Eur. J. Biochem. **268**, 3558–3565 (2001) © FEBS 2001

Site-directed mutagenesis of the ionizable groups in the active site of *Zymomonas mobilis* pyruvate decarboxylase

Effect on activity and pH dependence

Chang-Yi Huang, Alan K. Chang, Peter F. Nixon and Ronald G. Duggleby

Centre for Protein Structure, Function and Engineering, Department of Biochemistry and Molecular Biology, The University of Queensland, Brisbane, Australia

Pyruvate decarboxylase (PDC, EC 4.1.1.1) is a thiamin diphosphate-dependent enzyme about which there is a large body of structural and functional information. The active site contains several absolutely conserved ionizable groups and all of these appear to be important, as judged by the fact that mutation diminishes or abolishes catalytic activity. Previously we have shown [Schenk, G., Leeper, F.J., England, R., Nixon, P.F. & Duggleby, R.G. (1997) *Eur. J. Biochem.* **248**, 63–71] that the activity is pH-dependent due to changes in k_{cat}/K_m while k_{cat} itself is unaffected by pH. The effect on k_{cat}/K_m is determined by a group with a pK_a of 6.45; the identity of this group has not been determined, although H113 is a possible candidate. Here we mutate five crucial residues in the active site with ionizable side-chains (D27, E50, H113, H114 and E473) in turn, to residues that are nonionizable or should have a substantially altered pK_a. Each protein was purified and characterized kinetically. Unexpectedly, the pH-dependence of k_{cat}/K_m is largely unaffected in all mutants, ruling out the possibility that any of these five residues is responsible for the observed pK_a of 6.45. We conjecture that the k_{cat}/K_m profile reflects the protonation of an alcoholate anion intermediate of the catalytic cycle.

Keywords: catalytic mechanism; pH-dependence; pyruvate decarboxylase; site-directed mutagenesis; thiamin diphosphate.

Pyruvate decarboxylase (PDC, EC 4.1.1.1) is the archetypal member of a family of enzymes that use thiamin diphosphate (ThDP) as a cofactor. In all of these enzymes, the initial step of catalysis (Fig. 1) is believed [1] to be dissociation of the C2 proton (Fig. 1, reaction 1) of the thiazole ring (I) to produce an ylide (II). This then attacks the carbonyl group of pyruvate (reactions 2a and 2b) to yield enzyme-bound lactyl-ThDP (IIIb), perhaps via a transiently formed alcoholate anion (IIIa) that is then protonated (reaction 2b). Carbon–carbon bond cleavage ensues (reaction 3), releasing CO_2 and producing the resonating α-carbanion (IVa) and enamine (IVb). Similar steps are common to all ThDP-dependent enzymes with the exception of N^2-(2-carboxyethyl)arginine synthase [2] in which there is no carbon–carbon bond cleavage. The remaining steps of the catalytic cycle vary depending on the enzyme concerned, and can involve simple release of the product (e.g. 2-ketoacid decarboxylases, benzaldehyde lyase), oxidation (e.g. 2-ketoacid dehydrogenases, pyruvate oxidase), or condensation with a second substrate (e.g. acetohydroxyacid synthase, transketolase). In the case of pyruvate decarboxylase, the α-carbanion/enamine is protonated (reaction 4) to hydroxyethyl-ThDP (V) then acetaldehyde is released (reaction 5) to regenerate enzyme-bound ThDP.

Three steps in the catalytic cycle of PDC (reactions 1, 2b and 4) involve proton addition or release and it might be expected that there would be acidic and basic amino acids in the active site to facilitate these steps. In addition, it is believed that catalysis involves closure of the active site by the C-terminal α helix [3,4], which will bury the carboxyl group of pyruvate from solvent. Presumably, formation of an ion pair with a positively charged group near the active site is needed to facilitate this process.

X-ray crystallographic studies of the yeast [5] and *Zymomonas mobilis* [3] enzyme have identified a number of ionizable groups in the active site (Fig. 2) although the precise role of these groups is unclear. Probably the best studied of these residues is E50, which is conserved in the sequence of all ThDP-dependent enzymes [6] and occupies a similar position in those ThDP-dependent enzymes whose structure has been determined [3,7–11]. This residue has been proposed [7] to initiate the ionization of C2 and it has been shown that mutation of this residue greatly diminishes the exchange of the C2 proton with solvent [12,13]. However, this finding is difficult to reconcile with the observation that mutation of this glutamate in transketolase [14], *Z. mobilis* PDC [15], yeast PDC [16] and human pyruvate dehydrogenase [17] results in enzymes with a significant residual activity.

Correspondence to R. G. Duggleby, Department of Biochemistry and Molecular Biology, The University of Queensland Brisbane, QLD 4072, Australia. Fax: + 617 33654699, Tel.: + 617 33654615, E-mail: duggleby@biosci.uq.edu.au

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

Enzyme: pyruvate decarboxylase (EC 4.1.1.1).

Note: a website is available at http://www.biosci.uq.edu.au

(Received 19 February 2001, revised 23 April 2001, accepted 26 April 2001)

