cycle of *Z. mobilis* PDC (Scheme 1) proposed by Sun et al. [37]. All mutations result in a major decrease in the specific activity (and therefore in k_{cat}) with relatively little effect on the K_m for pyruvate. The model shows

$$E \underset{k_2 = 75\ s^{-1}}{\overset{k_1 = 200\ mM^{-1}s^{-1}}{\rightleftharpoons}} E{\bullet}pyruvate \xrightarrow{k_3 = 300\ s^{-1}} E{\bullet}acetaldehyde \xrightarrow{k_5 = 188\ s^{-1}} E$$

$$k_{cat} = k_3\,k_5 / (k_3 + k_5) \qquad K_m = (k_2\,k_5 + k_3\,k_5) / (k_1\,k_3 + k_1\,k_5)$$

Scheme 1 Quantitative model for the catalytic cycle of *Z. mobilis* PDC

This model is adapted from [37]; in that model, rate constants refer to the tetramer and these values have been divided by 4 to represent the rate constants per active site.

that there is no single rate-determining step, with k_{cat} dependent to similar extents on k_3 (decarboxylation) and k_5 (acetaldehyde release). However, a large decrease in k_5 would result in a nearly proportional decrease in K_m, so this focuses attention on k_3 as the rate constant that is most affected by the mutations. If k_3 decreases from 300 to 0.06 s^{-1} and there is also a modest decrease (from 200 to 160 $mM^{-1} \cdot s^{-1}$) in the rate constant for pyruvate binding (k_1), this would result in a specific activity that was approx. 0.05 % of wild-type and in a 35 % decrease in K_m. Such properties are very similar to those observed for the mutant D27N. It would be of interest to measure ^{13}C-substrate isotope effects to substantiate this conjecture.

Wild-type *Z. mobilis* PDC contains approx. 1 mol per subunit of each cofactor [24] but these can be removed by treatment with EDTA at pH 8.5. This procedure has been shown to be effective for most of the mutants that we have prepared previously [11,13,14,23,38]. The only exceptions have been the mutations at Asp-440 or Asn-467 (Figure 1), in which cofactor affinity decreases to such an extent that they cannot bind effectively and no special treatment is needed for their removal. Three of the mutations reported here (D27E, D27N and E473D) could be converted to apoenzyme by the same procedure (Table 2). However, E473Q is unique: it apparently binds cofactors with very high affinity so that neither can be removed.

The three variants that could be converted to apoenzyme showed little activity when assayed in the absence of one of the cofactors. However, they could be reactivated by adding back both cofactors and this method was used to measure cofactor activation constants (Table 1). The mutations resulted in a small increase (approx. 2–3.5-fold) in the K_c for ThDP but the largest effect was on the K_c for Mg^{2+} of D27N, due mainly to a substantial increase in the rate of dissociation of this cofactor. This was an unexpected result given that Asp-27 is some distance from where Mg^{2+} binds (Figure 1). However, we have shown previously that binding of the two cofactors is a highly co-operative process [24] in which the prior binding of one cofactor increases the affinity for the other by several hundred-fold. As a result, the effect of certain mutations is manifested more on the 'wrong' cofactor by influencing this cooperation between them. For example, Asn-467 provides one of the ligands to Mg^{2+} (Figure 1) but does not interact directly with ThDP [6,9]. However, mutation to an Asp residue has no discernible effect on the affinity for Mg^{2+} but weakens ThDP binding to 1/580 [14].

The unusually high affinity of E473Q for cofactors is a puzzling observation for which no definitive explanation can be offered. Possibly it is due to a loss of charge repulsion between the carboxylate and the active carbanionic form of ThDP. However, we are conscious that Lobell and Crout [17] have argued that Glu-473 is normally protonated (at pH 6) and is therefore neutral, whereas Kern et al. [12] have some evidence that the dominant form of ThDP in the enzyme (also at pH 6) is also protonated. Nevertheless both ThDP and Glu-473 might be ionized at pH 8.5 in wild-type PDC and this would assist in the release of ThDP; the E473Q mutation would therefore prevent the liberation of ThDP.

