The Strongly Conserved Lysine 256 of *Saccharomyces cerevisiae* Phosphoenolpyruvate Carboxykinase Is Essential for Phosphoryl Transfer[†]

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Received June 24, 1997; Revised Manuscript Received October 29, 1997

ABSTRACT: Lysine 256, a conserved amino acid of *Saccharomyces cerevisiae* phosphoenolpyruvate (PEP) carboxykinase located in the consensus kinase 1a sequence of the enzyme, was changed to alanine, arginine, or glutamine by site-directed mutagenesis. These substitutions did not result in gross changes in the protein structure, as indicated by circular dichroism, tryptophan fluorescence spectroscopy, and gel-exclusion chromatography. The three variant enzymes showed almost unaltered K_m for MnADP but about a 20 000-fold decrease in V_{max} for the PEP carboxylation reaction, as compared to wild-type PEP carboxykinase. The variant enzymes presented oxaloacetate decarboxylase activity at levels similar to those of the native protein; however, they lacked pyruvate kinase-like activity. The dissociation constant for the enzyme−MnATP complex was 1.3 ± 0.3 μM for wild-type *S. cerevisiae* PEP carboxykinase, and the corresponding values for the Lys256Arg, Lys256Gln, and Lys256Ala mutants were 2.0 ± 0.6 μM, 17 ± 2 μM, and 20 ± 6 μM, respectively. These results collectively show that a positively charged residue is required for proper binding of MnATP and that Lys[256] plays an essential role in transition state stabilization during phosphoryl transfer for *S. cerevisiae* PEP carboxykinase.

Phosphoenolpyruvate (PEP)[1] carboxykinases [ATP/GTP: oxaloacetate carboxylyase (transphosphorylating), EC 4.1.1.49/30] catalyze one of the first steps in the biosynthesis of carbohydrates from C_3 and C_4 precursors and, depending on the enzyme source, they require ATP or GTP as the phosphoryl donor (*1*). The reaction involves the decarboxylation and phosphorylation of oxaloacetate in the presence of a nucleoside triphosphate and a divalent metal ion to yield PEP, CO_2, and the corresponding nucleoside diphosphate:

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

In addition to this physiologically important reaction, the decarboxylation of OAA to pyruvate + CO_2 and a pyruvate kinase-like activity have been reported in *Saccharomyces cerevisiae* and other PEP carboxykinases (*2*−*4*). In all cases examined, the reaction catalyzed by PEP carboxykinases follows a sequential kinetic mechanism (*5*, *6*) and a direct transfer of the γ-phosphate of the nucleoside triphosphate to OAA has been proposed for the guinea pig liver mitochondrial and rat liver cytosolic enzymes (*8*, *9*).

The cDNAs of the ATP-dependent PEP carboxykinases from several microorganisms and plants have been cloned, and their amino acid sequences have been derived from the corresponding cDNA sequences (*10*, *11*). These aligned sequences all exhibit considerable amino acid identity. High sequence identity is also found among the similarly derived amino acid sequences of the GTP-dependent PEP carboxykinases (*12*, *13*); however, no significant homology between this group and the ATP-dependent carboxykinases can be found. The presence of consensus sequences for phosphoryl binding (*14*, *15*) is, however, consistently found in the primary structure of all PEP carboxykinases so far described. One of these sequences (Gly-X−X-X−X-Gly-Lys-Thr) is that designated kinase 1a by Traut (*16*). In the primary structure of the *S. cerevisiae* enzyme, the kinase 1a sequence corresponds to Gly[250]-Leu-Ser-Gly-Thr-Gly-Lys-Thr[257] (*17*). The function of the conserved Lys residue within the kinase 1a sequence has been investigated in several enzymes, and the general view is that it participates in nucleotide binding and/or phosphoryl transfer between the nucleotide and the accepting nucleophile (*14*, *18*).

According to the crystal and molecular structure of the *Escherichia coli* PEP carboxykinase—the only PEP carboxykinase for which a three-dimensional structure is available—the first five residues of the kinase 1a sequence (Gly[248]−Thr[252]) are within a loop formed between a β-strand and a short α-helix, and the remaining residues (Gly[253]-Lys-Thr[255]) are part of the α-helix (*10*). In that structure, the β-strand−

[†] This work was supported by Research Grants GM30480 from National Institute of General Medical Sciences, U.S. Public Health Service (P.A.F.), FONDECYT 1941073/1970670, and DICYT 029441CU (E.C.).