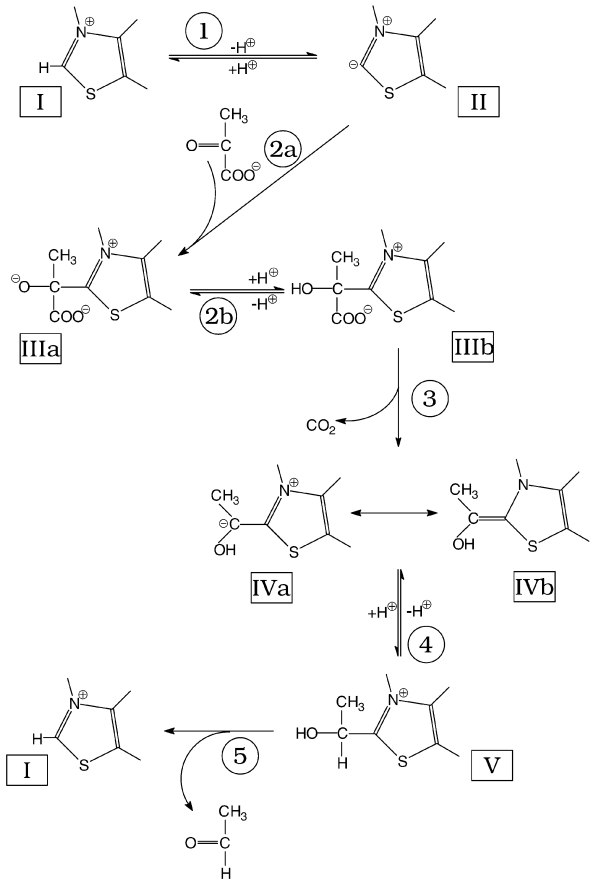

Fig. 1. The catalytic cycle of PDC. Roman numerals in square boxes are intermediates referred to in the text, while Arabic numerals in circles are individual steps in the catalytic cycle. The thiazole ring of ThDP (I) ionizes at C2 (reaction 1) and the resulting ylide (II) reacts with pyruvate (reaction 2a) and a proton (reaction 2b) to give lactyl-ThDP (IIIb) via the transiently formed alcoholate anion (IIIa). In reaction 3, CO_2 is released giving rise to the resonating α-carbanion (IVa) and enamine (IVb). Protonation (reaction 4) yields hydroxyethyl-ThDP (V) that then releases acetaldehyde (reaction 5) to regenerate I. Double arrows are ionizations, single arrows are steps involving reactant addition or release, and the double-headed arrow represents resonance. The single arrows indicate the prevailing reaction direction when pyruvate is converted to acetaldehyde and CO_2, and are not intended to imply that the step is irreversible.

The other residues shown in Fig. 2 all influence PDC activity and each is conserved in all PDCs, providing further evidence that they play important roles in substrate binding or catalysis. Mutation of D27 [18,19] or E473 [18] of *Z. mobilis* PDC to the corresponding amides results in enzymes with low but detectable activity ($< 0.2\%$ of wild-type). In these mutants, k_{cat}/K_m and k_{cat} decrease in parallel so that K_m is virtually unchanged. As k_{cat}/K_m includes rate constants for all steps up to and including decarboxylation (reaction 3), while k_{cat} includes rate constants for all steps including and subsequent to decarboxylation [20], it appears that it is the decarboxylation step that is impaired in these mutants. Interestingly, these mutants display an enhanced carboligase activity in which the α-carbanion/enamine (IV) reacts with acetaldehyde to form acetoin

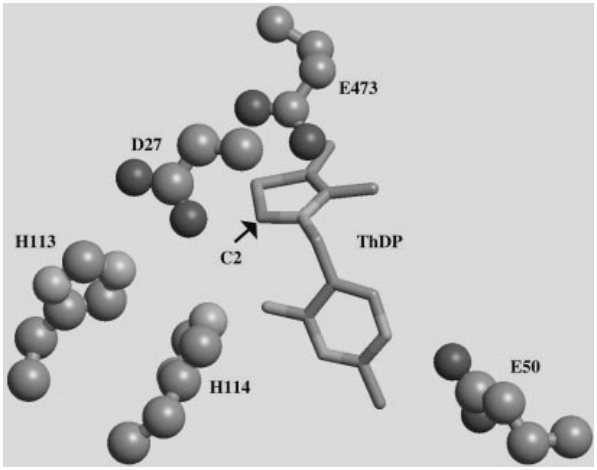

Fig. 2. The active site of *Z. mobilis* PDC. The thiamin portion of ThDP is shown in stick representation, together with ionizable residues in the active site as ball-and-stick. Coordinates were taken from [3]; the published structure is missing C2 of ThDP and its expected position was calculated from those of the remaining atoms of the thiazole ring.

[19,21]. Mutation of H113 to glutamine has an even more pronounced effect on catalysis, producing an enzyme with no detectable PDC activity [22]. However, this H113Q retains the ability to release acetaldehyde from hydroxyethyl-ThDP (reaction 5) suggesting that it is an earlier step in the catalytic cycle that requires H113. On the other hand, H114 plays a comparatively minor role because mutation to glutamine results in an enzyme that retains 37% of the wild-type activity [22].

Despite the possible roles of proton donors and acceptors in PDC catalysis, there has been little investigation of such residues by measurements of the influence of pH on the activity. The most complete and recent study of the pH-dependent kinetics of yeast PDC [23] was complicated by the fact that the enzyme displays sigmoidal kinetics towards pyruvate [24]. These properties result because activation of yeast PDC requires the binding of pyruvate to a separate allosteric site [25], making it difficult to determine the classical Michaelis–Menten kinetic parameters (V_m and K_m). The sigmoidal kinetics can be abolished by activating the enzyme with pyruvamide [25] or ketomalonate [26], or by using one of several PDC variants that are activated by mutation [27–29]. Unfortunately, the pH-dependent kinetic properties of the activated yeast enzyme have not been reported. The reported allosteric pH-dependent kinetics [23] show at least two pK_a values in the range 5–7 and the authors discuss the possibility that the yeast counterparts of D27, E50, H113, H114 and E473 could be responsible for the observed kinetics. However, no data have been adduced to test these possibilities.

In this laboratory, we have focussed our studies on the *Z. mobilis* enzyme. One of the main reasons for doing so is that it is the only known PDC that shows Michaelis–Menten kinetics [6,30]. We have shown [22] that the enzyme is strongly affected by pH. The maximum velocity is virtually constant in the pH range 5.8–8.5, but the K_m increases markedly as the pH increases. As a result k_{cat}/K_m decreases and the data are consistent with an ionizable

group with an estimated pK_a of 6.45, that must be protonated for the PDC reaction to occur. This observation, coupled with the fact that the H113Q mutant is totally inactive, led us to speculate that the observed pK_a could be ascribed to the protonation of H113. There is, however, no direct confirmation of this hypothesis. The fact that H113Q is inactive precludes pK_a measurements that are dependent on catalysis.

In this report we describe the preparation and characterization of two further H113 mutants (H113K and H113R). Both are active, which allowed us to measure the effect of pH on k_{cat} and on k_{cat}/K_m. For these mutants, both kinetic parameters are affected by pH, and the pK_a derived from the k_{cat}/K_m profile is elevated compared to that observed for the wild-type enzyme. However, the increase is small compared to that which would be expected if H113 is the source of the observed pK_a. The pH-dependent kinetics of D27, E50, H114, and E473 mutants were also studied and the results rule out the possibility that the side-chain of any of these residues is the ionizable group observed in the k_{cat}/K_m profile. We propose that the observed pK_a represents the protonation of an alcoholate anion intermediate (IIIa) that has been suggested [31] to be formed transiently in reaction 2 of the catalytic cycle.

