Published in *Journal of Biochemistry and Molecular Biology*, Vol. 32, No. 1, January 1999, pp. 39-44

Effect of mutagenesis of V111 and L112 on the substrate specificity of *Zymomonas mobilis* pyruvate decarboxylase

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Pyruvate decarboxylase (PDC) catalyses the conversion of pyruvate to acetaldehyde as the penultimate step in alcohol fermentation. The enzyme requires two cofactors, thiamin diphosphate (ThDP) and Mg^{2+}, for activity. *Zymomonas mobilis* PDC shows a strong preference for pyruvate although it will use the higher homologues 2-ketobutyrate and 2-ketovalerate to some extent. We have investigated the effect of mutagenesis of valine 111 and leucine 112 on the substrate specificity. V111 was replaced with glycine, alanine, leucine and isoleucine while L112 was replaced with alanine, valine and isoleucine. With the exception of L112I, all mutants retain activity towards pyruvate with k_{cat} values ranging from 40% to 139% of wild-type. All mutants show changes from wild-type in the affinity for ThDP, and several (V111A, L112A and L112V) show decreases in the affinity for Mg^{2+}. Two of the mutants, V111G and V111A show an increase in the K_m for pyruvate. The activity of each mutant towards 2-ketobutyrate and 2-ketovalerate was investigated and some changes from wild-type were found. For the V111 mutants, the most notable of these is a 3.7-fold increase in the ability to use 2-ketovalerate. However, the largest effect is observed for the L112V mutation which increases the ability to use both 2-ketobutyrate (4.3-fold) and 2-ketovalerate (5.7-fold). The results suggest that L112 and, to a lesser extent, V111 are close to the active site and may interact with the alkyl side-chain of the substrate.

Keywords: pyruvate decarboxylase; site-directed mutagenesis; substrate specificity; thiamin diphosphate; *Zymomonas mobilis*.

Many plants and fungi, and a few bacteria, are capable of fermenting carbohydrates to ethanol. Usually the penultimate step in this process is the conversion of pyruvate to acetaldehyde, catalysed by pyruvate decarboxylase (EC 4.1.1.1; PDC). Although the main physiological role of this enzyme dictates that it will act on pyruvate, the enzyme also has the capacity to act on other 2-keto acids. For example yeast PDC is capable of decarboxylating a range of linear and branched-chain aliphatic 2-keto acids, although none is used as effectively as pyruvate (Lehmann *et al.*, 1973). The enzyme from the bacterium *Zymomonas mobilis* is more specific, having only limited ability to accept analogues of pyruvate (Bringer-Meyer and Sahm, 1988; Thomas *et al.*, 1990).

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Introduction

The three-dimensional structure of PDC from the yeasts *Saccharomyces uvarum* (Dyda *et al.*, 1993) and *S. cerevisiae* (Arjunan *et al.*, 1996, Lu *et al.*, 1997) has been determined. These structures have provided only limited insight into how substrate specificity is controlled since there appears to be little to limit the size of potential substrates. The thiamin diphosphate (ThDP) cofactor is at the bottom of a shallow open pit with the reactive C2 atom of the cofactor accessible from solvent. However, the eight-residue sequence comprising amino acids 106-113 (yeast numbering, corresponding to *Z. mobilis* PDC residues 105-112), that must be near the active site in these structures, is partially or completely disordered. We have suggested (Schenk *et al.*, 1997; Candy and Duggleby, 1998) that this may form a loop that closes over the active site during catalysis.

