The roles of Tyr91 and Lys162 in general acid–base catalysis in the pigeon NADP+-dependent malic enzyme

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INTRODUCTION

Malic enzymes catalyse divalent metal-ion-dependent reversible oxidative decarboxylation of L-malate using either NAD+ or NADP+ as a coenzyme to produce pyruvate, CO2 and NAD(P)H. The reaction catalysed by malic enzyme consists of reduction/oxidation, decarboxylation and enol–keto tautomerization. The general acid–base chemical mechanism of malic enzyme has been proposed based on the pH-dependence of kinetic and isotope studies [1–3]. In this mechanism, a general base is required to accept a proton from the 2-hydroxyl group of L-malate to facilitate studies [1–3]. In this mechanism, a general base is required to accept a proton from the 2-hydroxyl group of L-malate to facilitate oxidation, decarboxylation and enol–keto tautomerization. The bell-shaped profile pattern of wild-type enzyme as it lacked a basic pH, value. Oxaloacetate, in the presence of NADPH, can be converted by malic enzyme into L-malate by reduction and into enolpyruvate by decarboxylation activities. Compared with wild-type, the K162A mutant preferred oxaloacetate reduction to decarboxylation. These results are consistent with the function of Lys162 as a general acid that protonates the C-3 of enolpyruvate to form pyruvate. The Tyr91 residue could form a hydrogen bond with Ly162 to act as a catalytic dyad that contributes a proton to complete the enol–keto tautomerization.

Key words: chemical rescue, enol–keto tautomerization, general acid–base catalysis, malic enzyme, pH profile, site-directed mutagenesis.
Expression and purification of recombinant malic enzymes

The plasmids were expressed in *E. coli* strain BL21(DE3), and the recombinant wild-type and mutant malic enzymes were purified using Q-Sepharose and 2′,5′-ADP-Sepharose columns. The transformed bacteria were grown in Luria–Bertani medium containing 0.1 mg/ml ampicillin at 37°C to an attenuation of 0.5–0.6 at 660 nm. Expression was induced with 1.0 mM IPTG (isopropyl β-D-thiogalactoside). The culture was then allowed to grow overnight at 25°C. The cells were harvested by centrifugation at 5000 g for 15 min. Cells were resuspended and sonicated in buffer containing 25 mM Tris/HCl (pH 7.5) and 2 mM 2-mercaptoethanol. The recombinant proteins were purified on a Q-Sepharose column pre-equilibrated with the same buffer. The malic enzymes were eluted with buffer containing 150 mM NaCl. The fractions containing malic enzymes were further purified on a 2′,5′-ADP-Sepharose column. The malic enzymes were then eluted with 0.23 mM NADP⁺. A Centricon 30 ultrafiltration unit was used to remove NADP⁺ and concentrate the proteins. The concentration of remaining NADP⁺ was determined by measuring NADPH formation in standard malic enzyme assay conditions without addition of NADP⁺. The purity of the proteins was assessed by SDS/PAGE. The protein concentrations were determined by the Bradford method using BSA as a standard [14]. A molecular mass of 62 kDa was used to calculate the concentration of enzyme subunits.

Enzyme assay

Malic enzyme activity was assayed according to the method of Hsu et al. [15]. The standard assay solution (1 ml) contained 66.7 mM TEA buffer, 5 mM L-malate, 0.23 mM NADP⁺ and 4 mM Mn²⁺, unless otherwise stated. The activity of the enzyme was monitored continuously, by reducing NADP⁺ to NADPH (ε₉₆₅ = 6.22 × 10³ M⁻¹ cm⁻¹), at 25°C with a PerkinElmer Lambda 3B spectrophotometer. One unit of enzyme activity was defined as the initial rate of 1 µmol NADPH formed per min under the assay conditions. Specific activity was defined as µmol NADPH formed per min per mg of protein.

