Effects of Deletions at the Carboxyl Terminus of Zymomonas mobilis Pyruvate Decarboxylase on the Kinetic Properties and Substrate Specificity†

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Abstract: The three-dimensional structure of Zymomonas mobilis pyruvate decarboxylase shows that the carboxyl-terminal region of the protein occludes the active site. This observation is consistent with earlier suggestions that the active site is inaccessible to solvent during catalysis. However, the carboxyl-terminal region must move aside to allow entry of the substrate, and again to permit the products to leave. Here we have examined the role of the carboxyl terminus by making 15 variants of the enzyme with serial deletions. The activity is largely unaffected by removal of up to seven residues but deletion of the next two, R561 and S560, results in a drastic loss of activity. Five of these deletion mutants were purified and fully characterized and showed progressive decreases in activity, in the ability to discriminate between pyruvate and larger substrates, and in cofactor affinity. Several substitution mutants at residues R561 and S560 were prepared, purified, and fully characterized. The results indicate important roles for the side-chain of R561 and the backbone atoms of S560. It is suggested that the carboxyl-terminal region of pyruvate decarboxylase is needed to lock in the cofactors and for the proper closure of the active site that is required for discrimination between substrates and for decarboxylation to occur.

Pyruvate decarboxylase (PDC, EC 4.1.1.1) is a member of a large family of enzymes that use thiamin diphosphate (ThDP) as a cofactor (1). The three-dimensional structures of several members of this family have now been determined, including PDC from yeast (2–4) and Zymomonas mobilis (5). In all cases, the active site of ThDP-dependent enzymes is at a domain interface and, with the exception of pyruvate:ferredoxin oxidoreductase (6), these domains are derived from different subunits. One domain contains the ThDP motif first identified by Hawkins et al. (7) and which binds a metal ion that in turn anchors ThDP to the enzyme via coordination to two of the phosphate oxygen atoms. The other domain contains the catalytic glutamate that is believed to promote the reactivity of the C2 atom of the thiazole ring (8).

Several workers have suggested that the active site of PDC is inaccessible to solvent-derived protons during catalysis (9–11). This notion of a closed active site agrees with earlier studies (12, 13) in which it was proposed that a hydrophobic environment is required for the decarboxylation step in the reaction. Modeling (14) and kinetic (15, 16) studies have further reinforced the idea of a cyclic opening and closing of the active site during catalysis. Active site closure would provide the right environment for catalysis to occur and might also prevent decomposition (17) of ThDP adducts that are intermediates in the PDC reaction.

On the basis of the first yeast PDC structure (2), Lobell and Crout (14) suggested that residues 106–113, 292–301, or the carboxyl terminus might participate in closure of the active site by acting as a “lid” since each of these regions is invisible in the structure and therefore presumed to be flexible. This was a reasonable proposal because the residues at the boundaries of these regions (e.g., 105 and 114) were not far from the position of ThDP in the structure. Candy and Duggleby (1) nominated residues 106–113 as the critical “lid” region but the determination of the structure of Z. mobilis PDC (5) suggests a more likely alternative.

This structure shows that the carboxyl-terminal region consists of an α-helix (E546–S560) connected to a tail (R561–K566) that together span the subunit interface (Figure 1A). ThDP is wedged in this interface but is inaccessible to solvent with only C2 of the ThDP thiazole ring, the atom that attacks the substrate, protruding from the underlying surface (Figure 1B). However, it should be noted that the position of C2 has been deduced from the locations of the remaining atoms of the thiazole ring; in the published structure, ThDP has undergone some degradation so that it is missing C2. For this reason, the enzyme cannot possibly be active. It is conceivable that these two features are connected and that the carboxyl-terminal region does not close over the active site when the true cofactor is present. However, as noted above, there are several lines of evidence that the active site is closed during catalysis and that the carboxyl-terminal region moves aside to allow substrate access and then closes during catalysis. Presumably, it then opens again to release the reaction products.
For sequencing we used the Prism Ready Dye Deoxy
and deoxyribonucleotides from Perkin-Elmer (Norwalk, CT). 
Enzymes were obtained from Promega (Sydney, Australia) 
England Biolabs (Beverly, MA). Exonuclease III and S1 
polymerase, and Klenow fragment were purchased from New 