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REFERENCES

1 Boiteux, A. and Hess, B. (1970) FEBS Lett. **9**, 293–296
2 Alvarez, F. J. and Schowen, R. L. (1991) in Biochemistry and Physiology of Thiamin Diphosphate Enzymes (Bisswanger, H. and Ullrich, J., eds.), pp. 59–66, VCH, Weinheim
3 Hübner, G., Weidhase, R. and Schellenberger, A. (1978) Eur. J. Biochem. **92**, 175–181
4 König, S. (1998) Biochim. Biophys. Acta **1385**, 271–286
5 Candy, J. M. and Duggleby, R. G. (1998) Biochim. Biophys. Acta **1385**, 323–338
6 Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B. and Jordan, F. (1993) Biochemistry **32**, 6165–6170
7 Arjunan, P., Umland, T., Dyda, F., Swaminathan, S., Furey, W., Sax, M., Farrenkopf, B., Gao, Y., Zhang, D. and Jordan, F. (1996) J. Mol. Biol. **256**, 590–600
8 Lu, G., Dobritzch, D., König, S. and Schneider, G. (1997) FEBS Lett. **403**, 249–253
9 Dobritzch, D., König, S., Schneider, G. and Lu, G. (1998) J. Biol. Chem. **273**, 20196–20204
10 Pohl, M. (1997) Adv. Biochem. Eng. Biotechnol. **58**, 15–43
11 Candy, J. M., Koga, J., Nixon, P. F. and Duggleby, R. G. (1996) Biochem. J. **315**, 745–751
12 Kern, D., Kern, G., Neef, H., Tittman, K., Killenberg-Jabs, M., Wikner, C., Schneider, G. and Hübner, G. (1997) Science **275**, 67–70
13 Diefenbach, R. J., Candy, J. M., Mattick, J. S. and Duggleby, R. G. (1992) FEBS Lett. **296**, 95–98
14 Candy, J. M. and Duggleby, R. G. (1994) Biochem. J. **300**, 7–13
15 Harris, T. K. and Washabaugh, M. W. (1995) Biochemistry **34**, 14001–14011
16 Lobell, M. and Crout, D. H. G. (1996) J. Chem. Soc. Perkin Trans. **1**, 1577–1581
17 Lobell, M. and Crout, D. H. G. (1996) J. Am. Chem. Soc. **118**, 1867–1873
18 Baburina, I., Dikdan, G., Guo, F., Tous, G. I., Root, B. and Jordan, F. (1998) Biochemistry **37**, 1245–1255
19 Baburina, I., Li, H., Bennion, B., Furey, W. and Jordan, F. (1998) Biochemistry **37**, 1235–1244
20 Jordan, F., Baburina, I., Gao, Y., Guo, F., Kahyaoglu, A., Nemeria, N., Volkov, A., Yi, J.-Z., Zhang, D., Machado, R. et al. (1996) in Biochemistry and Physiology of Thiamin Diphosphate Enzymes (Bisswanger, H. and Schellenberger, A., eds.), pp. 53–69, Wissenschaftlicher Verlag, Prien, Germany
21 Jordan, F., Nemeria, N., Guo, F., Baburina, I., Gao, Y., Kahyaoglu, A., Li, H., Wang, J., Yi, J., Guest, J. R. and Furey, W. (1998) Biochim. Biophys. Acta **1385**, 287–306
22 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23 Schenk, G., Leeper, F. J., England, R., Nixon, P. F. and Duggleby, R. G. (1997) Eur. J. Biochem. **248**, 73–81
24 Diefenbach, R. J. and Duggleby, R. G. (1991) Biochem. J. **276**, 439–445
25 Duggleby, R. G. (1984) Comput. Biol. Med. **14**, 447–455
26 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
27 Sedmac, J. J. and Grossberg, S. E. (1977) Anal. Biochem. **79**, 544–552
28 Neale, A. D., Scopes, R. K., Wettenhall, R. E. H. and Hoogenraad, N. J. (1987) Nucleic Acids Res. **15**, 1753–1761
29 Mihalyi, E. (1968) J. Chem. Eng. Data **13**, 179–182
30 Vaccaro, J. A., Crane, III, E. J., Harris, T. K. and Washabaugh, M. W. (1995) Biochemistry **34**, 12636–12644
31 Gounaris, A. D., Turkenkopf, I., Buckwald, S. and Young, A. (1971) J. Biol. Chem. **246**, 1302–1309
32 Hübner, G., König, S., Schellenberger, A. and Koch, M. H. J. (1990) FEBS Lett. **266**, 17–20
33 Eppendorfer, S., König, S., Golbik, R., Neef, H., Lehle, K., Jaenicke, R., Schellenberger, A. and Hübner, G. (1993) Biol. Chem. Hoppe-Seyler **374**, 1129–1134
34 Pohl, M., Grötzinger, J., Wollmer, A. and Kula, M.-R. (1994) Eur. J. Biochem. **224**, 651–661
35 König, S., Svergun, D. I., Volkov, V. V., Feigin, L. A. and Koch, M. H. J. (1998) Biochemistry **37**, 5329–5334
36 Pohl, M., Siegert, P., Mesch, K., Bruhn, H. and Grötzinger, J. (1998) Eur. J. Biochem. **257**, 538–546
37 Sun, S., Duggleby, R. G. and Schowen, R. L. (1995) J. Am. Chem. Soc. **117**, 7317–7322
38 Huang, C.-Y., Nixon, P. F. and Duggleby, R. G. (1999) J. Biochem. Mol. Biol., in the press