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The Role of Tyrosine 207 in the Reaction Catalyzed by Saccharomyces cerevisiae phosphoenolpyruvate carboxykinase

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ABSTRACT

The functional significance of tyrosine 207 of *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase was explored by examining the kinetic properties of the Tyr207Leu mutant. The variant enzyme retained the structural characteristics of the wild-type protein as indicated by circular dichroism, intrinsic fluorescence spectroscopy, and gel-exclusion chromatography. Kinetic analyses of the mutated variant showed a 15-fold increase in K_m CO_2, a 32-fold decrease in V_{max}, and a 6-fold decrease in K_m for phosphoenolpyruvate. These results suggest that the hydroxyl group of Tyr 207 may polarize CO_2 and oxaloacetate, thus facilitating the carboxylation/decarboxylation steps.

Key terms: Phosphoenolpyruvate carboxykinase, *Saccharomyces cerevisiae*, CO_2 interaction.

INTRODUCTION

Enzymes have evolved different strategies to catalyze carboxylation and decarboxylation reactions, some enzymes use organic or inorganic cofactors, and others are cofactor independent (Liu & Zhang, 2005). Phosphoenolpyruvate (PEP) carboxykinases (GTP/ATP, oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32/49) catalyze the reversible GTP/ATP-dependent decarboxylation of oxaloacetate (OAA) to yield PEP, CO_2 and the corresponding GDP/ADP. They have an absolute requirement for divalent cations for activity. One cation, preferentially a transition metal like Mn^{2+}, interacts with the enzyme at metal binding site 1 to elicit activation, while the second cation (Mg^{2+} or Mn^{2+}), at metal binding site 2, interacts with the nucleotide to serve as the metal-nucleotide substrate (Matte et al., 1997). Two classes of PEP carboxykinases exist in nature: ATP-dependent enzymes, which are found in plants, yeast, trypanosomatids, and some bacteria (Matte et al., 1997), and GTP-dependent PEP carboxykinases found in animals and some bacteria (Hanson & Patel, 1994; Fukuda et al., 2004). There is no significant overall sequence identity between the two groups of enzymes; however, active site residues are strictly conserved.

Kinetic experiments suggested that PEP carboxykinases have a binding site for CO_2 (Arnelle & O'Leary, 1992; Cheng & Nowak 1989). This proposal was confirmed by the structure of the *Escherichia coli* enzyme complexed with CO_2-ATP-Mg^{2+}-Mn^{2+}, which indicates that CO_2 is hydrogen bonded to Arg65 and Tyr207 (Cotelesage et al., 2007). A proposed reaction mechanism for PEP production indicates the formation of a hydrogen bond between the C_4 carboxylate group of oxaloacetate and the hydroxyl group of Tyr207. Thus Tyr207 would polarize CO_2 and oxaloacetate, facilitating catalysis (Cotelesage, 2007). Structural (Dunten et al., 2002) and kinetic studies (Dharmarajan et al., 2008) indicate that the equivalent Tyr 235 of human cytosolic PEP carboxykinase establishes an anion-quadrupole interaction with PEP carboxylate. A similar role has been proposed for Tyr 254 from the Mn^{2+}-PEP complex of mitochondrial PEP carboxykinase (Holyoak & Sullivan, 2006).

In this work, we use site directed mutagenesis to evaluate the contribution of Tyr 207 to substrate affinity and catalysis of *Saccharomyces cerevisiae* PEP carboxykinase.

MATERIALS AND METHODS

The ATP was obtained from Sigma Chemical Company, Saint Louis, USA and the OAA from Boehringer. Germany. All other reagents and auxiliary enzymes were of the highest purity commercially available, and their origin has been described before (Krautwurst et al., 2002).

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Mutagenesis and expression of recombinant wild-type and mutant enzymes

A specific substitution was introduced at Tyr207 triplet to code for Leu (TAC to TTA) in the cloned *S. cerevisiae* PEP carboxykinase gene (pMV7 plasmid) by Retrogen, USA. To check the mutated site and verify that no additional mutations had been introduced, the altered gene in the recombinant plasmid was completely sequenced by the manufacturer. The mutant plasmid was transformed into the PEP carboxykinase-deficient yeast strain PUK-3B (MATa *pck ura*3) and the mutant PEP carboxykinase was purified as previously described (Llanos et al., 2001).