**EXPERIMENTAL PROCEDURES**

**Materials.** Restriction enzymes, T4 DNA ligase, T4 DNA 
polymerase, and Klenow fragment were purchased from New 
England Biolabs (Beverly, MA). Exonuclease III and S1 
nuclease were obtained from Promega (Sydney, Australia) 
and deoxyribonucleotides from Perkin-Elmer (Norwalk, CT). 
For sequencing we used the Prism Ready Dye Deoxy 
Terminator Cycle Sequencing Kit from Perkin-Elmer Applied 
Biosystems.

**Mutagenesis.** Mutants with serial deletions at the carboxyl 
terminal were constructed using a protocol developed from 
Promega’s Erase-a-Base System. pPLZM-PDC, which 
contains a XhoI site 22 bp downstream of the PDC gene stop 
codon, was cut with XhoI and the linearized DNA with 5’- 
overhangs was subjected to limited digestion with exo-
nuclease III to generate a family of fragments that have 20– 
100 bases removed. The resulting collection of DNA 
fragments was treated with S1 nuclease to remove the 5’- 
overhangs and yield blunt-ended DNA fragments. These were 
then ligated in the presence of a double-stranded linker DNA 
with the palindromic sequence 5’TGACTCGAGTC-3’ to 
provide a stop codon (TGA). DNA sequencing of clones 
obtained in this way identified those that contained the stop 
codon in the correct reading frame, and these were used for 
PDC expression without further subcloning.

Variants with seven residues deleted and the next residue 
(R561) replaced by another amino acid were constructed 
using the complementary pair of primers shown below. SacII, 
EcoRV, and SphI recognition sites are underlined, the residue 
561 codon is double-underlined, and lowercase letters 5’ to 
the 561 codon indicate a change from the wild-type sequence.

\[
\text{SacII} 5’-561 \quad \text{EcoRV} \quad \text{SphI} \\
5’-\text{GTTGCGC CGGCAACACGCAATATCGCATGCACG}-3’ \\
\text{TATAACGTTACGCGC}-5’
\]

The 5’-overhang was filled in using the Klenov fragment of 
E. coli DNA polymerase I. The resulting DNA was cut 
with SacII and SphI and then ligated to SacII/SphI-digested 
pPLZM-PDC. Positive clones were identified by the presence 
of the introduced EcoRV site in the primer. DNA sequencing 
identified the actual base changes at the degenerate positions.

The additional deletion mutants Δ7 and Δ9 were con-
structed by PCR, using the primers shown in Table 1. A 
DNA fragment, which comprises the second half of the PDC 
gene, was generated by PCR using the forward primer F/P1 
with R/Δ7 or R/Δ9 as the reverse primer. The PCR product, 
which contains a single NcoI and XhoI site, was cut with 
these enzymes and used to replace the corresponding 
fragment of the wild-type gene in pPLZM-PDC. The mutants 
Δ8, Δ8+E, and Δ8+L were constructed in a similar way 
using the forward primer F/P2 and the reverse primer R/Δ8, 
R/Δ8+E, or R/Δ8+L, respectively.

**Expression, Protein Purification, and Preparation 
of Apoenzyme.** For expression of PDC on a 2 L scale, the cells 
were grown in 2YT medium (18) containing 100 μg/mL 
ampicillin at 30 °C. When the cell culture reached an A600 
of 0.5 the temperature was increased rapidly to 42 °C and 
the induction was maintained for 3 h. The cells were 
harvested by centrifugation at 4 °C for 15 min at 2500g and
the cell pellet was stored at −20 °C. The purification of PDC was based on the protocol described previously (20). The purified enzyme was stored at −20 °C. Removal of cofactors to yield the apoenzyme was performed using our published procedure (21, 22).