MATERIALS AND METHODS

Restriction endonucleases and molecular biology products

Restriction enzymes, T4 DNA polymerase and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). The Big-Dye Terminator PCR sequencing kit was from Perkin-Elmer Applied Biosystems (Norwalk, CT, USA). ADH, pyruvate, deoxyribonuclease I, lysozyme, dithiothreitol and ThDP were obtained from Sigma (St Louis, MO, USA). Agarose and protein molecular weight markers were supplied by Progen (Brisbane, Australia). The 'Altered Sites' *in vitro* mutagenesis system was purchased from Promega (Sydney, Australia). Mutagenic primers were obtained from Pacific Oligos (Lismore, Australia) and Gibco BRL (Life Technologies, Melbourne, Australia) as the cartridge-purified grade. DNA purification kits were bought from Life Technologies and Boehringer-Mannhein (Castle Hill, Australia).

Bacterial strains and plasmids

Bacterial culture, plasmid isolation, restriction endonuclease digestions, ligations, DNA electrophoresis, transformations and SDS polyacrylamide gel electrophoresis were carried out according to standard protocols [32]. All PCR sequencing reactions were carried out using a Perkin-Elmer DNA Cycler, Model 480. *Escherichia coli* strain DH5α was from Gibco BRL. The ampicillin-resistant pPLZM plasmid for heat-inducible expression of *Z. mobilis* PDC was described previously [15]. *E. coli* cells containing pPLZM wild-type or mutant constructs were maintained on Luria-Bertani broth agar plates containing 100 $\mu g \cdot mL^{-1}$ ampicillin for short-term storage. For long-term storage, 15% glycerol was added to liquid cultures, which were then stored at −70 °C.

Site-directed mutagenesis

Construction of the D27N, E50Q, H114Q and E473Q mutants has been described previously [15,18,22]. Other mutants (H113K, H113R and H114A) were constructed by the 'Altered Sites' *in vitro* mutagenesis system (Promega) with slight modifications. The template used for constructing mutants contained the H113Q [22] substitution, which possesses a *Sph*I site spanning codons 113, 114 and 115 (underlined in CAGCATGCC) involving the last base of codon 113 (CAG). Alteration of this codon for H113 mutations (K, AAA; R, CGA) eliminates the *Sph*I site allowing easy identification of mutants. A similar protocol was employed for H114A, with a simultaneous change in the H114 codon (CAT→GCT) and correction of codon 113 to that for histidine (CAG→CAC). The template was prepared using a 0.85-kb *Eco*RI/*Nco*I fragment of the PDC gene that was ligated into *Eco*RI/*Nco*I-digested pALTER-*Ex*1 (Promega). Ligation mixtures were transformed into DH5α competent cells and colonies were screened by *Sph*I and *Eco*RI/*Nco*I digestions of plasmid DNA. The construct was confirmed by DNA sequencing. Mutagenic primers were designed to avoid any possible false priming, dimer formation and hairpin loop structure. Mutagenic primers were phosphorylated and stored at −20 °C [32]. Mutagenesis was conducted by combining approximately 400 ng of template DNA, 100 pmol of the mutagenic primer and 20 pmol of an oligonucleotide that repairs a disabled ampicillin-resistance gene in the vector. These were mixed with annealing buffer (Promega) in a total volume of 20 μL and the mixture was boiled in a water bath for 3 min then left on ice for 15 min. T4 DNA polymerase, T4 DNA ligase, 5 mM dNTP, 10 mM ATP and synthesis buffer (Promega) were added and the mixture was then incubated at 37 °C for 90 min. The mutagenesis reaction mixture was used to transform *E. coli* BMH competent cells, which were then grown at 37 °C overnight in Luria-Bertani broth with 100 $\mu g \cdot mL^{-1}$ ampicillin. Plasmid DNA was purified and digested with *Sph*I to check that the mutagenesis was successful. After recovering the mutagenized *Eco*RI/*Nco*I fragment of the PDC gene, it was cloned back into the expression vector pPLZM to construct the full-length coding sequence. Mutations were confirmed by DNA sequencing.

Expression, protein purification, and preparation of apoenzyme

The *E. coli* cells, transformed by the appropriate expression vector, were grown at 30 °C and expression was induced by rapidly shifting the temperature to 42 °C, as described previously [15,22]. Enzyme purification, and the removal of cofactors to produce apoenzyme, also followed our published procedures [4,33].

Kinetic studies

PDC activity was measured at 30 °C in a coupled enzyme assay using alcohol dehydrogenase (ADH). Reaction mixtures contained 5 mM pyruvate, 0.1 mM ThDP, 5 mM $MgCl_2$ 10 units$\cdot mL^{-1}$ ADH, 0.15 mM NADH and buffer. The buffer system (50 mM Mes, 100 mM Tris and 50 mM acetic acid) was adjusted to the

appropriate pH with HCl or NaOH. This buffer system allows the ionic strength to remain constant over a wide range of pH values [34]. Enzyme was added to the mixture and the decline in NADH concentration was followed at 340 nm for 5 min. For kinetic characterization, the pyruvate concentration was varied over a range that depended on the pH and the particular mutant concerned, but usually involved at least 12 concentrations, in duplicate. Determination of pK_a values from pH-activity profiles was as described previously [22], and is explained below.

Cofactor activation kinetics were determined by measuring the activity of the reconstituted holoenzyme on addition of cofactors to the apoenzyme. The apoenzyme was preincubated at 30 °C for 15 min at a saturating concentration of one cofactor, with various concentrations of the other. The reaction was initiated by adding a mixture containing pyruvate, ADH and NADH, and the rate was measured as above.

Cofactor binding assay

For an inactive mutant, the affinity for cofactors cannot be determined as described in the previous section. For such mutants, cofactor binding can be measured by following the quenching of tryptophan fluorescence [30,35]. The apoenzyme was preincubated at 30 °C for 15 min with a saturating concentration of one cofactor only in a fluorescence cuvette. Various concentrations of the other cofactor were added and fluorescence (excitation, 300 nm; emission, 340 nm) was measured over time using a Jasco model FB-770 spectrofluorimeter. Collected data were analyzed as described below.