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                                    ‡‡
Zmo  (X59558)  99 GAPNNNDHAAGHVLHHALGKTD120
Sce1 (X77316)    GVPSISSQAKQLLLHHTLGNGD
Sce5 (X15668)    GVPSISSQAKQLLLHHTLGNGD
Sce6 (X55905)    GVPSISAQAKQLLLHHTLGNGD
Spo  (Z81312)    GSPNTNDLSSGHLLHHTLGTHD
Huv  (U13635)    GVPSLASQAKQLLLHHTLGNGD
Kla  (X85968)    GVPSVSSQAKQLLLHHTLGNGD
Kma  (L09727)    GVPSISSQAKQLLLHHTLGNGD
Ncr  (U65927)    GSPNTNDPSQYHILHHTLGHPD
Apa  (U00967)    GTPMRASQESRALIHHTFNDGD
Nta1 (X81854)    GGPNSNDYGTNRILHHTIGLQD
Nta2 (X81855)    GGPNTNDYGTNRILHHTIGLPD
Psa1 (Z66543)    GGPNSNDYGTNRILHHTIGLPD
Ath1 (U71121)    GGPNSNDYGTNRILHHTIGLPD
Ath2 (U71122)    GGPNSNDYGTNRILHHTIGLPD
Zma1 (X59546)    GGPNSNDYGTNRILHHTIGLPD
Osa1 (U26660)    GGPNSNDYGTNRILHHTIGLPD
Osa2 (U27350)    GGPNSNDYGTNRILHHTIGLPD
Osa3 (U07338)    GGPNSNDYGTNRILHHTIGLPD
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Fig. 1. Alignment of a selected region of pyruvate decarboxylases. The sequence near V111 and L112 (indicated by ‡) of *Z. mobilis* PDC is shown with the eight-residue segment corresponding to a region that is disordered in the yeast PDC structure underlined. Species abbreviations are: Apa, *Aspergillus parasiticus*; Ath, *Arabidopsis thaliana*; Huv, *Hanseniaspora uvarum*; Kla, *Kluyveromyces lactis*; Kma, *Kluyveromyces marxianus*; Ncr, *Neurospora crassa*; Nta, *Nicotiana tabacum*; Osa, *Oryza sativa*; Psa, *Pisum sativum*; Sce, *S. cerevisiae*; Spo, *Schizosaccharomyces pombe*; Zma, *Zea mays*; and Zmo, *Z. mobilis*. Sequences were aligned using ClustalW (Thompson *et al.*, 1994) then manually adjusted to remove any anomalies.

Protein sequence alignment of this loop region in PDC from several species (Fig. 1) reveals that no residue is totally conserved. However, immediately beyond the loop are two conserved histidines (113 and 114) that we have shown are involved in catalysis (Schenk *et al*., 1997), with mutation of H113 to glutamine resulting in a complete loss of catalytic activity. Immediately preceding these histidines are two residues that are invariably one of the aliphatic amino acids valine, leucine and isoleucine. This has led us to examine the possibility that these residues (L112 and L113 in yeast PDC, V111 and L112 in *Z. mobilis* PDC) may be involved in controlling substrate specificity.

Very recently the three-dimensional structure of *Z. mobilis* PDC has been determined (Dobritzsch *et al.*, 1998). This structure confirms the hypothesis that there is a mobile loop that closes over the active site during catalysis. However, rather than involving residues 105-112, it is the C-terminal helix that is proposed to perform this function. Nevertheless, as Dobritzsch *et al*. (1998) point out, residues 101-112 interact with a second loop (residues 287-304) to form part of the active site. This leaves open the question of whether residues 111 and 112 could influence substrate specificity. To test this possibility, we have now replaced these residues in *Z. mobilis* PDC with others that have aliphatic side-chains. The results confirm that these two amino acids affect substrate specificity with position 112 being more influential than position 111.

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, USA) and deoxyribonucleotides from Perkin Elmer (Norwalk, CT, USA). 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 carried out according to standard protocols (Sambrook *et al*., 1989). *E. coli* strain DH5α was obtained from Gibco BRL (Life Technologies, Melbourne, Australia). The pPLZM plasmid for heat-inducible PDC expression has been described previously (Candy and Duggleby, 1994). All *E. coli* cultures containing the wild-type 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% glycerol and stored at -70°C.