Activity Assay for PDC. PDC activity was measured in a coupled enzyme assay in which the rate of acetaldehyde production from 5 mM pyruvate was determined by following the oxidation of NADH at 340 nm and 30 °C in the presence of alcohol dehydrogenase (21). Reactions were started by addition of PDC using an amount of enzyme that could be monitored over 5 min. In cell extracts and during enzyme purification, assays were performed with and without addition of alcohol dehydrogenase. The latter was taken as a measure of the lactate dehydrogenase activity that is present addition of alcohol dehydrogenase. The latter was taken as a measure of the lactate dehydrogenase activity that is present in cell extracts and is separated during purification (22). The kinetic parameters were determined by fitting the experimental data to the Michaelis-Menten equation (22).

Measurement of Cofactor Activation and 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 NADH/alcohol dehydrogenase and a saturating concentration of one cofactor while varying the concentration of the other. The reaction was started by addition of 5 mM pyruvate and the data obtained were analyzed as described below.

Cofactor binding was measured by monitoring the quenching of tryptophan fluorescence of PDC (21) as a function of time using a Jasco model FB-770 spectrofluorimeter. Excitation was at 300 nm (band width 5 nm) and emission was measured at 340 nm (band width 5 nm).

Protein Analysis. SDS-PAGE was performed as described by Laurent (23) and proteins were detected by staining with 0.1% (w/v) Coomassie blue. For routine measurements of protein concentrations, the dye-binding method of Sedmac and Grossberg (24) was used. Gel filtration chromatography was performed on a Pharmacia FPLC system using a Superdex 200 HR 10/30 column in 50 mM sodium phosphate buffer (pH 6.6) containing 0.15 M NaCl. The column was calibrated using as standards apoferritin (443 kDa), β-amylose (200 kDa), ADH (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa).

Data Analysis. Most experiments were repeated two or three times, and we observed good agreement between replicate experiments. The results reported represent the combined results from these replicates.

Kinetic parameters were determined by fitting the appropriate equation to the data by nonlinear regression using InPlot (GraphPad Software, San Diego, CA), GraFit (Erithacus Software, Staines, U.K.) or an adaptation of the DNRP53 program (25). The best fit values and standard errors obtained from these analyses are reported. Substrate and cofactor saturation curves were fitted to eq 1 to obtain values of \( V_m \) and \( K_m \), where the latter represents the Michaelis constant, while \( K_a \) and \( K_c \) represent the Michaelis constant and the cofactor concentration, respectively. Values of \( k_{cat} \) were calculated from \( V_m \) using the known concentration of enzyme (see below) that was present in the assay.

\[
\nu = \frac{V_m[X]}{K + [X]} \tag{1}
\]

Results of tryptophan fluorescence quenching experiments were analyzed according to Diefenbach and Duggleby (21). Briefly, the fluorescence values \( F_i \) as a function of time \( t \) were fitted using exponential decay curve (eq 2) from an initial fluorescence \( F_0 \) to a final value \( F_\infty \) to obtain an apparent first-order rate constant \( k' \) at a given cofactor concentration.

\[
F_i = (F_0 - F_\infty) \exp(-k't) + F_\infty \tag{2}
\]

The slope of the linear relationship (eq 3) between this rate constant and the cofactor concentration \( [C] \) represents the rate constant for association \( k_{on} \), while the rate constant for cofactor dissociation \( k_{off} \) is obtained from the intercept on the ordinate.

\[
k' = k_{on}[C] + k_{off} \tag{3}
\]
The specific activities and kinetic properties of these enzymes are summarized in Table 2. Removal of the cofactors, ThDP and Mg$^{2+}$, abolishes activity and this can be fully restored by addition of the cofactors. The dependence of activity on the concentration of each cofactor added can be used to calculate a cofactor activation constant ($K_c$) that is an approximate measure of the affinity of the enzyme for ThDP and Mg$^{2+}$ (Table 2). Very clear trends are obvious in the data; $\Delta 7$ is similar to wild-type but $\Delta 8$, $\Delta 9$, $\Delta 11$, and $\Delta 15$ show progressive increases in the $K_c$ values. For the last member of this series, the values were too high to measure accurately but are certainly several hundred-fold higher than those of wild-type.