Data analysis

Kinetic parameters were determined by fitting the appropriate equation to the data by nonlinear regression using GRAFIT (Erithacus Software, Staines, England) or INPLOT (GraphPad Software, San Diego, USA). The best fit values and standard errors obtained from these analyses are reported. Substrate saturation curves were fitted using the Michaelis − Menten equation to obtain V_m and K_m. The ratio k_{cat}/K_m and k_{cat} were then calculated from the fitted parameters using the known enzyme concentration in the assay. Cofactor activation curves were fitted using Eqn. (1). In this equation, the quantity [C] represents the cofactor concentration in the reaction, v is the measured reaction rate, and v_o is the small amount of residual activity, resulting from traces of cofactors remaining in the apoenzyme preparation. The data were then corrected for v_o and fitted using Eqn (1), but omitting the v_o term, to obtain the cofactor activation constant K_c.

$$v = v_o + V_m[C]/(K_c + [C]) \quad (1)$$

Data from the tryptophan fluorescence quenching cofactor binding assay were analyzed as follows. When both cofactors are present, PDC apoenzyme shows a decrease of tryptophan fluorescence that lasts for several minutes. Provided that the concentration of enzyme subunits ($[E]_o$) is much smaller than the added concentration of cofactor ($[C]_o$), the data (fluorescence, F_t versus time, t) follow a first-order decay curve [Eqn (2)].

$$F_t = F_o - (F_o - F_\infty)(1 - e^{-k't}) \quad (2)$$

In this equation, the fitted parameters are F_o, F_∞ (the initial and final fluorescence, respectively), and k' (the apparent first-order rate constant). The latter is defined by Eqn (3).

$$k' = k_{on}[C]_o + k_{off} \quad (3)$$

The rate constant k_{on} is the second-order rate constant for the formation of reconstituted holoenzyme and k_{off} is the first-order rate constant for the dissociation of cofactor from the enzyme. Values for these rate constants were obtained by linear regression, and used to calculate the k_{off}/k_{on} ratio, which equals the cofactor dissociation constant K_d.

For pK_a determination, values of k_{cat} and k_{cat}/K_m were determined within the pH range 5.0–8.5. The negative logs (base 10) of these k_{cat} and k_{cat}/K_m values (i.e. pk_{cat} and pk_{cat}/K_m) were analyzed by fitting a single [Eqn (4)] or double [Eqn (5)] titration curve, to obtain values for pK_a [Eqn (4)] or pK_{a1} and pK_{a2} [Eqn (5)].

$$Y = A + \log_{10}(1 + 10^{-pK_a}/10^{-pH}) \quad (4)$$

$$Y = A + \log_{10}(1 + 10^{-pH}/10^{-pK_{a1}} + 10^{-pK_{a2}}/10^{-pH}) \quad (5)$$

In these equations, Y represents pk_{cat} or pk_{cat}/K_m at different pH values and A represents the maximum value of Y.

RESULTS

Expression and purification of mutants

All mutants (D27N, E50Q, H113K, H113R, H114Q, H114A and E473Q) were prepared successfully. Mutants showed similar behavior to wild-type during purification, which suggests they have not undergone major structural changes. Each appeared to be pure, or nearly so, in SDS polyacrylamide gel electrophoresis (data not shown) and indicated a subunit size near the expected value of 60 kDa. H114A is inactive, while other mutants show specific activities that range from 0.025% (E473Q) to 37% (H114Q) of wild-type PDC (Table 1). H113 mutants have very low specific activities, which may be because H113 plays an important role in the active site. As mentioned previously, H113Q is inactive and even the replacement of this histidine by other basic amino acids greatly impairs the catalytic activity. Position 114 appears to be less important because the enzyme retains 37% activity upon replacement of H114 by glutamine [22]. However, the more radical substitution by alanine abolishes the activity.

Cofactor kinetics

The dependence of activity on ThDP and Mg^{2+} added to the apoenzyme was used to determine activation constants (half-saturating concentrations) for each cofactor (Table 1). Usually, the activity in the absence of one cofactor (at a saturating concentration of the other) is 5% or less of the activity of the fully reconstituted holoenzymes. For Mg^{2+}, each of the mutants requires higher concentrations for activation but in all cases the concentration used in the

Table 1. Kinetic properties of wild-type and mutants of *Z. mobilis* PDC. The specific activities were determined using the standard assay. The cofactor activation constants were determined by reconstitution of apoenzyme activity or by tryptophan fluorescence quenching measurements on apoenzymes (H113Q and H114A). Some data reported previously [15,18,22] have been included for comparison. ND, not determined.

Enzyme	Specific activity (% of wild-type)	Cofactor activation constant	
		ThDP (μM)	Mg^{2+} (μM)
Wild-type	100	3.35 ± 0.45	6.58 ± 1.01
D27N	0.072	3.30 ± 0.28	64.5 ± 4.6
E50Q	0.46	10.7 ± 4.0	194 ± 65
H113Q	< 0.007	75 ± 9	105 ± 15
H113K	0.90	6.71 ± 0.49	45.4 ± 6.2
H113R	1.40	13.9 ± 1.2	37.3 ± 4.9
H114Q	37	1.25 ± 0.12	14.9 ± 2.8
H114A	< 0.007	0.82 ± 0.15	25.7 ± 5.3
E473Q	0.025	ND	ND

standard assay (5 mM) will be close to saturating. For ThDP, activation constants of the active mutants range from 1.25 to 13.9 μM, spanning the value for wild-type (3.35 μM). Again, the concentration used in the standard assay (0.1 mM) will be close to saturating. For the inactive mutants (H113Q and H114A), binding of cofactors could not be assessed by activity stimulation so the tryptophan fluorescence quenching assay [30] was used. The half-saturating concentration for Mg^{2+} of both mutants is elevated, but the values fall within the range observed for other mutants. The value for ThDP of H113Q is relatively high; in contrast, that of H114A is lower than for wild-type and for all the other mutants. These measurements demonstrate that the reduced or absent activity of these mutants cannot be ascribed to impairment of cofactor binding, as is the case for other mutants such as D440N [35] and N467Q [36].

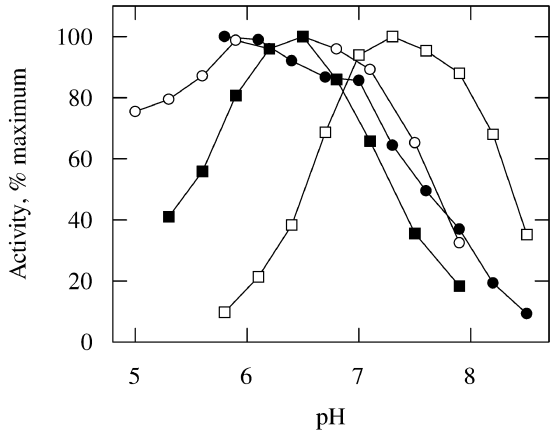

Fig. 3. Effect of pH on the activity of wild-type and mutants of *Z. mobilis* PDC. Activity was measured as described in the text, at a fixed pyruvate concentration of 5 mM. To facilitate comparison between enzymes that have widely differing activities, the curves have been normalized to a maximum activity set at 100%. Data are shown for wild-type (●), D27N (○), E50Q (■) and H113K (□).