Mutagenesis All mutants were constructed using the 'Altered Sites' *in vitro* mutagenesis system (Promega, Sydney, Australia) as described previously (Diefenbach *et al*., 1992). Mutagenic primers were 27 to 33 bases in length with a G+C content of 50 % or higher. Silent mutations were introduced where possible to remove a restriction endonuclease site to facilitate in the screening of transformants. An *Eco*RI/*Nco*I fragment of the PDC gene with the desired mutations was subcloned into *Eco*RI/*Nco*I 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 large scale expression of PDC the cells were grown in 2YT medium containing 100 μg /ml ampicillin at 30°C. When the cell culture reached an A_{600} of 0.5 the temperature was rapidly increased to 42°C and the induction was maintained for three hours. The cells were harvested by centrifugation at 4°C for 15 min at 2500 x g and the cell pellet was stored at -20°C. The purification of PDC was based on the protocol described previously (Schenk *et al*., 1997) and the enzyme was stored at -20°C after mixing with an equal volume of glycerol. Removal of cofactors to yield the apoenzyme was performed using our published procedure (Schenk *et al*., 1997).

Activity assay for PDC PDC activity was measured in a coupled enzyme assay at 30°C. The rate of acetaldehyde production from 10 mM pyruvate was determined by following the oxidation of NADH at 340 nm in the presence of alcohol dehydrogenase (Diefenbach and Duggleby, 1991). Reactions were started by addition of PDC and the amount of enzyme was chosen so that a steady decrease of NADH concentration could be monitored over 5 min. During purification, assays were performed with and without addition of alcohol dehydrogenase. The latter was taken as a measure for lactate dehydrogenase activity that is present in cell extracts but is separated during purification. The K_m values for pyruvate, 2-ketobutyrate and 2-ketovalerate were determined in the standard assay mixture with varying concentrations of substrate.

Measurement of cofactor binding Cofactor activation studies were carried out 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 varying the concentration of the other. The reaction was started by adding a pyruvate/NADH/alcohol dehydrogenase mixture and the data obtained were analyzed as described below.

Data analysis Kinetic parameters were determined by fitting the appropriate equation to the data by nonlinear regression using InPlot (GraphPad Software, San Diego, CA, USA), GraFit (Erithacus Software, Staines, UK) or an adaptation of DNRP53 (Duggleby, 1984). The best fit values and standard errors obtained from this analysis are reported. Substrate saturation curves were fitted to the Michaelis-Menten equation to obtain V_{max} and K_m, then k_{cat} was calculated from V_{max} using the measured molar concentration of PDC that was present ($k_{cat} = V_{max}/[E]_o$). The value and standard error of k_{cat}/K_m was estimated in a similar way by fitting Eq. 1 to the same data, treating k_{cat}/K_m and k_{cat} as the two fitted parameters.

$$v = \frac{[E]_o[S]}{1/(k_{cat}/K_m) + [S]/(k_{cat})} \quad (1)$$

Cofactor saturation curves were first fitted to Eq. 2, in which v_o represents the small amount of residual activity resulting from incomplete removal of cofactors from the apoenzyme.

$$v = v_o + \frac{V_{max}[C]}{K_c + [C]} \quad (2)$$

The data were then corrected for v_o and fitted to the same equation but omitting the v_o term to obtain the cofactor activation constant K_c.

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

Results

Kinetic properties of wild-type In addition to pyruvate, wild-type *Z. mobilis* PDC is capable of utilizing 2-ketobutyrate and 2-ketovalerate as substrates although the Michaelis constant (K_m) for each of these substrates is higher than that for pyruvate (Fig. 2A and Table 1). Moreover, the activity (k_{cat}, Table 2) is lower reaching only 51% of that with pyruvate for 2-ketobutyrate and 8% for 2-ketovalerate. We tested 2-ketoisovalerate also but this is not active as a substrate and is probably not bound to the enzyme, as indicated by the observation that this compound does not inhibit the decarboxylation of pyruvate. The K_m value for pyruvate determined here (0.59 ± 0.10 mM) is similar to that of 0.52 ± 0.05 mM which we have reported previously (Schenk *et al*., 1997).