Substitution Mutations at R561 and S560. The results described above established that the last seven residues of PDC are unimportant and that the next few are critical for activity, cofactor binding and substrate specificity. We therefore went on to prepare specific mutants at positions 560 and 561 to determine the influence of the particular residues. As with the deletion series described earlier, we first assayed for PDC activity in cell extracts (Table 3). In the nomenclature that we have adopted for these mutated enzymes, $\Delta 8$+$X$ represents deletion of eight residues but with amino acid X added at the carboxyl-terminus. In this case, however, we used a codon (e.g., CAG) which can be mistaken for PDC. Corrections for this activity gave very similar results to the wild-type and therefore go on to prepare specific mutants at positions 560 and 561 to determine the influence of the particular amino acids.

The $K_m$ value for pyruvate shows only small variations across the series of mutants and ranged from 0.5 ($\Delta 15$) to 1.39 ($\Delta 8$) mM. The data show a discernible trend with $\Delta 7$ and $\Delta 8$ having a $K_m$ that is approximately double the wild-type value followed by a gradual decrease through $\Delta 9$, $\Delta 11$, and $\Delta 15$ to a value that is 74% of wild-type. Although these changes are relatively small, they were quite reproducible. The $k_{cat}/K_m$ values of the truncation mutants are all lower than that of wild-type with $\Delta 7$ and $\Delta 8$ showing values that are 61 and 12% of wild-type, respectively. $\Delta 9$ and $\Delta 11$ are each about 4% of wild-type while $\Delta 15$ is a mere 0.2%. For the most part, the effects on $k_{cat}$ dominate the changes in $k_{cat}/K_m$ because the $K_m$ values of the mutants are fairly similar to that of wild-type.

We have shown previously (26) that wild-type Z. mobilis PDC is capable of catalyzing the decarboxylation of both 2-ketobutyrate and 2-ketovalerate, albeit with lower efficiency than its activity with pyruvate. The ability of the various truncation mutants (except $\Delta 15$) to use these alternative substrates was therefore tested. The $K_m$ value for 2-ketobutyrate tends to increase through the deletion series while $k_{cat}$ rises above the wild-type value ($\Delta 7$) and then falls below ($\Delta 8$, $\Delta 9$, and $\Delta 11$). Although the $k_{cat}/K_m$ for 2-ketobutyrate is in all cases lower than that for pyruvate, it is of interest that $\Delta 8$ has the same $k_{cat}$ for 2-ketobutyrate as it has for pyruvate, while $\Delta 9$ and $\Delta 11$ each has a higher $k_{cat}$ for 2-ketovalerate than it has for pyruvate by factors of 1.9 and 6.8, respectively. Trends were less obvious for 2-ketovalerate although the $k_{cat}/K_m$ for this substrate, compared to that for pyruvate, is higher for all mutants than it is for wild-type. For example, wild-type shows a preference for pyruvate over 2-ketovalerate (as judged by the ratios of $k_{cat}/K_m$) by a factor of 155, while the corresponding values for $\Delta 7$, $\Delta 8$, $\Delta 9$, and $\Delta 11$ are 103, 63, 64, and 27.

Removal of the cofactors, ThDP and Mg$^{2+}$, abolishes activity and this can be fully restored by addition of the cofactors. The dependence of activity on the concentration of each cofactor added can be used to calculate a cofactor activation constant ($K_c$) that is an approximate measure of the affinity of the enzyme for ThDP and Mg$^{2+}$ (Table 2). Very clear trends are obvious in the data; $\Delta 7$ is similar to wild-type but $\Delta 8$, $\Delta 9$, $\Delta 11$, and $\Delta 15$ show progressive increases in the $K_c$ values. For the last member of this series, the values were too high to measure accurately but are certainly several hundred-fold higher than those of wild-type.