Table 2. pK_a values and lowest observed K_m values of wild-type and mutants of *Z. mobilis* PDC. The pK_a values were determined as described in the text using data such as those illustrated in Figs 4 and 5. The lowest observed (limiting) K_m values were those determined at the lowest pH investigated. NA, not applicable.

Enzyme	k_{cat}/K_m (pK_a)	k_{cat} (pK_{a1}, pK_{a2})	Limiting K_m (mM)
Wild-type	6.23 ± 0.07	NA	0.330 ± 0.026
D27N	6.46 ± 0.05	NA	0.246 ± 0.012
E50Q	6.52 ± 0.11	6.37 ± 0.06, 8.19 ± 0.18	0.388 ± 0.052
H113K	6.59 ± 0.14	6.94 ± 0.06, 8.36 ± 0.11	0.007 ± 0.002
H113R	7.05 ± 0.06	7.39 ± 0.09, 8.39 ± 0.18	0.016 ± 0.010
H114Q	6.06 ± 0.09	NA	0.420 ± 0.048
E473Q	6.72 ± 0.02	NA	0.669 ± 0.049

Effect of pH upon activity

Initially, the effect of pH on wild-type PDC and the various mutants was assessed by measuring the activity at a fixed concentration (5 mM) of pyruvate (Fig. 3). Three distinct patterns were seen. The first pattern, displayed by D27N, H114Q (not shown) and E473Q (not shown) has a maximum near pH 6.0 with a sharp decrease in activity on the alkaline side and a smaller decrease on the acidic side. Wild-type also shows a similar pattern, except that below pH 5.8 reliable data were not obtained. The second pattern, which was observed for E50Q only, is similar to the first pattern on the alkaline side but there is a much sharper drop in activity on the acidic side. This results in a clearly defined maximum of activity at pH 6.5. The third pattern, which was found for both H113K and H113R (not shown), is quite similar to that of E50Q except that the entire curve is shifted 0.8 pH units towards the alkaline side. These experiments were conducted at a constant concentration of pyruvate (5 mM) and the shape of the curves can be due to changes in K_m, k_{cat}, or both.

Previous studies [22] have shown that for wild-type PDC the effects of pH on activity are dominated by changes in k_{cat}/K_m with little effect on k_{cat} itself. We have confirmed this result (data not shown) and obtain a pK_a of 6.23 ± 0.07 (Table 2) from the k_{cat}/K_m profile (cf. 6.45 in [22]). Nevertheless, it does not follow necessarily that the similar pH profile to wild-type found in the mutants D27N, H114Q and E473Q (Fig. 3) is a result of comparable effects on k_{cat} and k_{cat}/K_m. The effect of pH on k_{cat} and k_{cat}/K_m for each of these mutants was determined. In each case, the pattern observed is similar to wild-type, as illustrated for E473Q in Fig. 4. The value of k_{cat} (and therefore pk_{cat}) is virtually constant while pk_{cat}/K_m increases as pH increases but reaches a plateau at low pH. The K_m reaches a lower limiting value on this plateau. The pK_a, estimated using Eqn (4), is 6.72 ± 0.02 and the limiting K_m is 0.669 ± 0.049; pK_a values and limiting K_m values determined for each of the mutants are listed in Table 2.

E50Q (not shown), H113K (Fig. 5A) and H113R (Fig. 5B) showed a similar pattern to wild-type for the

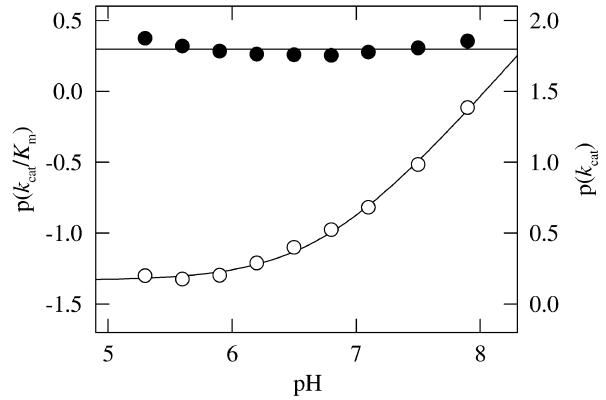

Fig. 4. Effect of pH on the kinetics of the E473Q mutant of *Z. mobilis* PDC. Substrate saturation curves were determined as described in the text, and used to calculate pk_{cat}/K_m (○, left ordinate) and pk_{cat} (●), right ordinate. The units of k_{cat} and k_{cat}/K_m are s^{-1} and $M^{-1}\cdot s^{-1}$, respectively. The lines represent the best fit of Eqn (4) to the pk_{cat}/K_m data (pK_a = 6.72 ± 0.02) or the average of the pk_{cat} data.

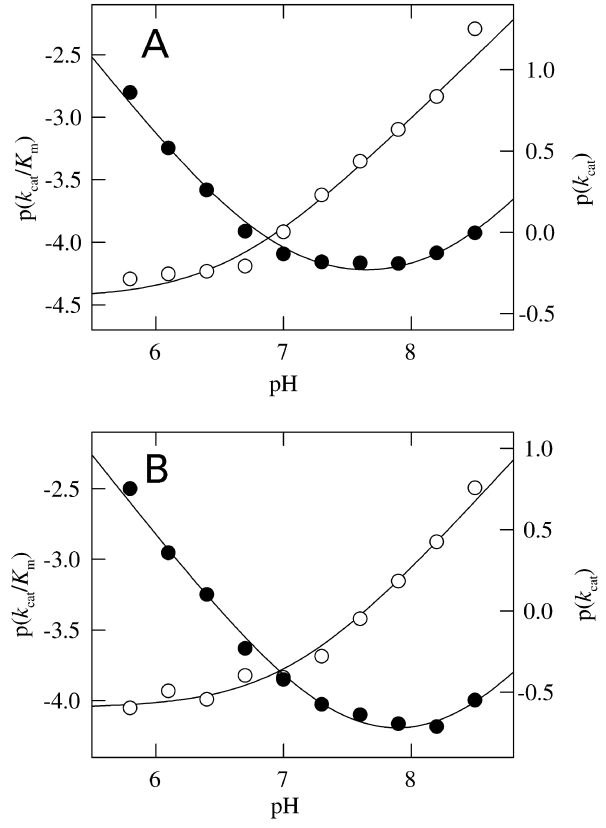

Fig. 5. Effect of pH on the kinetics of the H113 mutants of *Z. mobilis* PDC. Substrate saturation curves were determined as described in the text, and used to calculate pk_{cat}/K_m (○, left ordinate) and pk_{cat} (●), right ordinate. The units of k_{cat} and k_{cat}/K_m are s^{-1} and $M^{-1}\cdot s^{-1}$, respectively. (A) Shows the result for H113K and (B) shows H113R. The lines represent the best fit of Eqn (4) to the pk_{cat}/K_m data (H113K: pK_a = 6.59 ± 0.14; H113R: pK_a = 7.05 ± 0.06) or of Eqn (5) to the pk_{cat} data (H113K: pK_{a1} = 6.94 ± 0.06, pK_{a2} = 8.36 ± 0.11; H113R: pK_{a1} = 7.39 ± 0.09, pK_{a2} = 8.39 ± 0.18).

