Mutation Arg336 to Lys in *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinase originates an enzyme with increased oxaloacetate decarboxylase activity

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Received 27 November 2000; revised 13 January 2001; accepted 19 January 2001

First published online 6 March 2001

Edited by Pierre Jolles

Abstract ***Saccharomyces cerevisiae*** **phosphoenolpyruvate (PEP) carboxykinase catalyzes one of the first reactions in the biosynthesis of carbohydrates. Apart from the physiologically important reaction, the enzyme also presents low oxaloacetate decarboxylase and pyruvate kinase-like activities. Data from the crystalline structure of homologous** ***Escherichia coli*** **PEP carboxykinase suggest that Arg^{333} may be involved in stabilization of enolpyruvate, a postulated reaction intermediate. In this work, the equivalent Arg^{336} from the** ***S. cerevisiae*** **enzyme was changed to Lys or Gln. Kinetic analyses of the varied enzymes showed that a positive charge at position 336 is critical for catalysis of the main reaction, and further suggested different rate limiting steps for the main reaction and the secondary activities. The Arg336Lys altered enzyme showed increased oxaloacetate decarboxylase activity and developed the ability to catalyze pyruvate enolization. These last results support the proposal that enolpyruvate is an intermediate in the PEP carboxykinase reaction and suggest that in the Arg336Lys PEP carboxykinase a proton donor group has appeared.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Phosphoenolpyruvate carboxykinase; Pyruvate enolization; *Saccharomyces cerevisiae*

1. Introduction

Phosphoenolpyruvate (PEP) carboxykinase catalyzes an early step in gluconeogenesis, the decarboxylation and phosphorylation of oxaloacetate (OAA) in the presence of a nucleoside triphosphate (ATP or GTP, depending on the enzyme source) and a divalent metal ion, as shown below [1]:

$$\mathrm{ATP/GTP + OAA} \overset{Me^{2+}}{\Leftrightarrow} \mathrm{PEP + CO_2 + ADP/GDP} \quad (1)$$

In addition to the physiologically important reaction, OAA decarboxylase (Eq. 2) and pyruvate kinase (PK)-like activities (Eq. 3) have been described in *Saccharomyces cerevisiae* and other PEP carboxykinases [2–5]:

$$\mathrm{OAA} \overset{Me^{2+}}{\Rightarrow} \mathrm{pyruvate + CO_2} \quad (2)$$

$$\mathrm{PEP + ADP} \overset{Me^{2+}}{\Rightarrow} \mathrm{pyruvate + ATP} \quad (3)$$

These secondary reactions are thought to represent the decarboxylation and phosphorylation steps of the main reaction of the enzyme, presumably linked through enolpyruvate, a putative reaction intermediate [2,5]. Recently, we showed in *S. cerevisiae* PEP carboxykinase that Lys^{256} is an essential amino acid residue for phosphoryl transfer but not for decarboxylation [2].

The three-dimensional structure of the ATP–pyruvate–Mg^{2+}–Mn^{2+} quinary complex of homologous *Escherichia coli* PEP carboxykinase has been solved at 1.9 Å resolution [6]. From this structure the locations and possible functions of active site amino acid residues have been inferred. It was suggested that Arg^{333} (Arg^{336} in the *S. cerevisiae* PEP carboxykinase) bridges ATP and pyruvate, neutralizing electrostatic repulsions and facilitating enolpyruvate formation through direct ionic interactions with the enolate oxygen [6]. It is conceivable, then, that this arginyl residue would play an important role both in the phosphoryl transfer and decarboxylation steps of the enzymatic reaction.

In the present work, we have evaluated the role in catalysis of Arg^{336} in *S. cerevisiae* PEP carboxykinase. Our results indicate that a positively charged residue in position 336 is critical for catalysis of the main reaction but not for the secondary activities. Furthermore, the analysis of a Arg336Lys mutant showed increased OAA decarboxylase activity, an effect possibly related to the capacity of the altered enzyme to catalyze isotopic exchange of the methyl protons of pyruvate.