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nomenclature, Δ8+R is the same as Δ7 because the last residue (561) of Δ7 is arginine. Each of these mutated enzymes appeared to be similar to one another, with activities (compared to wild-type) ranging from 20 to 30% (Δ8+P) to 66–81% (Δ8+S); these activities span the value observed for Δ8 (38–42%). Four of these mutants (Δ8+E, Δ8+L and Δ8+P, and Δ9+L) were selected for further study; in each case, these represent a radical substitution in the wild-type sequence (R to E, L or P at position 561, and S to L at position 560).

Each of the proteins was purified and judged to be close to purity, as indicated by SDS-PAGE (data not shown). Each of them eluted very close to wild-type in gel filtration chromatography, suggesting that each is homotetrameric. Their kinetic properties were determined and these are shown in Table 4; to assist in comparison with Δ7, Δ8, and Δ9, data from these mutants that were shown in Table 2 are reproduced in Table 4. No two of these substitution mutants are identical in all respects but, for the most part, they are quite similar to Δ8 and clearly different from both Δ7 and Δ9. Thus, any substitution of R561 is approximately equivalent to deleting R561 entirely, showing that the side-chain of this residue plays an important role. In contrast, the substitution at S560 made little difference, consistent with the proposition that it is backbone atoms rather than the side chain of this serine that control the difference between Δ8 and Δ9.

**Rate Constants for Cofactor Binding and Release.** The cofactor activation constants reported in Tables 2 and 4 are combined constants that depend on the rate constants for binding (k_{on}) and release (k_{off}), as well as those of other steps in the catalytic cycle. Cofactor binding in the absence of substrate is accompanied by quenching of the fluorescence of W487 (21, 27) due to a change in the local environment at the subunit interface (28). Measurements of the time course of fluorescence quenching (Figure 3A) allow an apparent first-order rate constant to be determined from the data. A series of such analyses, at various cofactor concentrations, allow k_{on} and k_{off} to be determined from the resulting linear relationship (eq 3; Figure 3B). Two of the