dependence of k_{cat}/K_m on pH. However, for these mutants, k_{cat} is also pH-dependent reaching a maximum at a slightly alkaline pH and decreasing at the extremes. These pk_{cat} data were used to estimate the two pK_a values [Eqn (5)], which are shown in Table 2. The group responsible for pK_{a1} must be deprotonated for full activity while that for pK_{a2} must be protonated.

DISCUSSION

The activity of wild-type *Z. mobilis* PDC is affected by pH (Fig. 3). The effect is due almost entirely to decreases in k_{cat}/K_m as the pH increases, with virtually no effect on k_{cat}. As k_{cat} is constant, K_m increases with pH; it approaches a plateau at a value of 0.33 mM (Table 2) at a pH of 5.8, but is increased to 35.5 mM at pH 8.5. Further increases at higher pH value would undoubtedly occur but this could not be investigated due to instability of the enzyme [37].

The effect of pH on k_{cat}/K_m of wild-type *Z. mobilis* PDC is well described by a simple titration curve with a pK_a of 6.23 (Table 2), which is similar to the value of 6.45 reported previously [22]. The identity and function of this ionizing group, which must be protonated for maximal k_{cat}/K_m, is not known. Its function could be to form an ion pair with the carboxyl group of pyruvate, or to act as a proton donor in one of the steps in the catalytic cycle (Fig. 1). Involvement in reaction 4 is not expected because pH changes do not affect k_{cat}; however, the ionizing group could be involved in reaction 2 as it affects k_{cat}/K_m [20].

There are several ionizing groups in the vicinity of the ThDP cofactor (Fig. 2) and any one of these could be the group that controls k_{cat}/K_m. Previously we have suggested [22] that the group is the side-chain of H113, based on the observations that: (a) the pK_a is similar to that expected for a histidine; (b) mutation to glutamine renders the enzyme inactive without preventing it from binding the cofactors and (c) the enzyme is still competent in reaction 5 (Fig. 1). Direct testing of this hypothesis, by measuring the pK_a of the H113Q mutant, was not possible because it is totally inactive. Here we have produced two other H113 mutants that possess some activity and therefore can be used in kinetic studies. If H113 is the residue responsible for the observed pK_a it is expected that these mutants would have much higher pK_a values than the wild-type. In addition, we have mutated each of the other residues shown in Fig. 2 to nonionizable amino acids that are otherwise similar in properties. If one of these amino acids is responsible for the observed pK_a then it is anticipated that the pH-dependence of k_{cat}/K_m would be abolished.

Contrary to our expectations, none of the mutations had a major effect on the pK_a in the k_{cat}/K_m profile (Table 2). The largest change, for H113R, is by about 0.8 pH units, which is substantially less than would be anticipated if H113 is responsible for the observed pK_a. For all other mutations, the change is less than 0.5 pH units. Thus, it may be concluded that none of the mutated residues is giving rise to the pK_a value observed for wild-type PDC.

In most cases, the K_m at low pH is comparable to that of the wild-type. The exceptions are the two H113 mutants where the K_m, at the lowest pH at which it was measured, is at least 20-fold less than that of the wild-type. A possible interpretation of this result is that arginine and lysine at position 113 are able to form a salt bridge with the carboxyl

group of pyruvate. It is also necessary to propose that the histidine residue in wild-type PDC is less able to form such a salt bridge because it does not reach the appropriate position in the active site due to its smaller size. As the reaction catalyzed by PDC involves a single substrate only, it has not been possible to crystallize the enzyme with bound pyruvate. Therefore, the exact location of the substrate in the active site, and the residues with which it interacts, have not been determined experimentally.

The failure of any of the mutations to substantially affect the pK_a leaves open the question of what group this represents. There are two possibilities. The first is that it is some other amino acid side-chain, possibly but not necessarily located in the vicinity of the active site. This could only be examined by systematically mutating every acidic and basic residue in the entire protein, a formidable undertaking. However, there is a second possibility that should be entertained; this is that the observed pK_a is that of ThDP ionization (Fig. 1, reaction 1).

The pK_a for the ionization of the C2 proton in free thiamin is very high; for example, Washabaugh and Jencks [38] give a value in the range 17–19. A substantial change in the properties of enzyme-bound ThDP would be needed if it were to have a pK_a in the range 6–7. Kern *et al*. [13] have reported that bound ThDP is not ionized at pH 6 but this conclusion was based on a comparison of the NMR spectrum of free and bound ^{13}C2-labelled ThDP. This interpretation has been challenged [39] because no data were provided on the expected chemical shift or peak height if some or all of the ThDP was ionized. Thus, it is impossible to judge whether ThDP is partially ionized in this experiment and a pK_a in the range 6–7 cannot be ruled out. Jordan *et al*. [39] have compared the ionization of the benzyl analog of V (Fig. 1), free and bound to yeast PDC. They find a pK_a shift from 15.4 (in water) to < 6.0 (in PDC). They suggest that the pK_a for the ionization of the C2 proton of enzyme-bound ThDP might also be shifted to a similar extent. Thus, a pK_a near neutrality is feasible.

The difficulty with this proposal in the context of the present work is that k_{cat}/K_m decreases as pH increases, whereas ionization of C2 (and therefore a higher k_{cat}/K_m) should be favored at higher pH. Thus, the behavior of the enzyme is exactly the opposite of that which would be expected if the observed pK_a represents the ionization of C2. There is a third possibility that is consistent with the response of k_{cat}/K_m to changes of pH. As mentioned earlier, reaction 2 (Fig. 1) involves addition of pyruvate and a proton, and this may involve the transient formation of an alcoholate anion (IIIa) that is then protonated [31]. If this is correct, the protonation (reaction 2b) is a required step in the catalytic cycle so k_{cat}/K_m would increase as pH decreases, as observed. Thus we suggest that the observed pK_a represents reaction 2b of Fig. 1. Although a pK_a of 6–7 is very low for an alcoholate anion in water, the environment in the active site of PDC is quite different from that of water and a value of this order is no less likely than a pK_a near neutrality for the C2 proton, as proposed by Jordan *et al*. [39].