Enzyme kinetics

Enzyme activity was measured at 30 ºC in 1 mL final volume. The standard assay mixture in the OAA formation direction contained 100 mM MOPS buffer (pH 6.6), 0.20 mM NADH, 50 mM $KHCO_3$, 15 mM PEP, 1.25 mM ADP, 5.0 mM $MgCl_2$, 0.1 mM $MnCl_2$ and 4 units malate dehydrogenase. The standard assay mixture in the PEP forming direction contained 100 mM MOPS buffer (pH 7.0), 0.20 mM NADH, 50 mM KCl, 5.0 mM $MgCl_2$, 0.1 mM $MnCl_2$, 1 mM ATP, 0.5 mM of OAA, and 5 units of both lactate dehydrogenase and pyruvate kinase. Activities were corrected for nonenzymatic OAA decarboxylation. Maximal velocity and apparent K_m were determined by fitting initial velocities to the Michaelis-Menten equation with the Microcal Origin™ program. K_a for Mn^{2+} was measured as a function of free Mn^{2+}, while keeping the concentration of components at fixed standard saturating levels.

The concentrations of free Mn^{2+}, $MnADP^-$, $MgADP^-$ and other species were calculated using the program COMPLEX version 6 (1986) written by Dr. Athel Cornish-Bowden. The dissociation constants were obtained from Martel and Smith (1998).

Circular dichroism and fluorescence spectroscopy

CD spectra (recorded from 200 nm to 260 nm) and fluorescence emission (lexcit 295 nm) were carried out as previously described (Castillo et al., 2009).

Electron Paramagnetic Resonance Spectroscopy

Mn^{2+} binding was measured by EPR spectroscopy on a Bruker EMX X-band EPR spectrometer at a frequency of 9.5 GHz. The enzyme used for the EPR experiments was first incubated for 10 min at 2 °C in 50 mM MOPS buffer (pH 7.0) with 1.25 mM ADP, 2 mM PEP, and 2 mM $MnCl_2$. The enzyme solution was then passed through a Bio-Gel P-6 (1.5 × 25 cm) column with a 2-cm layer of Chelex-100 on top. Without this preincubation, a tight binding, contaminating cation is not totally removed to form apoenzyme. Enzyme solutions (40 and 113 μM of enzyme subunits for wild-type, and Tyr207Leu PEP carboxykinases, respectively) were prepared in 50 mM KCl and 100 mM MOPS buffer (pH 7.0). The total Mn^{2+} concentration varied from 0 to 250 μM. Each sample was prepared in a 100 μL volume and $[Mn^{2+}]$ free was measured. The dissociation equilibrium constants (K_D) were calculated by curve fitting to equation 1.

$$\nu = [Mn^{2+}] / (K_D + [Mn^{2+}]) \quad (1)$$

where n is the number of moles of Mn^{2+} bound per mole of enzyme monomer.

RESULTS AND DISCUSSION

To better understand the importance of the invariant Tyr207 of *S. cerevisiae* PEP carboxykinase, this residue was changed to leucine, so as to entirely eliminate the ability to form an anion-quadrupole interaction, or an H- bond.

Cell growth, gene expression, enzyme purification, and structural characteristics of the Tyr207Leu mutant enzyme

The Tyr207Leu PEP carboxykinase plasmid was sequenced to confirm the absence of any spurious mutations in the coding region outside the area of the mutation. Expression of the mutated PEP carboxykinase was achieved in the PEP carboxykinase-deficient *S. cerevisiae* strain, PUK-3B, containing the pMV7 plasmid. The cells containing the Tyr207Leu mutation were unable to grow on medium containing ethanol as the primary carbon source, indicating lack of *in vivo* functional activity of the altered PEP carboxykinase. These cells were grown on glucose medium instead, and the medium was changed to ethanol to achieve the induction of the PEP carboxykinase gene. Eight liters of glucose medium yielded 50-60 g of cells. The enzyme was purified using the procedure previously reported (Krautwurst et al., 1998). The final yield of mutated PEP carboxykinase was 6-8 mg from about 60 g of cells. The enzyme was judged to be at least 95% pure as determined by SDS-PAGE.

The apparent mass of the variant enzyme was the same as that of the wild-type enzyme, as determined in a calibrated (R = 0.95) Superose-12 column (results not shown). The calculated molecular mass of wild type PEP carboxykinase in this column was 251 kDa, and the molecular mass of the variant enzyme was within 10% of this value.