After removal of the cofactors, the enzyme exhibits very little activity when either one of them is omitted from the assay. Adding back both cofactors fully restores activity and from the dependence of rate on the concentration of each cofactor, the activation constant for each was determined (Fig. 2B and Table 1). The activation constants for ThDP and Mg^{2+} (3.35 ± 0.45 μM and 6.58 ± 1.01 μM, respectively) are comparable to the values of 2.53 ± 0.16 μM (ThDP) and 5.87 ± 0.42 μM (Mg^{2+}) reported previously (Schenk *et al*., 1997).

Activity of mutants We have mutated V111 to glycine, alanine, leucine and isoleucine, and L112 to alanine, valine and isoleucine. Each of the mutants behaves normally during purification with respect to its binding to, and elution from, hydroxylapatite and DEAE-Sephacel. With the exception of L112I, all mutants are active; the inactivity of L112I is surprising since it may be considered to be the most conservative of all substitutions tested.

The cofactor activation constants show some substantial variations from wild-type (Table 1). There is no significant effect of the V111 mutations on the affinity for Mg^{2+} except for the 2-fold weaker binding by V111A. The effects of the L112 mutations are far more marked with binding weakened by 3.3- and 8.9-fold for L112V and L112A respectively. In contrast, most of the mutations caused tighter binding of ThDP by factors ranging from 2.2-fold (L112V) to 12.4-fold (V111L). The only exception is L112A for which ThDP binding is weakened by 3.5-fold. While no obvious pattern emerges from these results that might allow a correlation between the nature of the substitution and the effect on cofactor binding, it is clear that V111 and L112 are sufficiently close to the cofactor-binding site that mutations at these residues perturb its function.