For three of the mutants (E50Q, H113K and H113R), k_{cat} is pH-dependent with a maximum at pH values of 7.28, 7.65 and 7.89, respectively. These results indicate the presence of two ionizable groups governing k_{cat} (Table 2) of these mutants, one that must be protonated and the other deprotonated for PDC activity. k_{cat} is dependent on the second half of the catalytic cycle (reactions 3, 4 and 5) only. Thus, it is of interest that the group responsible for the lower of these two pK_a values must be deprotonated, but reactions 3, 4 and 5 do not involve a step in which there is net removal of a proton. This rules out the possibility that the residue contributing this ionizing group acts as a proton acceptor. Possibly, it is an ionized carboxyl group that stabilizes the positive charge on the nitrogen atom in IVa thereby allowing protonation to give V. The group responsible for the higher of the two pK_a values in the k_{cat} profile must be protonated for activity, and this may be the group that supplies the proton to IVa. However, it is unclear why these events should be detectable in the E50Q, H113K and H113R mutants, but not in wild-type. Apparently, steps that are not rate-limiting in wild-type become so for these mutants allowing observation of pH effects that are masked in the wild-type.

It has been suggested previously [22,31] that the function of H114 is to orient catalytic intermediates (e.g. V) by hydrogen bonding to them. Substitution by another residue of similar size that is capable of hydrogen bonding (glutamine) would have only minor effects on the properties of the enzyme, as observed (Tables 1 and 2). However, the more radical substitution to a residue incapable of hydrogen bonding (alanine) abolishes activity (Table 1); this supports the idea that H114 is involved in orienting catalytic intermediates.

In summary, the activity of wild-type *Z. mobilis* PDC is pH-dependent due mainly to an ionizable group with a pK_a of 6.23 that affects k_{cat}/K_m but not k_{cat}. Five ionizable amino acids in the active site of the enzyme have been mutated to residues that are nonionizable or have a substantially altered pK_a value. In all cases, the pK_a that affects k_{cat}/K_m shows a small change at most, thereby ruling out these residues as the ionizable group concerned. It is suggested that the observed pK_a represents the ionization of an alcoholate anion intermediate of the catalytic cycle.

ACKNOWLEDGEMENT

This work supported by the Australian Research Council (Grant A09800834).

REFERENCES

1. Schellenberger, A. (1998) Sixty years of thiamin diphosphate biochemistry. *Biochim. Biophys. Acta* **1385**, 177–186.
2. Khaleeli, N., Li, R. & Townsend, C.A. (1999) Origin of the β-lactam carbons in clavulanic acid from an unusual thiamine pyrophosphate-mediated reaction. *J. Am. Chem. Soc.* **121**, 9223–9224.
3. Dobritzsch, D., Konig, S., Schneider, G. & Lu, G.G. (1998) High resolution crystal structure of pyruvate decarboxylase from *Zymomonas mobilis* – implications for substrate activation in pyruvate decarboxylases. *J. Biol. Chem.* **273**, 20196–20204.
4. Chang, A.K., Nixon, P.F. & Duggleby, R.G. (2000) Effects of deletions at the carboxyl terminus of *Zymomonas mobilis* pyruvate decarboxylase on the kinetic properties and substrate specificity. *Biochemistry* **39**, 9430–9437.
5. Lu, G.G., Dobritzsch, D., Baumann, S., Schneider, G. & Konig, S. (2000) The structural basis of substrate activation in yeast pyruvate decarboxylase – a crystallographic and kinetic study. *Eur. J. Biochem.* **267**, 861–868.