<table>
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<th>enzyme</th>
<th>Δ7 (=Δ8+R)</th>
<th>Δ8+E</th>
<th>Δ8+L</th>
<th>Δ8+P</th>
<th>Δ8 (=Δ9+S)</th>
<th>Δ9+L</th>
<th>Δ9</th>
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<tr>
<td>specific activity (units/mg)</td>
<td>103</td>
<td>33.1</td>
<td>27.3</td>
<td>17.2</td>
<td>24.3</td>
<td>21.9</td>
<td>4.5</td>
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<td></td>
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</tr>
<tr>
<td>k_{on} (mM)</td>
<td>135 ± 1</td>
<td>41.7 ± 0.3</td>
<td>32.5 ± 0.2</td>
<td>22.9 ± 0.1</td>
<td>28.6 ± 0.1</td>
<td>27.0 ± 0.1</td>
<td>5.29 ± 0.02</td>
</tr>
<tr>
<td>k_{off}/k_{on} (mM^{-1} s^{-1})</td>
<td>101 ± 1</td>
<td>33.0 ± 0.5</td>
<td>28.6 ± 0.4</td>
<td>22.3 ± 0.4</td>
<td>20.5 ± 0.2</td>
<td>27.2 ± 0.3</td>
<td>6.43 ± 0.08</td>
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<td>2-ketobutyrate</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>k_{on} (mM)</td>
<td>4.44 ± 0.07</td>
<td>6.60 ± 0.22</td>
<td>9.19 ± 0.23</td>
<td>10.3 ± 0.3</td>
<td>8.86 ± 0.18</td>
<td>11.7 ± 0.4</td>
<td>16.1 ± 0.4</td>
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<tr>
<td>k_{off}/k_{on} (mM^{-1} s^{-1})</td>
<td>18.5 ± 0.2</td>
<td>6.05 ± 0.15</td>
<td>5.82 ± 0.10</td>
<td>4.14 ± 0.10</td>
<td>3.40 ± 0.05</td>
<td>4.75 ± 0.10</td>
<td>0.640 ± 0.010</td>
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<td>2-ketovalerate</td>
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<tr>
<td>k_{on} (mM)</td>
<td>14.6 ± 0.1</td>
<td>6.34 ± 0.08</td>
<td>9.60 ± 0.08</td>
<td>7.04 ± 0.12</td>
<td>4.81 ± 0.05</td>
<td>7.42 ± 0.04</td>
<td>1.97 ± 0.03</td>
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<tr>
<td>k_{off}/k_{on} (mM^{-1} s^{-1})</td>
<td>15.0 ± 0.3</td>
<td>15.5 ± 0.6</td>
<td>15.1 ± 0.4</td>
<td>15.7 ± 0.8</td>
<td>14.8 ± 0.4</td>
<td>16.9 ± 0.2</td>
<td>19.3 ± 0.7</td>
</tr>
<tr>
<td>ThDP K_{i} (μM)</td>
<td>0.980 ± 0.010</td>
<td>0.410 ± 0.010</td>
<td>0.640 ± 0.010</td>
<td>0.640 ± 0.010</td>
<td>0.350 ± 0.002</td>
<td>0.440 ± 0.005</td>
<td>0.101 ± 0.002</td>
</tr>
<tr>
<td>Mg^{2+} K_{i} (μM)</td>
<td>1.55 ± 0.03</td>
<td>7.42 ± 0.15</td>
<td>4.13 ± 0.07</td>
<td>6.51 ± 0.12</td>
<td>8.66 ± 0.19</td>
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<td>Mγ</td>
<td>10.3 ± 0.3</td>
<td>308 ± 11</td>
<td>53.6 ± 1.2</td>
<td>142 ± 4</td>
<td>193 ± 5</td>
<td>55.6 ± 1.0</td>
<td>410 ± 6</td>
</tr>
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</table>
Figure 4: Alignment of the carboxyl-terminal region of various PDC protein sequences. The species abbreviations are Apa, Aspergillus parasiticus; Ath, Arabidopsis thaliana; Cre, Chlamydomonas reinhardtii; Huv, Hanseniaspora uvarum; Kla, Kluyveromyces lactis; Kna, K. marxianus; Ncr, Neospora crassa; Nta, Nicotiana tabacum; Oryza sativa; Psa, Pisum sativum; Sce, Saccharomyces cerevisiae; Sfo, Saccharum officinarum; Spz, Schizosaccharomyces pombe; Zma, Zea mays; Zmo, Z. mobilis. Arabic numerals following species abbreviation indicate the products of different genes. Totally conserved residues are shown by an asterisk (*), while the residue equivalent values of relatively small variations from wild-type. However, the altered enzymes were chosen for such an analysis, representing a moderate (Δ8+L) and large (Δ9) decrease in affinity for cofactors, and compared with the wild-type enzyme (Table 5).

The rate constants for release of each cofactor (k_{cat}) showed relatively small variations from wild-type. However, the values of k_{on} changed substantially and showed clear trends which indicate that the dominant factor in decreased cofactor affinity in the mutants is a reduction in the rate of binding.

**DISCUSSION**

PDC genes and/or cDNAs have been isolated and sequenced from a number of organisms and an alignment of the carboxyl-terminal region is shown in Figure 4. There is little overall conservation of sequence with only two residues (D543 and L548) identical in all PDCs although sub-groups, such as the plant sequences, are very similar to one another. However, it is noteworthy that every sequence extends to or beyond a position equivalent to R561 of Z. mobilis PDC, and the residue at this position is invariably arginine or lysine. These observations are fully consistent with our data in which residues beyond R561 are unnecessary for PDC function, and deletion or radical substitution of this residue affects significantly the properties of the enzyme. Although we have not made the Δ8+K mutant of Z. mobilis PDC, we anticipate that it would be more similar to Δ7 than to Δ8.

