

## Effect of Mutagenesis of V111 and L112 on the Substrate Specificity of *Zymomonas mobilis* Pyruvate Decarboxylase

Chang-Yi Huang, Peter F. Nixon and Ronald G. Duggleby\*

Centre for Protein Structure, Function and Engineering, Department of Biochemistry,  
University of Queensland, Brisbane 4072, Australia

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Pyruvate decarboxylase (PDC) catalyzes 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 by glycine, alanine, leucine, and isoleucine while L112 was replaced by 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*.

### Introduction

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, catalyzed 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 Sahn, 1988; Thomas *et al.*, 1990).

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.

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

\* To whom correspondence should be addressed.  
Tel: 61-7-3365-4615; Fax: 61-7-3365-4699  
E-mail: duggleby@biosci.uq.edu.au

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Zmo (X59558)	<sup>99</sup> GAPNNNDHAAGHVLHHALGKTD <sup>120</sup>	
Sce1 (X77316)	GVPSISSQAKQLLLHHTLGNGD	
Sce5 (X15668)	GVPSISSQAKQLLLHHTLGNGD	
Sce6 (X55905)	GVPSISAQAKQLLLHHTLGNGD	
Spo (Z81312)	GSPNTNDLSSGHLHHTLGTHD	
Huv (U13635)	GVPSIASQAKQLLLHHTLGNGD	
Kla (X85968)	GVPSVSSQAKQLLLHHTLGNGD	
Kma (L09727)	GVPSISSQAKQLLLHHTLGNGD	
Ncr (U65927)	GSPNTNDPSQYHILHHTLGHPD	
Apa (U00967)	GTPMRASQESRALIHHTFNDGD	
Nta1 (X81854)	GGPNSNDYGTNRILHHTIGLQD	
Nta2 (X81855)	GGPNTNDYGTNRILHHTIGLDP	
Psa1 (Z66543)	GGPNSNDYGTNRILHHTIGLDP	
Ath1 (U71121)	GGPNSNDYGTNRILHHTIGLDP	
Ath2 (U71122)	GGPNSNDYGTNRILHHTIGLDP	
Zma1 (X59546)	GGPNSNDYGTNRILHHTIGLDP	
Osa1 (U26660)	GGPNSNDYGTNRILHHTIGLDP	
Osa2 (U27350)	GGPNSNDYGTNRILHHTIGLDP	
Osa3 (U07338)	GGPNSNDYGTNRILHHTIGLDP	

**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.

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, USA) and deoxyribonucleotides from Perkin Elmer (Norwalk, USA). For DNA 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 DH5 $\alpha$  was obtained from Gibco BRL (Life Technologies, Melbourne, Australia). The pPLZM plasmid for heat-inducible PDC expression has been previously described (Candy and Duggleby, 1994). All *E. coli* cultures containing the wild-type and the mutant constructs were maintained on Luria broth plates with 100  $\mu$ g/ml ampicillin. For long-term storage, liquid cultures were kept in 15% glycerol and stored at  $-70^{\circ}\text{C}$ .

**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 to remove a restriction endonuclease site to facilitate in the screening of transformants. An *EcoRI/NcoI* fragment of the PDC gene with the desired mutations was subcloned into *EcoRI/NcoI* 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  $\mu$ g/ml ampicillin at  $30^{\circ}\text{C}$ . When the cell culture reached an  $A_{600}$  of 0.5, the temperature was rapidly increased to  $42^{\circ}\text{C}$  and the induction was maintained for 3 h. The cells were harvested by centrifugation at  $4^{\circ}\text{C}$  for 15 min at  $2500 \times g$  and the cell pellet was stored at  $-20^{\circ}\text{C}$ . The purification of PDC was based on the protocol described previously (Schenk *et al.*, 1997) and the enzyme was stored at  $-20^{\circ}\text{C}$  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  $30^{\circ}\text{C}$ . 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, 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  $K_m$ , and then  $k_{cat}$  was calculated from  $V_{max}$  using the measured molar concentration of PDC that was present ( $k_{cat} = V_{max}/[E]_0$ ). 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.

$$v = \frac{[E]_0[S]}{1/(k_{cat}/K_m) + [S]/(k_{cat})} \quad (1)$$

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.