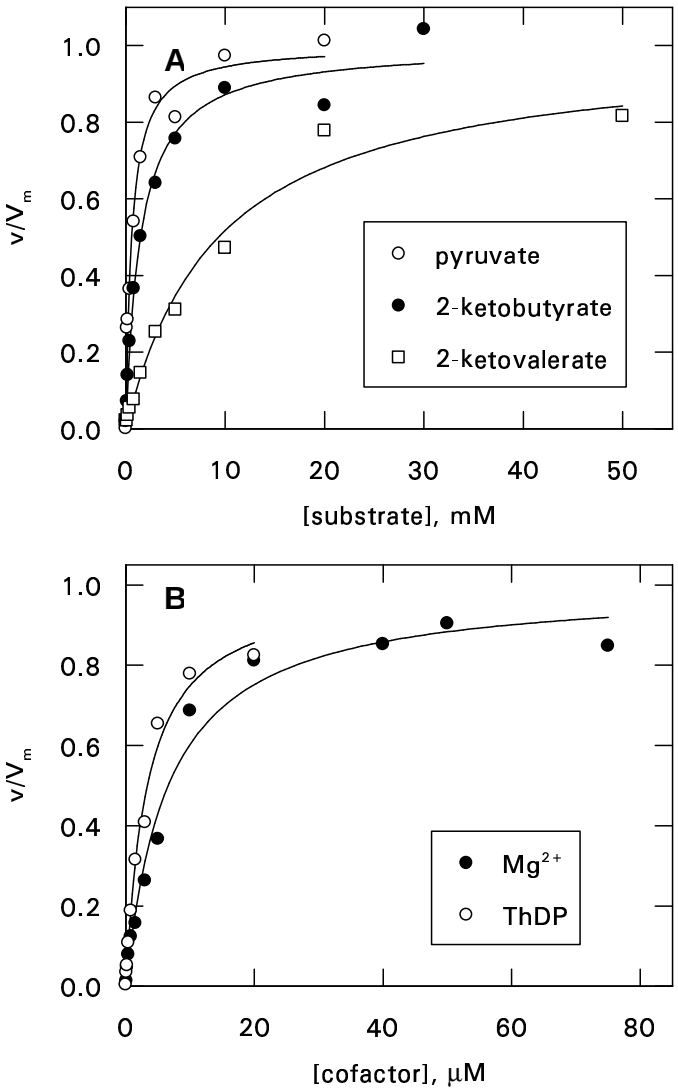

Fig. 2. Substrate saturation and cofactor activation curves for wild-type *Z. mobilis* PDC. To assist comparison between the curves, they have been converted to a common ordinate scale by dividing the observed rate by the maximum velocity (V_{max}) obtained for each curve. The lines represent the best-fit of the appropriate equation to the data. **A**. Substrate saturation curves using pyruvate, 2-ketobutyrate or 2-ketovalerate. **B**. Cofactor activation curves for ThDP and Mg^{2+}. The non-varied cofactor was held at a constant and saturating concentration of 0.1 mM (ThDP) or 5.0 mM (Mg^{2+}).

Kinetic properties of mutants For each of the six active mutants we have measured the K_m (Table 1), k_{cat} and k_{cat}/K_m (Table 2) for each of the three substrates, as well as the activation constant for the two cofactors ThDP and Mg^{2+} (Table 1).

Table 1. K_m for substrates and K_c for cofactors of wild-type and various *Z. mobilis* PDC mutants . Values were judged to be different from those of wild-type PDC at the 95% (*) and 99% (**) confidence level by applying the variance-ratio test (Duggleby, 1990).

Enzyme	pyruvate (mM)	2-ketobutyrate (mM)	2-ketovalerate (mM)	ThDP (μM)	Mg^{2+} (μM)
Wild-type	0.59 ± 0.10	1.48 ± 0.19	9.33 ± 1.19	3.35 ± 0.45	6.58 ± 1.01
V111G	4.51 ± 0.32**	3.93 ± 0.37**	34.81 ± 4.12**	1.08 ± 0.15**	6.54 ± 0.84
V111A	1.33 ± 0.10**	4.59 ± 0.84**	24.15 ± 1.70**	1.38 ± 0.16**	12.58 ± 1.43**
V111L	1.06 ± 0.12	2.55 ± 0.20*	13.05 ± 0.87*	0.27 ± 0.03**	5.30 ± 0.43
V111I	0.42 ± 0.03	0.75 ± 0.03**	10.72 ± 0.90	0.44 ± 0.07**	6.90 ± 0.64
L112A	0.45 ± 0.05	0.91 ± 0.05**	7.23 ± 0.44	11.65 ± 0.94**	58.40 ± 5.00**
L112V	0.68 ± 0.11	0.67 ± 0.03**	4.47 ± 0.42**	1.50 ± 0.20**	21.62 ± 4.05**

Table 2. Values for k_{cat} and k_{cat}/K_m of wild-type and various *Z. mobilis* PDC mutants for substrates. Values were judged to be different from those of wild-type PDC at the 95% (*) and 99% (**) confidence level by applying an adaptation of the variance-ratio test (Duggleby, 1990). PYR = pyruvate, 2KB = 2-keto-butyrate, 2KV = 2-ketovalerate.