2. Materials and methods

All materials and reagents were from previously cited sources [2]. 100% deuterated water was from Aldrich. Lyophilized rabbit muscle PK was from Sigma.

2.1. Site-directed mutagenesis and enzyme purification

Specific substitutions were introduced at arginine triplet 336 in the cloned *S. cerevisiae* PEP carboxykinase gene (pMV7 plasmid) by the method of Künkel [7] for Arg336Lys, or by using recombinant PCR

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Abbreviations: CD, circular dichroism; HEPES, *N*-(2-hydroxyethylpiperazine-*N*′-[2-ethanesulfonic acid]); MOPS, 3-(*N*-morpholino)propanesulfonic acid; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PK, pyruvate kinase

PII: S0014-5793(01)02158-5

for Arg336Gln. To obtain the Arg336Lys substitution, synthetic oligonucleotide 5′-GTAGGCAAAT**T**TAGTATTTTC-3′ (base substitution is shown in bold and underlined) was used, and all procedures were as previously described [2,8]. To obtain the Arg336Gln substitution, 10 ng of template DNA were amplified with 10 pmol of the mutagenic oligonucleotide, oligo M1: 5′-GGTAGGCACAT**TG**AGTATTTTCAG-3′ and 10 pmol of the non-mutagenic oligonucleotide, oligo A: 5′-GTTTCGTTTACTCGAATTG-3′. In parallel, the 3′ moiety was amplified using 10 pmol of a mutagenic oligonucleotide, oligo M2, which is complementary to oligo M1, and 10 pmol of the non-mutagenic oligonucleotide B: 5′-CCGGAAGATCTCCAAAGGAC-3′. The PCR products were purified from an agarose gel, mixed, and subjected to a second PCR reaction in the presence of 10 pmol of the non-mutagenic primers described above. The purified product was digested with restriction enzymes *Sac*I and *Bam*HI, cloned into pMV7, and transformed into *E. coli* JM109 competent cells. Mutant pMV7 plasmids were transformed into the PEP carboxykinase-deficient yeast strain PUK-3B (*MAT* α pck ura3) as previously described [2,8]. To check the mutated sites and verify that no additional mutations had been introduced, the altered genes in the recombinant plasmids were completely sequenced. Recombinant wild type, Arg336Lys, and Arg336Gln PEP carboxykinases were purified as described [2], except that the last step in AMP–Sepharose was omitted and replaced, if necessary, by a hydroxylapatite chromatography step. This was done using a 23×2.5 cm column in 10 mM phosphate buffer pH 7 plus 0.1 mM EDTA at a flow rate of 50 ml h^{-1}. The enzyme was eluted with a linear gradient (200 ml) from 10 to 150 mM of the same phosphate buffer. Pooled enzyme fractions were concentrated and exchanged into 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0) by passage through a Bio Gel P10 column or by repeated concentration and dilution in an Amicon ultrafiltration unit. PEP carboxykinase concentration was determined spectrophotometrically at 280 nm using the extinction coefficients $\varepsilon^{1\%} = 12.3$ cm^{-1}, $\varepsilon = 7.5\times10^4$ M^{-1} cm^{-1}, and a MW of 60 983 for the enzyme subunit [2].