The apparent Michaelis constants for the substrates were determined by varying one substrate concentration around its *Kₐₜ* value and holding the other components constant. To determine the Michaelis and dissociation constants for L-malate and NADP⁺, initial velocity studies were performed by varying the concentrations of L-malate at different fixed concentrations of NADP⁺. The concentration of Mn²⁺ was held at a saturated concentration. Data were analysed using the following equation (Eqn 1), which describes a sequential initial velocity pattern:

\[
v = VA/v\cdot (KₐₜKₐ + KₐB + KA + AB)
\]

where *v* and *V* represent the initial and maximum velocities respectively, *A* (variable substrate) and *B* (fixed changing substrate) represent different reactant concentrations, *Kₐₜ* and *Kₐ* are the Michaelis constants for *A* and *B* respectively, and *Kₐ* is the dissociation constant for *A*. Linear regression analysis was carried out using the commercial program Fit 6.0 (QuantumSoft).

Partial reaction analysis

The decarboxylation activity of malic enzyme was assayed according to the method of Tang and Hsu [16] using oxaloacetate as a substrate. The rate of oxaloacetate decarboxylation was measured by monitoring the disappearance of the enolic oxaloacetate absorbance at 260 nm in the presence of either Mn²⁺ or Mg²⁺. Different concentrations of oxaloacetate in 185 mM potassium acetate buffer (pH 4.5), were added to 50 mM EDTA.
and incubated at 25 °C for 10 min to reach keto–enol equilibrium. To initiate the reaction, the various concentrations of oxaloacetate were added to a total volume of 1 ml containing 4 mM MnCl₂ and 37 mM potassium acetate buffer (pH 4.5). The rate of decarboxylation in the presence of enzyme was corrected by subtracting the spontaneous decarboxylation of oxaloacetate. The partial reduction activity of malic enzyme was performed according to the method of Tang and Hsu [17] using pyruvate as the substrate. The rate of pyruvate reduction was measured by monitoring the decrease in absorbance at 340 mM for the oxidation of NADPH. A typical assay mixture contained 66.7 mM TEA buffer (pH 7.4), 0.23 mM NADPH, 4 mM MnCl₂, 1–50 mM pyruvate and an appropriate amount of enzyme.

**pH studies**

The pH dependencies of $k_{cat}$ and $k_{cat}/K_m$ for the wild-type and mutant enzymes were determined by initial velocity studies using a variable concentration of l-malate at different fixed concentrations of NADP⁺ as a function of pH over the range 5–10, which was maintained with a 60 mM Bis/Tris propane buffer. The pH values were measured and showed no significant changes before and after initial velocity measurements. The $K_i$ values were obtained by fitting the data to the following equations (Eqn 2 and Eqn 3):

\[
\log y = \log \left[ \frac{C}{(1 + H/K_{a1} + K_{a2}/H)} \right]
\]

\[
\log y = \log \left[ \frac{C}{(1 + H/K_{a1})} \right]
\]

where $y$ is the value of the parameter of interest ($k_{cat}$ or $k_{cat}/K_m$), $C$ is the pH-independent value of $y$, $H$ is the hydrogen ion concentration and $K_{a1}$ and $K_{a2}$ are the acid dissociation constants of the unprotonated and protonated forms of the functional groups of free enzyme and substrate when $y$ represents $k_{cat}/K_m$ or the enzyme–substrate complex when $y$ represents $k_{cat}$.

**Chemical rescue**

Stock solutions of exogenous bases were prepared at pH 7.4. To examine their rescue abilities, different exogenous bases, including ammonium chloride, methylamine, ethylamine and propylamine, were added to the reaction mixture containing 66.7 mM TEA buffer, 5 mM l-malate, 0.23 mM NADP⁺ and 8 mM Mg²⁺. To measure the effects of exogenous base on kinetic properties, all kinetic studies were performed in the presence of 100 mM ammonium chloride.