Enzyme	Substrate	k_{cat} (sec^{-1})	k_{cat}/K_m ($mM^{-1}sec^{-1}$)
Wild-type	PYR	56.66 ± 2.24	95.70 ± 13.80
	2KB	28.79 ± 0.96	19.51 ± 2.15
	2KV	4.64 ± 0.18	0.50 ± 0.06
V111G	PYR	78.61 ± 1.22**	17.44 ± 1.07**
	2KB	32.92 ± 0.78**	8.37 ± 0.67**
	2KV	3.55 ± 0.20*	0.10 ± 0.01**
V111A	PYR	35.92 ± 1.23**	27.01 ± 1.32**
	2KB	21.65 ± 1.15**	4.71 ± 0.69**
	2KV	3.57 ± 0.08**	0.15 ± 0.01**
V111L	PYR	22.45 ± 0.61**	21.28 ± 2.09**
	2KB	13.51 ± 0.30**	5.30 ± 0.33**
	2KV	5.38 ± 0.12**	0.41 ± 0.02
V111I	PYR	43.03 ± 0.46**	103.20 ± 6.72
	2KB	25.94 ± 0.22**	34.71 ± 1.30**
	2KV	4.89 ± 0.11	0.46 ± 0.03
L112A	PYR	52.58 ± 1.14	116.49 ± 11.60
	2KB	48.53 ± 0.65**	53.56 ± 2.33**
	2KV	9.66 ± 0.17**	1.34 ± 0.06**
L112V	PYR	30.37 ± 0.98**	44.85 ± 6.60*
	2KB	26.27 ± 0.23**	39.04 ± 1.59**
	2KV	6.00 ± 0.16**	1.34 ± 0.10**

All of the mutations affect the saturation curve for at least one of the substrates, as measured by the K_m values (Table 1). Reducing the size of the side-chain of residue 111 by mutation from valine to alanine increased the K_m for pyruvate by 2.3-fold and a further decrease in the size (to glycine) resulted in a 7.6-fold increase in the K_m (Table 1). Generally similar patterns were observed using 2-ketobutyrate and 2-ketovalerate as substrates although the changes were less pronounced. Based on these results, we had expected that increasing the size of the side chain of residue 111 to leucine might have the opposite effect. However, we were surprised to observe that this mutation also increased the K_m for each of the substrates, although to a lesser extent than the change to alanine. Thus it appears that a valine at position 111 has a side-chain of the optimum size in *Z. mobilis* PDC. Clearly, however, the data give support to the hypothesis that this residue is involved in controlling substrate binding in that any change affects the K_m for at least one of the substrates.

Mutations at L112 also can affect substrate specificity but in no case is there a significant increase in the K_m. For pyruvate, there is no effect on K_m while for the larger substrates, valine has a greater effect than alanine. Again, the data give support to the hypothesis that this residue is involved in substrate binding.

Specificity ratios The results presented above concern effects on the K_m but it is often argued that measurements of k_{cat}/K_m are a better indicator of substrate specificity (Table 2). The parameter R (Eq. 3) allows comparison between pyruvate and the other 2-keto acid (2KA) substrates for each of the enzymes.

$$R = (k_{cat}/K_m) \text{ for pyruvate} / (k_{cat}/K_m) \text{ for 2KA} \quad (3)$$

For example, the wild-type prefers pyruvate over 2-ketovalerate by an R factor of 95.7/0.50 = 191, while for V111L the factor is 52. However, comparison for any given substrate between mutants and wild-type is complicated by the fact that the k_{cat} is different in most cases. Therefore we have calculated a specificity ratio (Eq. 4) for the mutants with each of the alternative substrates. Values of this ratio greater that 1.0 indicate a relative preference for the alternative substrate, compared to the properties of wild-type PDC.

$$\text{Specificity ratio} = R_{\text{wild-type}} / R_{\text{mutant}} \quad (4)$$

The values for this ratio are illustrated in Fig. 3. Mutation of V111 has a relatively small effect on the specificity ratio for 2-ketobutyrate, with the largest change (2.35-fold) being observed

for the most radical substitution, namely V111G. More pronounced changes are induced by mutation at L112, with substitution by valine giving a 4.27-fold increase in the specificity ratio.