2.2. Kinetic studies

All assays were carried out in 1 ml final volume at 30°C. Maximal velocity and the apparent K_m were determined by fitting to the Michaelis equation with Microcal Origin™ 5.0, which provides the standard deviation of the values. The enzyme was assayed in the PEP carboxylation direction in a solution containing 100 mM MOPS buffer (pH 6.6), 0.20 mM NADH, 1.25 mM ADP, 2.5 mM PEP, 50 mM $KHCO_3$, 2 mM $MnCl_2$, and 4 units malate dehydrogenase, essentially as described before [9]. To obtain the kinetic parameters, enzyme activity was measured as the concentration of either substrate was varied in the range 0.01–0.125 mM (ADP), 0.04–0.44 mM (PEP), or 5–50 mM ($KHCO_3$). Because of the low activity of the Arg336Gln PEP carboxykinase, 0.8 mg of protein/assay was used, which was more than 4000 times the amount employed for the assay of the wild type or Arg336Lys enzymes. The OAA decarboxylase activity was determined in 100 mM MOPS buffer (pH 7.8), 0.5 mM OAA, 0.2 mM $MnCl_2$, 0.20 mM NADH, and 4 units lactate dehydrogenase, all in 1 ml at 30°C. The spontaneous decarboxylation of OAA was measured, and then PEP carboxykinase was added to start the reaction. For the determination of K_m for OAA, the substrate was varied from 0.25 K_m to 2.5 K_m for the wild type enzyme, from 0.2 K_m to 3.0 K_m for the Lys336Arg mutants, and from 0.2 K_m to 2.5 K_m for the Arg336Gln mutant. The upper concentration of OAA employed was limited for the spontaneous metal-dependent decarboxylation of OAA to pyruvate+CO_2 (Eqs. 2 and 3). The PK-like activity was determined in 100 mM MOPS buffer (pH 6.6), 0.20 mM NADH, 2 mM $MnCl_2$, 1.25 mM ADP, 2.5 mM PEP, and 4 units lactate dehydrogenase, all in 1 ml at 30°C. To obtain the kinetic parameters, enzyme activity was measured as the concentration of either substrate was varied in the range 0.04–0.56 mM (ADP) or 0.05–0.68 mM (PEP).

2.3. Proton exchange

The enolization of pyruvate was quantitated by measuring the loss of the ^1H NMR resonance for the methyl protons of pyruvate in deuterated water using a Brucker 200 MHz spectrometer. The samples contained 20 mM MOPS (pH 7.8), 0.1 mM $MnCl_2$, 5 mM $MgCl_2$, 31 mM pyruvate, 1 mM ATP, and PK or PEP carboxykinase. Concentrated stock solutions were prepared in 100% D_2O or repeatedly lyophilized and dissolved in D_2O. Lyophilized PK was dissolved in 20 mM MOPS (pH 7.8) containing 95 mM KCl. PEP carboxykinase stock solutions were exchanged into D_2O by repeated filtration/centrifugation using Millipore PTTK 30,00 NMWL Ultrafree-MC filter units. The reactions were initiated by adding the enzyme. The methylene protons at position 1 of MOPS served as an internal integration standard. Control samples lacking enzyme were used to quantitate the non-enzymatic exchange of the methyl protons of pyruvate.

2.4. Circular dichroism (CD) spectroscopy

CD spectra were recorded in an Aviv 62A DS spectrometer in 20 mM potassium phosphate buffer (pH 6.8) at 25°C using 0.10 cm cells. Protein concentration was between 0.09 and 0.22 mg ml^{-1}.

2.5. Urea denaturation experiments

The enzyme (0.1 mg ml^{-1} for wild type or Arg336Lys, and 0.7 mg ml^{-1} for Arg336Gln PEP carboxykinases) was incubated 15 h at 16°C in 50 mM *N*-(2-hydroxyethylpiperazine-*N*′-[2-ethanesulfonic acid]) (HEPES) buffer (pH 7.5) at several urea concentrations. Aliquots were assayed for remaining enzyme activity in the PEP carboxylation direction in the presence of the same urea concentration to avoid enzyme renaturation during the assay [10].

2.6. Computer-assisted three-dimensional homology modeling

The programs InsightII, Homology, and Discover 972 (Biosym/MSI) were used on SGI workstations to build a homology-based three-dimensional model of *S. cerevisiae* PEP carboxykinase (2). The reference protein used as a template to follow the local degree of structure conservation or divergence was the *E. coli* PEP carboxykinase–ATP–pyruvate–Mg^{2+}–Mn^{2+} complex (1AQ2) (6). All calculations were done with Discover_3 (MSI) and force field ESFF (MSI), that has all parameters needed for the octahedral Mn^{2+} coordination and amino acids. This program was also employed for energy minimization and molecular dynamics. Sequence conservation between *E. coli* and *S. cerevisiae* PEP carboxykinases shows 48% identity and 60% similarity. The relaxed structure was used to replace pyruvate for enolpyruvate. To build the enolpyruvate structure, bonds were reorganized in the pyruvate molecule. Then Discover_3 was used for energy minimization and molecular dynamics. Final relaxation was 100 ps. In this case, a zone of 20 Å around Pγ of ATP was used. The remaining areas were fixed. Cutoff values for van der Waals and Coulombic interactions were 10 and 12 Å, respectively.