These results agree with the expected molecular mass of 244 kDa for the wild-type enzyme tetramer (Krautwurst et al., 1995), and are in the range of those determined by other authors for this same protein (Muller et al., 1981; Jacob et al., 1992). Circular dichroism spectra were measured for the mutant and wild-type enzymes to examine whether mutation at position 207 induced changes in the secondary structure of the protein. The CD spectrum of the variant enzyme was very similar to that of the wild-type enzyme, with a minimum at 208 nm and a shoulder at 222 nm (not shown). Alterations in the tertiary structure were analyzed through the intrinsic fluorescence spectra of the enzymes that have a total of 8 Trp residues at positions 88, 89, 101, 127, 171, 275, 446, and 508 (Krautwurst et al., 1995). No alteration in the λ_{max} of emission (328 nm) was detected. The Tyr207Leu mutant enzyme showed a 15% increase in fluorescence intensity, suggesting that this mutation changes the microenvironment of some Trp residues. Changes in fluorescence emission of proteins can occur from even small movements of neighboring amino acid residues.

Steady-state kinetics studies on wild-type andTyr207Leu PEP carboxykinases

A summary of the resulting steady-state parameters for the PEP carboxylation reaction is listed in Table I. V_{max} decreased by 32-fold for the Tyr207Leu PEP carboxykinase compared to wild-type enzyme. The K_m value for CO_2 and Mn^{2+} were increased 15-fold and fivefold compared to values measured for the wild-type enzyme. There was a 6-fold decrease in K_m for PEP in the mutant enzyme, and the K_m value for $MgADP^-$ was not significantly altered. In the variant enzyme, the absence of the hydroxyl group abolished the proposed hydrogen bond between CO_2 and Tyr207, thus increasing the K_m for CO_2.

The kinetic parameters in the OAA decarboxylation direction (Table II) showed that mutation causes a 6-fold decrease in V_{max}, a 2.8- fold increase in the K_m value for $MnATP^{2-}$, and a 3.5-fold increase in K_m for OAA, suggesting that Tyr207 might be involved in OAA binding.

The relatively minor decrease in V_{max} seen in both directions of the reaction indicates that Tyr 207 is not a catalytically essential residue for the yeast PEP carboxykinase.

A recent kinetic analysis of site-directed mutants of Tyr235 in human GTP-dependent PEP carboxykinase (Dharmarajan et al., 2008), showed some similarities and some differences with our data. Thus, as compared to the wild-enzymes, both the yeast and the human variant enzymes did not change the K_m for the nucleotide, increase the K_m for Mn^{2+}, and did not greatly affect V_{max}. On the other hand, the Tyr235Ala and Tyr235Ser enzymes showed a 4- to 6-fold increase in K_m for PEP, thus confirming an edge-on interaction between the aromatic ring of Tyr235 and PEP carboxylate. In our studies, replacement of Tyr207 decreases the K_m for

Table I
Kinetic parameters of wild-type and Tyr207Leu PEP carboxykinases in the carboxylation direction.

	Wild-type	Tyr207Leu
K_m Mn^{2+} (μM)	8 ± 1	41 ± 9
K_m $MgADP^-$ (μM)	16 ± 3	13 ± 1
K_m PEP (μM)	3000 ± 100	500 ± 100
K_m CO_2 (mM)[a]	2 ± 0.2	29 ± 3
V_{max} (μmol min^{-1} mg^{-1})	16 ± 3	0.5 ± 0.1

Values given are the mean ± standard deviation for three independent experiments.
[a]Concentration of CO_2 is expressed as total bicarbonate.

Table II
Kinetic parameters of wild-type and Tyr207Leu PEP carboxykinases in the decarboxylation direction.

	Wild-type	Tyr207Leu
K_m $MgATP^{2-}$ (μM)	16 ± 2	44 ± 4[a]
K_m OAA (μM)	200 ± 40	700 ± 20
V_{max} (μmol min^{-1} mg^{-1})	20 ± 2	> 3 ± 0.6

Values given are the mean ± standard deviation for three independent experiments.
[a] Determined at 1.5 mM OAA

PEP, which is not consistent with such an interaction between Tyr 207 and this substrate. The Tyr207Leu change abolished the H-bond that existed between Tyr and CO_2 and in turn increased the K_m for CO_2. The replacement of Tyr235 in the human enzyme causes a 3-fold decrease in K_m for CO_2, suggesting no role in CO_2 binding.