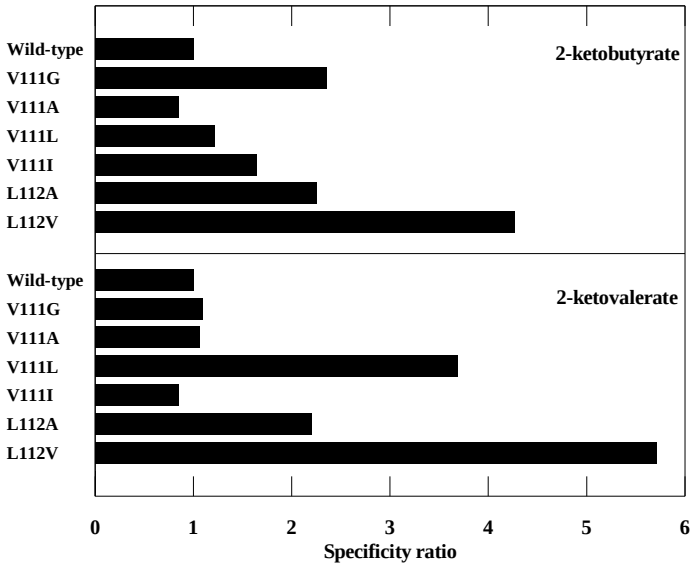

Fig. 3. Specificity ratios of *Z. mobilis* PDC mutants. The specificity ratio was calculated using Eqs. 3 and 4, and the values of k_{cat}/K_m listed in Table 2.

The effects of mutations at position 112 on the specificity ratio for 2-ketovalerate are similar, except for being even more pronounced in L112V (5.72-fold). However a somewhat different, and unexpected, pattern is observed for V111 mutations where only the substitution by leucine has any real effect.

Discussion

There have been several suggestions (Alvarez *et al*., 1995; Harris and Washabaugh, 1995; Lobell and Crout, 1996) that the active site of PDC is inaccessible to solvent during catalysis but examination of several crystal structures of the yeast enzyme gives few clues as to how closure might occur. Yeast PDC is inactive in the absence of an obligatory activator such as pyruvamide (Hübner *et al*., 1978) and it may be relevant that two of the yeast PDC structures (Dyda *et al*., 1993; Arjunan *et al*., 1996) are for the non-activated enzyme. Thus these structures may show some crucial differences from the active form. More recently, Lu *et al*. (1997) have determined the structure of yeast PDC crystallized in the presence of pyruvamide but it should be noted that this activator could not be located in the structure; thus it is still possible that this structure might not represent the active enzyme.

Z. mobilis PDC does not require activation and the very recent solution of its structure (Dobritzch *et al*., 1998) seems more likely to show the active form. Curiously, and so far inexplicably, the cofactor in this structure is incomplete having lost the vital C2 atom. Thus, it remains possible that none of the four structures is of active PDC, and that the positions of groups around the active site might be different from their locations during catalysis. Any amino acid side-chain in the immediate vicinity of the active site has the potential to be involved in active site closure. Such amino acids are likely to come into close contact with the alkyl group of the substrate and thereby affect substrate specificity.

In this study we have examined the possibility that mutation of V111 and L112 would alter the ability to accept 2-ketobutyrate and 2-ketovalerate as alternative substrates. Several mutants were prepared and all but one were found to be active. The exception, L112I, is surprising given that it might be thought of as the most conservative of the substitutions tested. Of the active mutants, none is seriously compromised in its catalytic capacity as judged by the k_{cat} values with pyruvate as substrate, which ranged from 40% (V111L) to 139% (V111G) of wild-type.

Every mutation tested affected the kinetic properties to some degree with the smallest effect on substrate kinetics being observed in the V111I mutant. In general, the effects of mutation at V111 are less pronounced that those at L112. In this context it should be noted that mutation of the adjacent H113 to glutamine completely abolishes catalytic activity (Schenk *et al*., 1997). Thus it is likely that in the active form of PDC, L112 is closer to the active site than V111 and may be involved more directly in active site closure and the control of substrate specificity.

Acknowledgement This work supported by the Australian Research Council, grant A09800834.

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