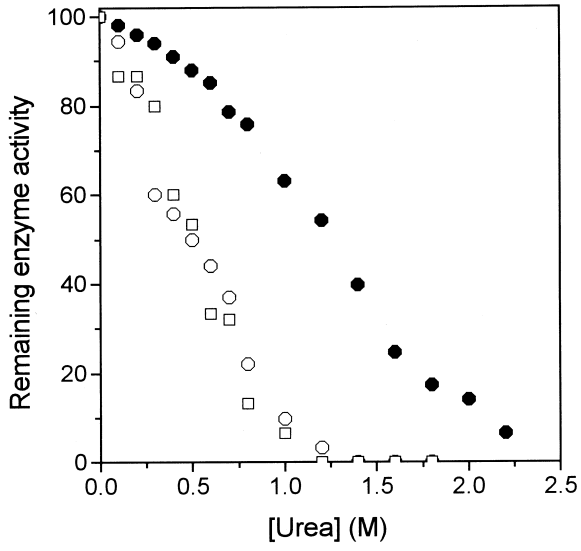

Fig. 1. Enzyme stability against urea denaturation. PEP carboxykinases were incubated in the presence of the indicated urea concentrations. After 15 h at 16°C, enzyme aliquots were withdrawn and assayed for remaining activity (wild type (●), Arg336Lys (□), and Arg336Gln (○) PEP carboxykinases) in the presence of the same urea concentration. Further details in Section 2.