The kinetic results here reported and the previous studies of Dharmarajan et al. (2008) support the different roles for the invariant active site Tyr in ATP-dependent and GTP-dependent PEP carboxykinases implied from structural studies (Dunten et al., 2002; Holyoak & Sullivan., 2006; Cotelesage et al., 2007). It is worth noting that active site differences in ATP- and GTP-dependent PEP carboxykinases have been inferred before. For example, the OAA decarboxylase activity of some GTP-dependent enzymes require the presence of the nucleoside diphosphate (Sullivan and Holyoak, 2007), meanwhile the ATP-dependent enzymes from *S. cerevisiae* and *Anaerobiospirillum succiniciproducens* (Jabalquinto et al., 1999) do not.

Mn^{2+} Binding

To evaluate the effect of mutation in the thermodynamic interaction of Mn^{2+} with the enzyme, the binding of Mn^{2+} to wild-type and Tyr207Leu enzymes was measured by EPR spectroscopy. Binding isotherms are shown in Figure 1. Quantitative estimates of binding parameters were obtained from curve fitting to equation 1. K_D values of 35 ± 3 and 157 ± 54 μM were obtained for wild-type and Tyr207Leu PEP carboxykinases, respectively. In both cases one binding site for Mn^{2+} per enzyme monomer was obtained. The 0.9 kcal/mol loss in Mn^{2+} binding affinity of *S. cerevisiae* PEP carboxykinase upon mutation Tyr207Leu correlate well with the increase in K_m value shown in Table 1, and also with the effect of mutation of Tyr235 of human cytosolic enzyme in K_m for Mn^{2+} (Dharmarajan et al., 2008). For the human enzyme it was proposed that mutation of Tyr235Phe, which lowers K_m for PEP, might cause a detrimental effect for the inner sphere coordination of PEP to Mn^{2+}, thus increasing K_m for Mn^{2+}. A similar situation might also occur upon mutation Tyr207Leu in the *S. cerevisiae* PEP carboxykinase. It is also possible that this mutation, which introduces a non-polar residue, might perturb the general water structure of the active site, thus affecting Mn^{2+} binding.

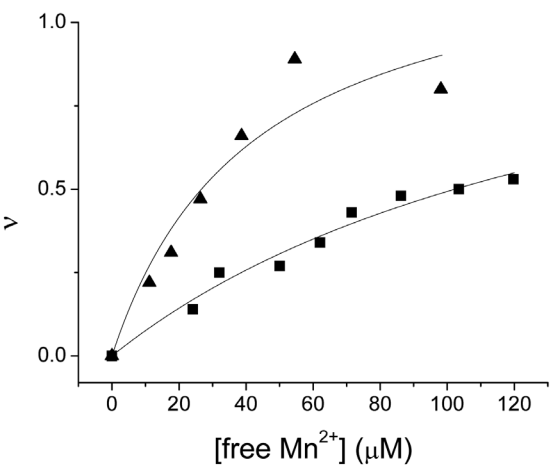

Figure 1. Mn2+ binding to PEP carboxykinases. The number of moles of Mn2+ bound per mole of enzyme monomer (ν,) is calculated from the signal intensity of the lowest-field transition and plotted against free Mn^{2+} concentration. Wild-type (⑨) and Tyr207Leu (⇥) enzymes. The lines show the best fit of the data to equation (1).

In conclusion, we show that the main effect of mutation Tyr207Leu in *S. cerevisiae* PEP carboxykinase is to substantially alter the enzyme kinetic affinity for CO_2. This suggests that the hydroxyl group of Tyr 207 is involved in CO_2 binding, as also inferred from the structure of the *E. coli* enzyme complexed with CO_2-ATP-Mg^{2+}-Mn^{2+}, where the hydroxyl group of Tyr 207 is at H bond distance to CO_2 (Cotelesage et al., 2007). The decrease in V_{max} upon mutation, although of a small magnitude, is in line with the suggestion that the hydroxyl group at position 207 may polarize CO_2 and OAA, thus facilitating the carboxylation/decarboxylation steps (Cotelesage, 2007; Cotelesage et al., 2007).

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