**Oxaloacetate partition**

The partition ratio of oxaloacetate was determined according to the method of Karsten and Cook [18]. The reaction mixture contained 100 mM Hepes (pH 7.0), 0.8 mM NADPH, 1 mM oxaloacetate and 20 mM Mn²⁺. The rate of decarboxylation of oxaloacetate was measured by monitoring the disappearance of the enolic oxaloacetate absorbance at 260 nm. For the disappearance of NADPH, the rate of malate formation was followed by the decrease in absorbance at 340 nm. The spontaneous decarboxylation of oxaloacetate was subtracted as background. The partition ratio (termed rH) expressed as pyruvate/malate was calculated as follows:

\[
rH = \frac{[\text{pyruvate}]/[\text{dt}]}{[\text{NADPH}]/[\text{dt}]},
\]

where $[\text{pyruvate}]/[\text{dt}] = (d[\text{oxaloacetate}]/[\text{dt}]) - (d[\text{NADPH}]/[\text{dt}])$.

**CD studies of the wild-type and mutant enzymes**

CD was conducted using a Jasco Model J-810 spectropolarimeter. Measurements of ellipticity as a function of wavelength at 0.1 nm increments between 250 and 200 nm were made using a cylindrical quartz cell with a 0.1-cm path length. Purified protein samples (0.15 mg/ml) were prepared in 100 mM NaH₂PO₄ (pH 7.5). Each scan was repeated five times. Background from the buffer was subtracted from all spectra. The mean molar ellipticity, $[\theta]$ (deg cm²/dnmol), was calculated from $[\theta] = \theta/10nCl$, where $\theta$ is the measured ellipticity (millidegrees), $C$ is the molar concentration of protein (molar), $l$ is the cell path length (cm) and $n$ is the number of residues per subunit of enzyme ($n$ = 557 for malic enzyme).

**RESULTS**

**Protein purity and CD spectra of the recombinant malic enzymes**

In the present study, the K162A mutant demonstrated a 27 000-fold decrease in $k_{cat}$ value. This is different from our previous studies in which the K162A mutant showed an approx. 1500-fold decrease in $k_{cat}$ value [6]. This difference could result from the contamination by E. coli or pigeon wild-type enzyme in our previous mutant preparations. Two isoforms of malic enzymes (SfCA and MaeB) have been identified and characterized in E. coli [19]. MaeB is a NADP⁺-dependent isofrom, whereas SfCA can use both NAD⁺ and NADP⁺ as coenzyme but prefers NAD⁺ under physiological conditions. The MaeB enzyme can be activated by potassium ions. To examine possible contamination by endogenous E. coli malic enzyme, the pET-21 plasmid without malic enzyme cDNA was transformed into BL21(DE3) cells and was expressed as described in the Experimental section. The results showed that the endogenous malic enzyme corresponds to 0.7% of the total activity but it was removed by both the Q-Sepharose and 2',5'-ADP-Sepharose columns. The E. coli malic enzyme was shown to be activated 40-fold in the presence of 10 mM potassium chloride [20]. The enzyme activity of our purified recombinant pigeon malic enzymes showed no enhancement of activity after treatment with potassium ion, suggesting no contamination with E. coli enzyme in the preparations used in the present study. For mutant–enzyme purification, separate columns were used to ensure that there was no crossover of wild-type enzyme. The purity of the recombinant pigeon NADP⁺-dependent wild-type and mutant enzymes was assessed by SDS/PAGE. All enzyme preparations were purified to greater than 95% purity.

The CD spectra were examined to evaluate the overall secondary structure of the mutant enzymes. In all cases, the far-UV CD spectra were superimposable on that of wild-type enzyme after protein concentration correction (results not shown). Therefore there were no significant changes in overall secondary structure. The kinetic variation we observed, therefore, must result from a local structural change in the active site.

**Kinetic parameters of the recombinant malic enzymes**

The three-dimensional structure of the pigeon malic enzyme did not show any involvement of Tyr⁹¹ and Lys⁶¹, in metal-ion binding [21]. As expected, all four mutants (Y91F, K162A, K162Q and K162R) had no effect on the apparent $K_m$ for Mn²⁺ (results not shown). Therefore initial velocity experiments were performed using various concentrations of l-malate at different fixed concentrations of NADP⁺ at a saturating concentration of Mn²⁺. The kinetic parameters of these mutants are summarized in Table 1. The Lys⁶¹ mutants exhibited a dramatic decrease in
$k_{cat}$ values compared with wild-type enzyme, whereas the $K_m$ and $K_i$ values for L-malate and NADP$^+$ remained unaltered compared with wild-type. Replacing lysine with alanine and glutamine resulted in an approx. 27 000- and 3500-fold decrease in $k_{cat}$ values respectively. The K162R mutant, as expected, exhibited the smallest decrease (125-fold) in $k_{cat}$ value. These results are similar to those from kinetic studies of mutant A. suum malic enzymes [5]. The Y91F mutant showed a 25-fold increase and a 3-fold decrease in the $k_{cat}$ values for L-malate and NADP$^+$ respectively. Its $k_{cat}$ value was decreased by 200-fold compared with that of wild-type enzyme.