The carboxyl-terminal region of PDC appears to play three roles. First, it allows proper closure of the active site and deletion results in drastic decreases in k_{cat} with little effect on K_m. This means that the effect on k_{cat}/K_m parallels that on k_{cat} itself, an observation that can be interpreted in two ways. One possibility is that several rate constants are affected and that there are compensatory changes which result in a reduction in k_{cat} while not affecting K_m. The simpler interpretation is that only the one rate constant common to k_{cat} and k_{cat}/K_m is affected; that is, decarboxylation of the lactyl-ThDP intermediate is the step that is impaired.

We had described previously (16, 20) a quantitative kinetic model of wild type Z. mobilis PDC (Figure 5) that agrees extremely well with the kinetic data reported in Table 2 and that would accommodate these results. The conclusion that active site closure is required for decarboxylation is entirely consistent with earlier studies (12, 13) in which it was deduced that the active site must be protected from solvent in order for this reaction to occur.

The second role of the carboxyl terminus is manifested in the affinity of the enzyme for its cofactors. As more of this region is deleted there is a progressive decrease in K_m. This is consistent with earlier studies (12, 13) in which it was shown that the active site must be protected from solvent in order for this reaction to occur.
been proposed by several groups based on solution X-ray scattering studies (29) and the kinetics of holoenzyme reconstitution (21, 30, 31). Although the nature of this conformational change has not been elucidated, we suggest that closure of the active site by the carboxyl-terminal region could represent an important component of the change.

The third effect of deletion of the carboxyl terminus is a broadening of substrate specificity so that the enzyme shows an improvement it its ability to decarboxylate 2-ketobutyrate and 2-ketovalerate, relative to the activity with pyruvate. As with pyruvate, the effects of truncation on the kinetics toward these larger substrates are reflected primarily in the values of \( k_{cat} \) (and \( k_{cat}/K_m \)), rather than in those of \( K_m \) itself. We suggest that in the wild-type enzyme there is tight closure of the active site that severely limits the ability of the enzyme to accommodate substrates larger than pyruvate. Truncation of the carboxyl terminus results in more flexibility and imposes fewer constraints on the size of the substrate. However, no deletion had a large effect on the \( K_m \) value for any one substrate, which indicates that there are no specific interactions between the substrate and any of the deleted residues. The possibility of specific interactions with residues more remote from the carboxyl terminus than K553 could not be tested due to instability of the proteins.

It is of interest that the largest deletion, of 15 residues, reduces \( k_{cat} \) by a factor of 600-fold only. This is rather a small change and implies that active site closure assists in, but is not essential for, catalysis. In general, PDC seems to be rather tolerant of changes around the catalytic center and, of the active site mutants whose properties have been properly characterized (1, 20, 22), only the H113Q mutant appears to be completely inactive. Even after mutation of the “catalytic glutamate” (28) the enzyme retains up to 3% of its activity. This seems to be a general feature of ThDP-dependent enzymes and mutations of the equivalent glutamate residue in transketolase (32) and the pyruvate dehydrogenase E1 component (33) also result in significant residual activity. Possibly this is because one of the main functions of the active site of these enzymes is to promote the ionization of the C2 proton of ThDP (8, 34). No single residue is indispensable in this process and most mutations result in an active, albeit crippled, enzyme.

The weight of evidence from the present study point toward a model in which the carboxyl-terminal region of PDC moves aside to allow substrate access, closes during catalysis, then opens again to release the reaction products.

However, we should stress that there is no direct evidence for this movement. Although the crystal structure of Z. mobilis PDC (5) clearly shows that access to the active site is impeded by the carboxyl terminus, it is possible that the structure of the enzyme in solution is slightly different from that in crystals. We are now attempting to obtain more direct evidence for movement of this region of PDC during catalysis.

REFERENCES

Pyruvate Decarboxylase C-Terminal Deletions


B10002683