6. Candy, J.M. & Duggleby, R.G. (1998) Structure and properties of pyruvate decarboxylase and site-directed mutagenesis of the *Zymomonas mobilis* enzyme. *Biochim. Biophys. Acta* **1385**, 323–338.
7. Muller, Y.A., Lindqvist, Y., Furey, W., Schulz, G.E., Jordan, F. & Schneider, G. (1993) A thiamin diphosphate binding fold revealed by comparison of the crystal-structures of transketolase, pyruvate oxidase and pyruvate decarboxylase. *Structure* **1**, 95–103.
8. Ævarsson, A., Chuang, J.L., Wynn, R.M., Turley, S., Chuang, D.T. & Hol, W.G.J. (2000) Crystal structure of human branched-chain α-ketoacid dehydrogenase and the molecular basis of multienzyme complex deficiency in maple syrup urine disease. *Struct. Fold. Des.* **8**, 277–291.
9. Ævarsson, A., Seger, K., Turley, S., Sokatch, J.R. & Hol, W.G.J. (1999) Crystal structure of 2-oxoisovalerate and dehydrogenase and the architecture of 2-oxo acid dehydrogenase multienzyme complexes. *Nat. Struct. Biol.* **6**, 785–792.
10. Chabrière, E., Charon, M.H., Volbeda, A., Pieulle, L., Hatchikian, E.C. & Fontecilla-Camps, J.C. (1999) Crystal structures of the key anaerobic enzyme pyruvate:ferredoxin oxidoreductase, free and in complex with pyruvate. *Nat. Struct. Biol.* **6**, 182–190.
11. Hasson, M.S., Muscate, A., McLeish, M.J., Polovnikova, L.S., Gerlt, J.A., Kenyon, G.L., Petsko, G.A. & Ringe, D. (1998) The crystal structure of benzoylformate decarboxylase at 1.6 angstrom resolution: diversity of catalytic residues in thiamin diphosphate-dependent enzymes. *Biochemistry* **37**, 9918–9930.
12. Hübner, G., Tittmann, K., Killenberg-Jabs, M., Schaffner, J., Spinka, M., Neef, H., Kern, D., Kern, G., Schneider, G., Wikner, C. & Ghisla, S. (1998) Activation of thiamin diphosphate in enzymes. *Biochim. Biophys. Acta* **1385**, 221–228.
13. Kern, D., Kern, G., Neef, H., Tittmann, K., Killenberg-Jabs, M., Wikner, C., Schneider, G. & Hübner, G. (1997) How thiamine diphosphate is activated in enzymes. *Science* **275**, 67–70.
14. Wikner, C., Meshalkina, L., Nilsson, U., Nikkola, M., Lindqvist, Y., Sundstrom, M. & Schneider, G. (1994) Analysis of an invariant cofactor–protein interaction in thiamin diphosphate-dependent enzymes by site-directed mutagenesis – glutamic-acid-418 in transketolase is essential for catalysis. *J. Biol. Chem.* **269**, 32144–32150.
15. Candy, J.M., Koga, J., Nixon, P.F. & Duggleby, R.G. (1996) The role of residues glutamate-50 and phenylalanine-496 in *Zymomonas mobilis* pyruvate decarboxylase. *Biochem. J.* **315**, 745–751.
16. Killenberg-Jabs, M., Konig, S., Eberhardt, I., Hohmann, S. & Hübner, G. (1997) Role of Glu51 for cofactor binding and catalytic activity in pyruvate decarboxylase from yeast studied by site-directed mutagenesis. *Biochemistry* **36**, 1900–1905.
17. Fang, R., Nixon, P.F. & Duggleby, R.G. (1998) Identification of the catalytic glutamate in the E1 component of human pyruvate dehydrogenase. *FEBS Lett.* **437**, 273–277.
18. Chang, A.K., Nixon, P.F. & Duggleby, R.G. (1999) Aspartate-27 and glutamate-473 are involved in catalysis by *Zymomonas mobilis* pyruvate decarboxylase. *Biochem. J.* **339**, 255–260.
19. Wu, Y.G., Chang, A.K., Nixon, P.F., Li, W. & Duggleby, R.G. (2000) Mutagenesis at Asp27 of pyruvate decarboxylase from *Zymomonas mobilis* – effect on its ability to form acetoin and acetolactate. *Eur. J. Biochem.* **267**, 6493–6500.
20. Sun, S., Duggleby, R.G. & Schowen, R.L. (1995) The linkage of catalysis and regulation in enzyme action. Carbon isotope effects, solvent isotope effects and proton inventories for the unregulated pyruvate decarboxylase of *Zymomonas mobilis*. *J. Am. Chem. Soc.* **117**, 7317–7322.
21. Pohl, M., Siegert, P., Mesch, K., Bruhn, H. & Grotzinger, J. (1998) Active site mutants of pyruvate decarboxylase from *Zymomonas mobilis* – a site-directed mutagenesis study of L112, I472, I476, E473 and N482. *Eur. J. Biochem.* **257**, 538–546.
22. Schenk, G., Leeper, F.J., England, R., Nixon, P.F. & Duggleby, R.G. (1997) The role of His113 and His114 in pyruvate decarboxylase from *Zymomonas mobilis*. *Eur. J. Biochem.* **248**, 63–71.
23. Guo, F.S., Zhang, D.Q., Kahyaoglu, A., Farid, R.S. & Jordan, F. (1998) Is a hydrophobic amino acid required to maintain the reactive V conformation of thiamin at the active center of thiamin diphosphate-requiring enzymes? Experimental and computational studies of isoleucine 415 of yeast pyruvate decarboxylase. *Biochemistry* **37**, 13379–13391.
24. Boiteux, A. & Hess, B. (1970) Allosteric properties of yeast pyruvate decarboxylase. *FEBS Lett.* **9**, 293–296.
25. Hübner, G., Weidhase, R. & Schellenberger, A. (1978) The mechanism of substrate activation of pyruvate decarboxylase: a first approach. *Eur. J. Biochem.* **92**, 175–181.
26. Alvarez, F.J. & Schowen, R.L. (1991) The kinetic consequences of the interaction of pyruvate decarboxylase with several close analogs of the substrate. In *Biochemistry and Physiology of Thiamin Diphosphate Enzymes* (Bisswanger, H. & Ullrich, J., eds), pp. 59–66. VCH, Weinheim, Germany.
27. Baburina, I., Gao, Y.H., Hu, Z.X., Jordan, F., Hohmann, S. & Furey, W. (1994) Substrate activation of brewers-yeast pyruvate decarboxylase is abolished by mutation of cysteine-221 to serine. *Biochemistry* **33**, 5630–5635.
28. Baburina, I., Li, H.J., Bennion, B., Furey, W. & Jordan, F. (1998) Interdomain information transfer during substrate activation of yeast pyruvate decarboxylase: the interaction-between cysteine 221 and histidine 92. *Biochemistry* **37**, 1235–1244.
29. Li, H., Furey, W. & Jordan, F. (1999) Role of glutamate 91 in information transfer during substrate activation of yeast pyruvate decarboxylase. *Biochemistry* **38**, 9992–10003.
30. Diefenbach, R.J. & Duggleby, R.G. (1991) Pyruvate decarboxylase from *Zymomonas mobilis* – structure and reactivation of apoenzyme by the cofactors thiamin diphosphate and magnesium-ion. *Biochem. J.* **276**, 439–445.
31. Harris, T.K. & Washabaugh, M.W. (1995) Solvent-derived protons in catalysis by brewers-yeast pyruvate decarboxylase. *Biochemistry* **34**, 14001–14011.
32. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
33. Huang, C.Y., Nixon, P.F. & Duggleby, R.G. (1999) Effect of mutagenesis of V111 and L112 on the substrate specificity of *Zymomonas mobilis* pyruvate decarboxylase. *J. Biochem. Mol. Biol.* **32**, 39–44.
34. Ellis, K.J. & Morrison, J.F. (1982) Buffers of constant ionic strength for studying pH-dependent processes. *Methods Enzymol.* **87**, 405–426.
35. Diefenbach, R.J., Candy, J.M., Mattick, J.S. & Duggleby, R.G. (1992) Effects of substitution of aspartate-440 and tryptophan-487 in the thiamin diphosphate binding region of pyruvate decarboxylase from *Zymomonas mobilis*. *FEBS Lett.* **296**, 95–98.
36. Candy, J.M. & Duggleby, R.G. (1994) Investigation of the cofactor-binding site of *Zymomonas mobilis* pyruvate decarboxylase by site-directed mutagenesis. *Biochem. J.* **300**, 7–13.
37. Pohl, M., Mesch, K., Rodenbrock, A. & Kula, R.-K. (1995) Stability investigations on the pyruvate decarboxylase from *Zymomonas mobilis*. *Biotechnol. Appl. Biochem.* **22**, 95–105.
38. Washabaugh, M.W. & Jencks, W.P. (1988) Thiazolium C(2)-proton exchange – structure reactivity correlations and the p*K*a of thiamin C(2)-h revisited. *Biochemistry* **27**, 5044–5053.
39. Jordan, F., Li, H.J. & Brown, A. (1999) Remarkable stabilization of zwitterionic intermediates may account for a billion-fold rate acceleration by thiamin diphosphate-dependent decarboxylases. *Biochemistry* **38**, 6369–6373.