In order to identify the possible roles of Tyr$^{91}$ and Lys$^{162}$, two partial reactions, the pyruvate reduction and the oxaloacetate decarboxylation, were examined. The kinetic parameters are summarized in Table 2. Both the Y91F and K162A mutants had no significant effect on $k_{cat}$ values for pyruvate reduction. The $K_m$ values for pyruvate and oxaloacetate were decreased by 74- and 153-fold respectively, for the K162A mutant. For oxaloacetate decarboxylation, the K162A mutant had a dramatically decreased $k_{cat}$ value (3250-fold) compared with wild-type, whereas the Y91F mutant demonstrated a 14-fold decrease. These results suggest that both Tyr$^{91}$ and Lys$^{162}$ have no contribution to the partial reduction reaction and, therefore, are unlikely to act as a general base.

**pH profiles**

The effect of pH on the oxidoreductive decarboxylation reaction catalysed by the malic enzyme was determined over the pH range of 5–10 by varying the concentrations of L-malate at different fixed concentrations of NADP$^+$ with saturated Mn$^{2+}$ ions at each pH. All of the initial velocity data were fitted best to the sequential mechanism is maintained over the pH range of 5.0–10. The p$K_a$ values estimated from the pH-log($k_{cat}$) and pH-log($k_{cat}/K_m$) profiles for both wild-type and mutant malic enzymes are summarized in Table 3. The wild-type enzyme showed a bell-shape pattern for both pH-log($k_{cat}$) and pH-log($k_{cat}/K_m$) profiles. In the pH-log($k_{cat}$) profile, the two p$K_a$ values were 6.29 ± 0.01 and 8.78 ± 0.09. Two p$K_a$ values of 5.82 ± 0.33 and 7.56 ± 0.20 in pH-log($k_{cat}/K_m$) were obtained for wild-type enzyme. These acidic and basic p$K_a$ values are similar to those of the native enzyme obtained by Schimerlik and Cleland [1]. Similar patterns and p$K_a$ values within acceptable experimental error were observed for the K162R and Y91F mutants, but the K162A mutant demonstrated a pH-independent pattern in the basic region of the pH-log($k_{cat}$) profile (Figure 1). This finding actually confirmed that the p$K_a$ in the basic region corresponds to the dissociation of the ε-amino group of the Lys$^{162}$ side chain in the enzyme.

**Chemical rescue**

Chemical rescue is a method using small exogenous molecules to restore lost enzymatic activity in mutant enzymes [22]. This method can be used to identify the amino acid residue(s) involved in the general acid–base mechanism. As illustrated in Figure 2, the K162A mutant can be chemically rescued by the addition of ammonium chloride and methylamine. The enzymatic activity of K162A was activated 28-fold in the presence of 1 mM ammonium chloride. Excess NH$_4$Cl (> 250 mM) inactivated the enzyme. For methylamine, the activity could be restored in a linear, dose–response pattern. A 14-fold increase in activity was observed up to 1 M methylamine. Neither ethylamine nor propylamine were able to rescue the activity of K162A. The kinetic parameters were determined in the presence of 100 mM ammonium chloride and are summarized in Table 4. Since the concentrations of Mn$^{2+}$ used may form a brownish Mn–malate complex to interfere with the enzyme assay, Mg$^{2+}$ was used for kinetic studies in the presence of ammonium chloride. The $K_m$ values for L-malate, NADP$^+$ and Mg$^{2+}$ showed moderate increases in the presence of ammonium chloride.