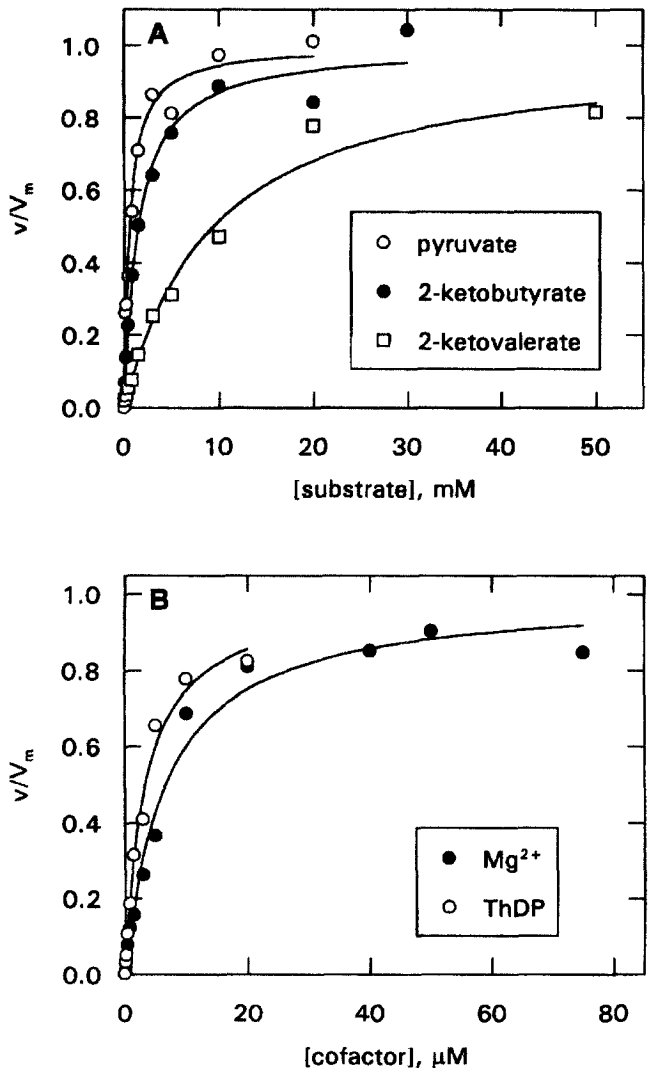
$$v = v_o + \frac{V_{max}[C]}{K_c + [C]} \quad (2)$$

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  $\epsilon_{267}$  of  $8520 \text{ M}^{-1}\text{cm}^{-1}$  (Diefenbach and Duggleby, 1991).  $\text{Mg}^{2+}$  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, 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 have also tested 2-ketoisovalerate 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 ( $0.59 \pm 0.10 \text{ mM}$ ) is similar to the value of  $0.52 \pm 0.05 \text{ mM}$  that we have reported previously (Schenk *et al.*, 1997).



**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  $\text{Mg}^{2+}$ . The non-varied cofactor was held at a constant and saturating concentration of 0.1 mM (ThDP) or 5.0 mM ( $\text{Mg}^{2+}$ ).

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  $\text{Mg}^{2+}$  ( $3.35 \pm 0.45 \mu\text{M}$  and  $6.58 \pm 1.01 \mu\text{M}$ , respectively) are comparable to the values of  $2.53 \pm 0.16 \mu\text{M}$  (ThDP) and  $5.87 \pm 0.42 \mu\text{M}$  ( $\text{Mg}^{2+}$ ) reported previously (Schenk *et al.*, 1997).

**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 ( $\mu$ M)	Mg <sup>2+</sup> ( $\mu$ M)
Wild-type	0.59 $\pm$ 0.10	1.48 $\pm$ 0.19	9.33 $\pm$ 1.19	3.35 $\pm$ 0.45	6.58 $\pm$ 1.01
V111G	4.51 $\pm$ 0.32**	3.93 $\pm$ 0.37**	34.81 $\pm$ 4.12**	1.08 $\pm$ 0.15**	6.54 $\pm$ 0.84
V111A	1.33 $\pm$ 0.10**	4.59 $\pm$ 0.84**	24.15 $\pm$ 1.70**	1.38 $\pm$ 0.16**	12.58 $\pm$ 1.43**
V111L	1.06 $\pm$ 0.12	2.55 $\pm$ 0.20*	13.05 $\pm$ 0.87*	0.27 $\pm$ 0.03**	5.30 $\pm$ 0.43
V111I	0.42 $\pm$ 0.03	0.75 $\pm$ 0.03**	10.72 $\pm$ 0.90	0.44 $\pm$ 0.07**	6.90 $\pm$ 0.64
L112A	0.45 $\pm$ 0.05	0.91 $\pm$ 0.05**	7.23 $\pm$ 0.44	11.65 $\pm$ 0.94**	58.40 $\pm$ 5.00**
L112V	0.68 $\pm$ 0.11	0.67 $\pm$ 0.03**	4.47 $\pm$ 0.42**	1.50 $\pm$ 0.20**	21.62 $\pm$ 4.05**

**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.

**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<sup>2+</sup> (Table 1).

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<sup>2+</sup> 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.

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

**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).

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

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.

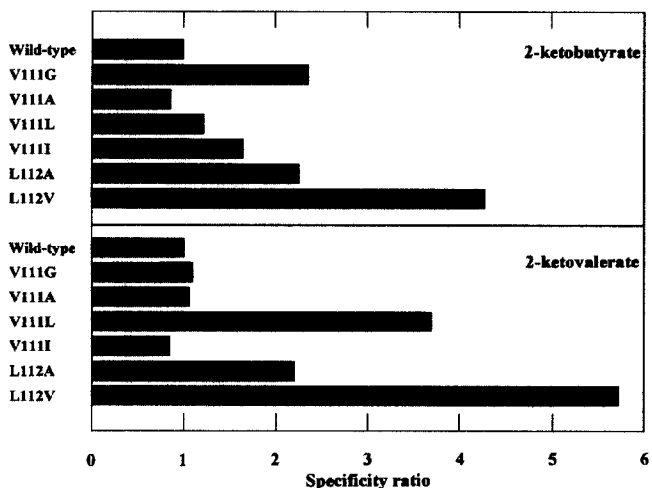
$$R = (k_{cat}/K_m) \text{ for pyruvate} / (k_{cat}/K_m) \text{ for 2KA} \quad (3)$$

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 than 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.

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.



**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.

## 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 (Dobritzsch *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 than 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 in the control of substrate specificity.

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