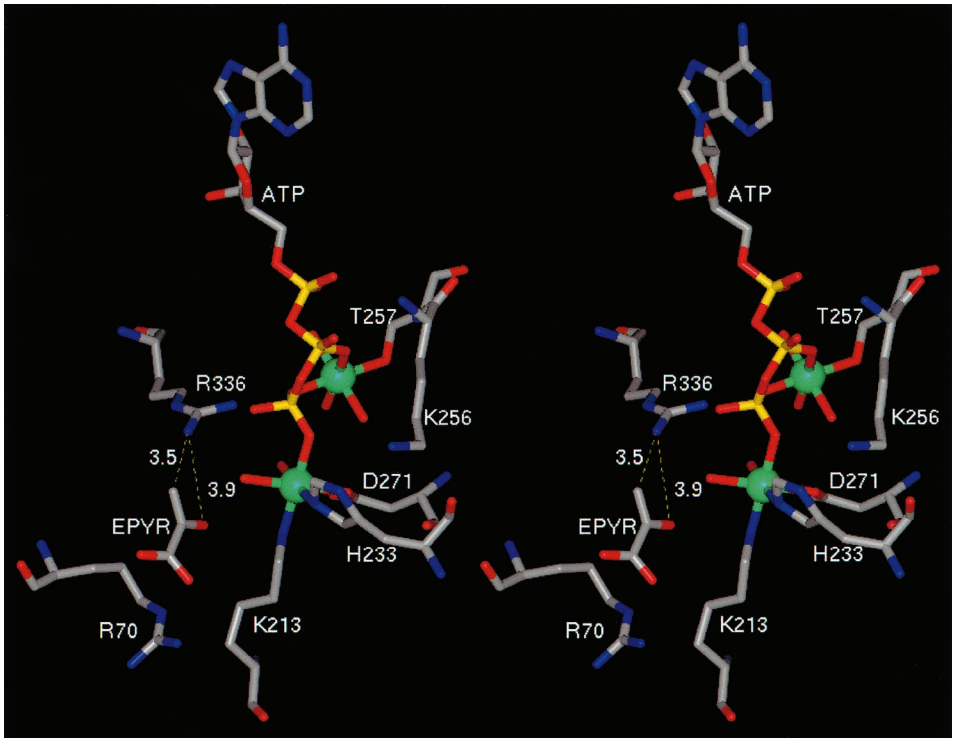

Fig. 2. Stereo view of a homology model of *S. cerevisiae* PEP carboxykinase–ATP–enolpyruvate–Mn^{2+} complex. The model was obtained using as reference the *E. coli* PEP carboxykinase–ATP–pyruvate–Mn^{2+}–Mg^{2+} complex (6). Distances are in Å. Mn^{2+} ions at the two metal sites are shown in green. The other heteroatoms are: oxygen, red; phosphorus, yellow; carbon, gray; nitrogen, blue.

3. Results and discussion

3.1. Basic structural and stability characteristics of Arg336Lys and Arg336Gln PEP carboxykinases

Expressed mutant proteins were homogeneous in SDS–PAGE, presenting a single protein band with the same rate of migration as the recombinant wild type enzyme (data not shown). The CD spectra of the wild type and mutated enzymes were very similar, with a negative peak at 208 nm and a negative shoulder at 222 nm. The ratio of the mean residue ellipticity at 208 and 222 nm was 0.78, 0.81, and 0.76 for the wild type, Arg336Lys, and Arg336Gln *S. cerevisiae* PEP carboxykinases, respectively, indicating that the secondary structure of the enzyme was not significantly altered by mutations at residue 336.

An assessment of the relative stability of enzyme active sites can be obtained through measurements of enzyme activity as a function of the concentration of protein denaturing agents [10,11]. When the activities of wild type and Arg^{336} altered enzymes were analyzed as a function of urea concentration, the activity profiles shown in Fig. 1 were obtained. The data show that replacement of Arg^{336} by either lysine or glutamine yields enzymes with decreased stability against urea denaturation, thus suggesting a specific role of Arg^{336} in stabilizing the active site conformation. This stabilizing effect is probably not related to the enzyme activity because, even when the Arg336Gln carboxykinase retains less than 0.05% of its original activity for the main reaction, the Arg336Lys mutant is fully active, as will be shown below. It is possible that specific interactions between the guanidinium group of Arg^{336} and the protein backbone [12], which can not be fulfilled by amino or amido groups, are required to assist the conformational stability of the *S. cerevisiae* PEP carboxykinase active site. Studies are being carried out to check the effect of Arg^{336} mutations in the overall protein stability, and will be the subject of a forthcoming publication.

3.2. Steady state parameters of wild type and mutant enzymes

The kinetic consequences of mutating Arg^{336} to Lys or Gln in yeast PEP carboxykinase in the main reaction (Eq. 1) are shown in Table 1. The substitution Arg336Lys caused only minor effects on V_{max} and K_m values; however, a loss in V_{max} of three orders of magnitude was seen for the Arg336Gln altered enzyme, with no variations in K_m values. The unaltered values for the kinetic constants in Arg336Lys PEP carboxykinase indicate that charge delocalization though the guanidinium group of Arg^{336} is not required for catalysis. However, the very low activity of the Arg336Gln mutant in-

Table 1
Kinetic parameters for wild type and mutant *S. cerevisiae* PEP carboxykinases in the PEP carboxylation reaction[a]

Enzyme	K_m			V_{max} (μmol min^{-1} mg^{-1})
	MnADP (μM)	PEP (μM)	CO_2 (mM)	
Wild type	34 ± 4	307 ± 40	18 ± 2	62 ± 10
Arg336Lys	38 ± 5	280 ± 60	24 ± 2	46 ± 10
Arg336Gln	31 ± 9	170 ± 70	18 ± 3	0.023 ± 0.002

[a]Kinetic constants were determined as indicated in Section 2. Values given are the mean ± S.D.