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**Table 1** Kinetic parameters for the recombinant NADP$^+$-dependent malic enzymes

<table>
<thead>
<tr>
<th></th>
<th>$K_{cat}$ (mM)</th>
<th>$K_{cat}$ (mM)</th>
<th>$K_{cat}$ (mM)</th>
<th>$K_{cat}$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.23 ± 0.01</td>
<td>0.28 ± 0.01</td>
<td>3.43 ± 0.43</td>
<td>8.85 ± 10.5</td>
<td>140.1 ± 2.4 (1-fold)</td>
</tr>
<tr>
<td>K162A</td>
<td>0.34 ± 0.01</td>
<td>0.34 ± 0.04</td>
<td>3.13 ± 0.01</td>
<td>7.77 ± 0.93</td>
<td>0.52 ± 0.02 × 10$^{-2}$ (26942-fold)</td>
</tr>
<tr>
<td>K162R</td>
<td>0.25 ± 0.02</td>
<td>0.46 ± 0.06</td>
<td>3.20 ± 0.52</td>
<td>12.9 ± 1.60</td>
<td>1.12 ± 0.03 (125-fold)</td>
</tr>
<tr>
<td>K162Q</td>
<td>0.14 ± 0.01</td>
<td>0.21 ± 0.00</td>
<td>3.13 ± 0.17</td>
<td>15.2 ± 2.12</td>
<td>0.04 ± 0.00 (3502-fold)</td>
</tr>
<tr>
<td>Y91F</td>
<td>5.89 ± 0.38</td>
<td>1.62 ± 1.20</td>
<td>1.05 ± 0.13</td>
<td>0.36 ± 0.17</td>
<td>0.7 ± 0.1 (200-fold)</td>
</tr>
</tbody>
</table>

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**Table 2** Kinetic parameters for the recombinant malic enzymes in pyruvate reduction and oxaloacetate decarboxylation reactions

<table>
<thead>
<tr>
<th></th>
<th>Reduction of pyruvate</th>
<th>Decarboxylation of oxaloacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>5.20 ± 0.04</td>
<td>1.40 ± 0.01</td>
</tr>
<tr>
<td>Y91F</td>
<td>8.77 ± 0.45</td>
<td>0.72 ± 0.01</td>
</tr>
<tr>
<td>K162A</td>
<td>(0.70 ± 0.01) × 10$^{-1}$</td>
<td>0.92 ± 0.01</td>
</tr>
</tbody>
</table>

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**Table 3** Summary of $k_{cat}$ and $k_{cat}/K_{cat}$ pH data for the recombinant malic enzymes

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_{cat}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH profile</td>
<td>$k_{cat}$</td>
<td>$K_{cat}$</td>
</tr>
<tr>
<td>Wild-type</td>
<td>6.29 ± 0.10</td>
<td>8.78 ± 0.09</td>
</tr>
<tr>
<td>Y91F</td>
<td>5.92 ± 0.11</td>
<td>8.63 ± 0.05</td>
</tr>
<tr>
<td>K162A</td>
<td>6.62 ± 0.05</td>
<td>–</td>
</tr>
<tr>
<td>K162R</td>
<td>6.28 ± 0.09</td>
<td>8.90 ± 0.08</td>
</tr>
</tbody>
</table>
Catalytic mechanism of malic enzyme

Figure 1  pH-dependence of $k_{cat}$ of wild-type (○), Y91F (●) and K162A (Δ) mutants of pigeon NADP$^+$-dependent malic enzyme

Enzyme activity was assayed in 60 mM Bis/Tris propane buffer as described in the Experimental section. Points represent the experimental data and traces are the results of curve-fitting of data for Eqn 2 and Eqn 3 (where $y$ is the value of $k_{cat}$).

Figure 2  Activation of the overall oxidoreduction decarboxylation of the wild-type (open symbols) and K162A (closed symbols) mutant malic enzymes as a function of the concentration of the addition of exogenous amine, ammonium chloride (circle) and methylamine (square)

Initial rates of overall oxidoreduction decarboxylation were measured at saturating concentrations of L-malate (5 mM), MgCl$_2$ (8 mM) and NADP$^+$ (0.23 mM).

Table 4  Kinetic parameters of the recombinant malic enzymes in the presence of 100 mM ammonium chloride

<table>
<thead>
<tr>
<th></th>
<th>$K_{cat}$ (mM)</th>
<th>$K_{M1}$ (mM)</th>
<th>$K_{cat2}$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.42 ± 0.03</td>
<td>0.04 ± 0.00</td>
<td>1.33 ± 0.12</td>
<td>147.20 ± 3.60 (1-fold)</td>
</tr>
<tr>
<td>K162A</td>
<td>2.50 ± 0.20</td>
<td>0.12 ± 0.01</td>
<td>14.90 ± 1.00</td>
<td>(0.52 ± 0.12) × 10$^{-1}$ (2830-fold)</td>
</tr>
</tbody>
</table>

chloride, whereas the $k_{cat}$ value was found to be 0.04 % of wild-type, corresponding to a 10-fold recovery of activity.

The effects of ammonium chloride on the partial pyruvate reduction and oxaloacetate decarboxylation activities are shown in

Figure 3  Activation of the partial dehydrogenation (A) and decarboxylation (B) reactions of wild-type (○) and K162A (●) mutant malic enzymes as a function of the concentration of ammonium chloride

Initial rates of dehydrogenation reaction using pyruvate as a substrate were measured at saturating concentrations of pyruvate (14 mM), MgCl$_2$ (8 mM) and NADPH (0.23 mM) in TEA buffer (pH 7.4). Initial rates of oxaloacetate decarboxylation were measured at saturating concentrations of oxaloacetate (6 mM) and MgCl$_2$ (8 mM) in acetate buffer (pH 4.5).

Figure 3. Ammonium chloride showed no rescue of the pyruvate reduction in the K162A mutant (Figure 3A). For oxaloacetate decarboxylation, ammonium chloride demonstrated a maximum restoration (3.5-fold) at a concentration of 1 mM, and its rescue efficiency decreased as the concentration increased (Figure 3B). These chemical rescue results also suggest that the ε-amino group of Lys$^{162}$ in the pigeon NADP-dependent malic enzyme does not play a general base role in the dehydrogenation of L-malate to form oxaloacetate. The relative low recovery by ammonium chloride for the overall oxidative decarboxylation and the partial decarboxylation reactions in the K162A mutant may be due to the inaccessibility of the exogenous amine in the active site. The crystal structure of the pigeon malic enzyme showed that the Lys$^{162}$ residue is located at the bottom of the active site [21].

Partition of oxaloacetate in the presence of NADPH

Malic enzyme may convert oxaloacetate into either pyruvate or L-malate in the presence of NADPH through oxaloacetate decarboxylase and reductase activities respectively. The partition ratios of pyruvate and L-malate formation were measured for wild-type and K162A and Y91F mutant enzymes. A partition ratio of 1.37 ± 0.13 was obtained for wild-type, in agreement with the value obtained by Grissom and Cleland [23]. The values of the partition ratio for the K162A and Y91F mutants were 0.17 ± 0.04 and 0.31 ± 0.09 respectively, consistent with the partial reaction studies in which these mutants showed preferential decreases in the rate of oxaloacetate decarboxylation with respect to the rate of hydride transfer. However, mutation of the homologous residues in A. suum [5] and maize [24] malic enzymes shifted the partition toward the decarboxylation reaction. The origin of these kinetic discrepancies is unclear.
DISCUSSION

Initial velocity studies showed that $k_{\text{cat}}$ values dramatically decreased (approx. 27 000-fold) in the K162A mutant compared with wild-type, and this activity could be partially rescued by the exogenous addition of ammonium chloride, which suggests that the ε-amino group of the Lys$^{162}$ residue is critical to the enzyme reaction. The pH profile of the wild-type malic enzyme showed a bell-shaped pattern for both pH-log($k_{\text{cat}}$) and pH-log($k_{\text{cat}}/K_m$) profiles. The basic limb of the pH-log($k_{\text{cat}}$) profile was abolished for the K162A mutant. The p$K_a$ values in the pH-log($k_{\text{cat}}$) plot reflect the dissociation of the catalytic groups with the substrate present. It is therefore reasonable to assign the observed basic p$K_a$ to the protonation and deprotonation of the ε-amino group of Lys$^{162}$. The descent on the basic side indicated that the protonated form of the ε-amino group of Lys$^{162}$ is essential for the enzymatic activity. Therefore Lys$^{162}$ may act as a general acid to donate a proton in the reaction.