dicates that the presence of a positive charge is critical for the enzyme reaction to occur. The unaltered K_m values for the varied enzymes suggest that Arg^{336} is not involved in substrate binding. These findings are consistent with the role envisioned for the equivalent Arg^{333} in the ATP–pyruvate–Mg^{2+}–Mn^{2+} complex of the homologous *E. coli* PEP carboxykinase [6]. As pointed out by those authors, Arg^{333} appears to fulfill a catalytic role neutralizing electrostatic repulsions between the anionic substrates and stabilizing enolpyruvate formation through direct ionic interaction with the enolate oxygen. An homology model of the *S. cerevisiae* PEP carboxykinase–ATP–enolpyruvate–Mn^{2+} complex (Fig. 2) shows the close vicinity of the guanidinium group of Arg^{336} to the enolate oxygen and methylene group of enolpyruvate. Along the reaction pathway to form PEP+ADP, the two water molecules coordinated to Mn^{2+}, at close distance to Arg^{336}, would be displaced by the enolate oxygen and carboxylic group of enolpyruvate (6).

To gain a more detailed understanding of the role of Arg^{336} in catalysis, the secondary activities were measured in the altered enzymes. As seen in Table 2, replacement of Arg^{336} by Gln caused 2–3 fold changes in K_m values, and about a 12 fold decrease in V_{max} for the OAA decarboxylase and PK-like activities. The small K_m effects are consistent with the absence of significant changes for this parameters seen in the main reaction, however the 12 fold decrease in V_{max} appears at first sight inconsistent with the 2700 fold decrease in V_{max} for the principal reaction. The possibility of contamination of *S. cerevisiae* PEP carboxykinases with *S. cerevisiae* PK can be discarded because the PK-like activities of the wild type and mutant carboxykinases show hyperbolic kinetics for PEP and lack of activation by fructose 1,6-bisphosphate (data not shown). *S. cerevisiae* PK displays cooperative behavior against PEP saturation and is activated by fructose 1,6-bisphosphate [3,13,14].

The presence of substantial secondary activities in Arg336Gln PEP carboxykinase could be explained if the rate limiting step for the main reaction differs from that for the secondary reactions. In the secondary reactions, the formation of pyruvate implies protonation of enolpyruvate, which is expected to arise directly from OAA decarboxylation (OAA decarboxylase activity) or upon phosphoryl transfer from PEP to ADP (PK-like activity). Considering that the secondary activities of PEP carboxykinase are of no physiological relevance, it might be expected that the rate limiting step for these activities would be protonation of enolpyruvate. From this point of view, a decrease in the rate of the decarboxylation or phosphoryl transfer steps of PEP carboxykinase would affect the main reaction but not the secondary reactions necessarily, unless decarboxylation or phosphoryl transfer become rate limiting steps for the secondary activities.

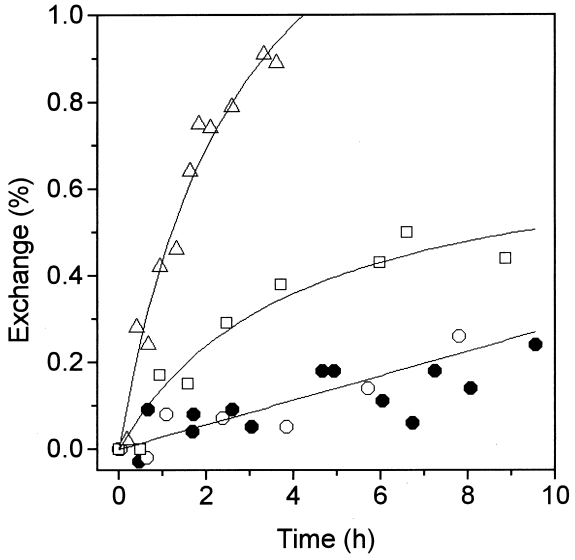

Fig. 3. Pyruvate enolization. The fractional exchange of the methyl protons of pyruvate in ^2H_2O was measured in 20 mM MOPS (pH 7.8) containing 0.1 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM ATP, and 31 mM pyruvate. Data are shown for a control in the absence of protein (●) and for solutions containing 0.12 mg rabbit muscle PK (△), 0.92 mg Arg336Lys PEP carboxykinase (□), or 0.96 mg wild type PEP carboxykinase (○). Further details in Section 2.