In order to further identify the function of the Lys$^{162}$ residue, the kinetic parameters of its partial reactions were determined. Malic enzyme catalyses the reduction of NADP$^+$ concomitant with L-malate oxidation to form oxaloacetate, oxaloacetate decarboxylation and enolpyruvate–pyruvate tautomerization. The general base-associated dehydrogenation and decarboxylation steps can be analysed independently. The oxidation–reduction reaction of L-malate is unable to be assayed directly due to a subsequent decarboxylation reaction. However, its reverse reaction can be analysed by using pyruvate and NADPH as substrates. Oxaloacetate decarboxylation can be monitored by measuring the disappearance of oxaloacetate in the presence of metal ion. There is no available method to analyse the general acid involved in enol–keto tautomerization. The K162A mutant demonstrated a 3250-fold decrease in the $k_{\text{cat}}$ value for the oxaloacetate decarboxylation reaction, which was partially rescued by ammonium chloride. However, this mutant showed no effect on the $k_{\text{cat}}$ value for pyruvate reduction. According to the mechanism shown in Scheme 1, a single residue responsible for the general base is required for both the partial dehydrogenation and decarboxylation reactions. We expect that the mutation of the general base residue should demonstrate decreased $k_{\text{cat}}$ values for both reactions. Thus Lys$^{162}$ is unlikely to act as a general base in these reactions. The decrease in the $k_{\text{cat}}$ value for the decarboxylation reaction may result from impairment in the subsequent enol–keto tautomerization. This is consistent with our previous studies on the conserved carboxylic amino acid residues in the active site, Glu$^{234}$, Asp$^{235}$, Asp$^{257}$ and Asp$^{258}$. The D258A mutant affects both the partial pyruvate reduction and oxaloacetate decarboxylation reactions by lowering their $k_{\text{cat}}$ values dramatically [10]. Both of these activities can be rescued by sodium azide. Therefore we suggest that the carboxyl group of Asp$^{258}$ acts as a general base in the catalytic mechanism of malic enzyme. Furthermore, the partitioning of oxaloacetate to pyruvate compared with L-malate in the presence of NADPH and Mn$^{2+}$ of the K162A mutant decreased 7-fold with respect to that of the wild-type, indicating that this mutant shows a preference for L-malate over pyruvate formation. These partitioning results are consistent with the results of the partial reaction studies in which the K162A mutant showed significant impairment in the oxaloacetate decarboxylation reaction. The ε-amino group of Lys$^{162}$ should act as a general acid to donate a proton in the enol–keto tautomerization (Scheme 2).

Compared with the K162A mutant, the Y91F mutant showed a similar, but lesser, effect on the $k_{\text{cat}}$ values for both the overall and decarboxylation partial reactions and on the partition ratio. The shapes of the pH-log($k_{\text{cat}}$) and pH-log($k_{\text{cat}}/K_m$) profiles for the Y91F mutant are essentially identical with those of the wild-type enzyme, which suggests that the phenolic group of Tyr$^{91}$ may have no direct role in the general acid–base mechanism. The phenolic group of Tyr$^{91}$ is within the hydrogen-bond distance of the ε-amino group of Lys$^{162}$. Therefore Tyr$^{91}$ and Lys$^{162}$ may form a catalytic dyad to facilitate the donation of a proton to oxaloacetate (Scheme 2).

Similar to the pigeon enzyme, the Y126F and K199A mutants of the A. suum NAD malic enzymes result in 10$^3$- and 10$^5$-fold decreases respectively, in the $k_{\text{cat}}$ values compared with wild-type [9]. However, the pH-$k_{\text{cat}}$ profile for the A. suum wild-type enzyme and K199R mutants showed only one p$K_a$ value at 4.9 and was pH-independent from pH 5.5 to pH 10. The pH-$k_{\text{cat}}$ profile for Y126F showed a partial activity loss above pH 6.6 [9]. The K255I mutant of the maize NADP$^+$-malic enzyme showed a 200-fold decrease in the $k_{\text{cat}}$ value [24]. The partition ratio of oxaloacetate for the K199A mutant enzyme in A. suum [5] and the K255I mutant in maize [24] was 10-fold higher than that of their respective wild-type enzymes, which are different from the pigeon mutant enzyme. Shifting the oxaloacetate partition toward L-malate formation suggests that this conserved lysine residue has a more critical role in dehydrogenation. The reasons for these kinetic discrepancies among pigeon, A. suum, and maize malic enzymes are not clear. The amino acid sequences of the A. suum and maize malic enzymes showed 65 % and 55 % identity with the pigeon malic enzyme respectively. The overall structures of pigeon and A. suum malic enzymes are similar. However, the pigeon cytosolic NADP$^+$-dependent enzyme is not an allosteric enzyme, whereas the A. suum mitochondrial enzyme is allosterically activated by fumarate [25,26]. A steady-state random kinetic mechanism has been documented for the A. suum enzyme [27]. In contrast, it has been suggested that the pigeon enzyme possesses an order kinetic mechanism [15,28]. Therefore some minor but important differences between their active site structures would be expected.
In general, the enol–keto tautomerization reaction is rapid. Therefore mutation of the residues involved in this process should have a minor effect on the $k_{cat}$ value. On the other hand, the K162A mutant showed dramatic decreases in the $k_{cat}$ values. If its general acid role were sustained, other factors would have to be involved in the tautomerization step. In the study of the mechanism of metal-ion-catalysed decarboxylation of oxaloacetate, lowering the dielectric constant of water by addition of dioxane (up to 50%) could increase the non-enzymatic reaction rate dramatically [29].

This result suggests that the hydrophobic environment might be an important factor for enzymatic catalysis. Yeast pyruvate kinase catalyses the phosphoryl transfer from phosphoenolpyruvate to ADP through an enolpyruvate intermediate to yield pyruvate and ATP. Comparing the model system with pyruvate kinase suggested that not only the hydrophobic environment but also the proton donation is required for the enzyme reaction [30]. The cleft of the active site of the pigeon NADP$^+$-dependent malic enzyme is formed by residues Tyr$^{91}$ and Asp$^{259}$ (domain A), Arg$^{144}$, Leu$^{162}$, Lys$^{162}$, and Asp$^{259}$ (domain B), and Lys$^{162}$, Asn$^{442}$ and Asn$^{259}$ (domain C) [21]. Although water is excluded from the active site during the transformation of the open–closed form, amino acid residues in the active site, except for Leu$^{442}$, are primarily polar and charged. The hydroxyl group of enolpyruvate then would be stabilized by a hydrophilic environment, shifting the enol–keto tautomerization reaction to the enol form. Therefore a proton donor is essential for enol–keto tautomerization.

Another possible explanation proposed previously by Cook and coworkers [5] is that isomerization of the malic enzyme is correlated with general acid function. The structure of the malic enzyme undergoes a transformation between the open and closed forms in the presence of L-malate. During this transformation, domain C moves toward domain B approx. 9°. This conformational change is correlated with general acid function. The structure of the malic enzyme from Escherichia coli [24,25] and pigeon NADP$^+$-dependent malic enzyme [26], which are both metal-ion-dependent decarboxylases, confirm that structural changes are also required to attain activity.

In conclusion, the protonated form of the ε-amino group of the Lys$^{162}$ residue in the pigeon NADP$^+$-dependent malic enzyme is essential for its enzymatic activity. Therefore Lys$^{162}$ should act as a general acid to provide a proton to enolpyruvate for tautomerization. The phenolic group of Tyr$^{91}$ could hydrogen bond with the ε-amino group of Lys$^{162}$ to form a catalytic dyad to facilitate proton transfer.

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