The catalytic role of Arg^{336} in *S. cerevisiae* PEP carboxykinase, although clearly important, is probably not as critical as the function performed by Lys^{256}. Our previous data indicate that alteration of Lys^{256} to Arg, Gln, or Ala, gives rise to enzymes with a four order of magnitude decrease in V_{max} for the main reaction and no measurable PK-like activity, suggesting that the function of Lys^{256} is critical for transition state stabilization in phosphoryl transfer [2].

3.3. Proton exchange

The kinetic parameters for the secondary reactions of the Arg336Lys PEP carboxykinase shown in Table 2 indicate that the only significant difference from those of the wild type enzyme is the increased V_{max} for the OAA decarboxylase activity, which is 10 fold higher after mutation. Considering the arguments presented in the above paragraph, we thought it possible that this increased activity might be due to an increased rate of protonation of enolpyruvate. Exchange of the methyl protons of pyruvate into solvent (pyruvate enolization) has been described in PK from several sources [15,16], and is considered to represent the proton transfer half-reaction of the enzyme reaction (i.e. protonation of the enolpyruvate intermediate). This reaction, however, is not catalyzed by chicken liver PEP carboxykinase [5]. Enzyme-catalyzed

Table 2
Kinetic parameters for the PK-like and OAA decarboxylase activities of wild type and mutant *S. cerevisiae* PEP carboxykinases[a]

Enzyme	K_m (μM)			V_{max} (μmol min^{-1} mg^{-1})	
	MnADP	PEP	OAA	PK-like	OAA decarboxylase
Wild type	151 ± 20	135 ± 28	140 ± 40	0.80 ± 0.01	0.27 ± 0.09
Arg336Lys	244 ± 30	170 ± 10	140 ± 20	0.71 ± 0.24	3.0 ± 0.3
Arg336Gln	80 ± 11	215 ± 39	46 ± 3	0.06 ± 0.01	0.025 ± 0.001

[a]Kinetic constants were determined as indicated in the text. Values given are the mean ± S.D.

proton exchange of the methyl protons of pyruvate in ^2H_2O was checked with the ^1H NMR assay described in Section 2 in the presence of wild type and Arg336Lys *S. cerevisiae* PEP carboxykinases, using rabbit muscle PK as a positive control (Fig. 3). The exchange rate for wild type PEP carboxykinase was indistinguishable from that for a non-enzymatic control, while the exchange rates for Arg336Lys PEP carboxykinase and PK were 0.076 μmol min^{-1} mg^{-1} and 2.4 μmol min^{-1} mg^{-1}, respectively. The absence of exchange catalysis by wild type *S. cerevisiae* PEP carboxykinase agrees with previous observations on the chicken liver enzyme [5], and the exchange rate for rabbit muscle PK is very similar to the value reported [16]. The isotopic exchange capacity developed upon Arg336Lys substitution in PEP carboxykinase suggests that a proton donor (the protonated ε-amino group of Lys^{336} or a different group) has appeared in the altered enzyme. This new catalytic activity offers further support to the proposal that enolpyruvate is an intermediate of the reactions catalyzed by PEP carboxykinases [5,17].

In conclusion, the work here described has shown that a positively charged amino acid residue at position 336 is essential for activity in *S. cerevisiae* PEP carboxykinase. The analysis of the Arg336Lys and Arg336Gln altered carboxykinases indicated different rate limiting steps for the principal reaction and the secondary activities of the enzyme. The development of the pyruvate enolization activity in Arg336Lys *S. cerevisiae* PEP carboxykinase supports the involvement of enolpyruvate as a reaction intermediate.

Acknowledgements: We thank Dr. Adrian Hagemann for his help in obtaining the ^1H NMR data. CD spectroscopy data were obtained at the University of Wisconsin-Madison Biophysical Instrumentation Facility (UW-Madison and BIR-9512577-NSF Grants). Access to the National Cancer Institute computational facilities were through the generous collaboration of Dr. Raúl Cachau. This work was supported by Grants FONDECYT 1000756 and DICYT-USACh 029941CU (to E.C.), and GM30480 from National Institute of General Medical Sciences (to P.A.F.).

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