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[1] Abbreviations: CD, circular dichroism; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; OAA, oxaloacetate; PEP, phosphoenolpyruvate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(97)01515-8 CCC: $15.00
Published on Web 04/17/1998

loop−α-helix motif lines one face of the active-site cleft, resulting in the P-loop being partially buried. The structure of the *E. coli* enzyme complexed with ATP−Mg^{2+}−oxalate has indicated that ATP binding induces a slight contraction in the kinase 1a loop, wrapping loop residues tightly around the ATP triphosphate group. In particular, Lys^{254} inserts its positively charged ϵ-amino group between the β- and γ-phosphates of the bound nucleotide (*19*).

Several lines of evidence indicate important structural similarities between the *E. coli* enzyme and the four identical *S. cerevisiae* PEP carboxykinase subunits. The yeast and bacterial PEP carboxykinases share 45% sequence identity (*10*, *17*), and are both composed of about 20−24% α-helix (*20*). Limited proteolysis experiments suggested a domain structure for both enzymes (*21*, *22*), and this was recently verified by the X-ray diffraction data for the *E. coli* carboxykinase (*10*). By using pyridoxal 5′-phosphate, Bazaes et al. (*23*) have identified the conserved amino acid residues Lys^{288} and Lys^{290} in *E. coli* and *S. cerevisiae* PEP carboxykinases, respectively, as two residues of high chemical reactivity, the modification of which led to loss of the enzyme activity The observed protection of inactivation and labeling afforded by the substrates agree with the observation that Lys^{288} is located within about 10 Å of the active site of the *E. coli* enzyme (*10*) and suggest a similar location for the corresponding residue in the *S. cerevisiae* carboxykinase. Recently, Chávez et al. (*24*) have demonstrated that the replacement $Lys^{290} \rightarrow$ Gln within *S. cerevisiae* PEP carboxykinase generates a variant enzyme with slightly altered K_m and V_m values, as could be expected for a residue near to but not within the active site proper. Similarly, chemical modification by the histidine-specific reagent diethyl pyrocarbonate has shown that the modifier inactivates both enzymes by reaction with the conserved residues His^{271} and His^{273} of *E. coli* and *S. cerevisiae* PEP carboxykinases, respectively (*25*). All these data point to significant structural similarities between these two carboxykinases, and further suggest that Lys^{256} and Lys^{254} of the consensus kinase 1a sequences of *S. cerevisiae* and *E. coli* PEP carboxykinases, respectively, may be structurally and functionally similar or equivalent. Reactive lysyl residues have been identified in chicken liver PEP carboxykinase through chemical modification techniques; however, their functions are not known (*26*, *27*).

In this work we have prepared variants of *S. cerevisiae* PEP carboxykinases in which Lys^{256} has been replaced by Ala, Arg, or Gln. We report an analysis of the steady-state kinetics and nucleotide binding characteristics of the native and mutant enzymes with the goal of investigating the roles of Lys^{256} in substrate binding and catalysis.

MATERIALS AND METHODS

All materials and reagents were from sources previously cited (*17*). [8-^{14}C]ATP was from New England Nuclear. Specific polyclonal rabbit anti-*S. cerevisiae* PEP carboxykinase antibodies were obtained and purified by standard procedures (*28*). Nitrocellulose transfer membranes were from Sigma. Oligonucleotides were synthesized by BiosChile S. A.

Site-Directed Mutagenesis. Mutagenesis was carried out according to Künkel et al. (*29*), as previously described (*17*).

Leu Ser Gly Thr Lys Thr Thr Leu Ser Ala

5' GGA TAA AGT GGT **TTT** CCC GGT ACC ACT TAG 3'

3' CCT ATT TCA CCA AAA GGG CCA TGG TGA ATC 5'

Lys256Ala 5' GGA TAA AGT GGT **TGC** CCC GGT ACC ACT T 3'

Lys256Arg 5' TAA AGT GGT **TCT** CCC GGT ACC 3'

Lys256Gln 5' TAA AGT GGT **TTG** CCC GGT ACC 3'

FIGURE 1: Nucleotide sequence and encoded protein sequence of the *S. cerevisiae* PEP carboxykinase gene in the region of Lys^{256} (upper panel) and the synthetic oligonucleotides used for site-directed mutagenesis at position 256 (lower panel). The position 256 codons are in boldface type.

Uridine-rich ssDNA was isolated from M13mp19 PEP carboxykinase clone-transformed CJ236 cells (*dut*$^-$*ung*$^-$ genotype). The oligonucleotides used to anneal onto the template ssDNA and create the mutants are shown in Figure 1. The synthesized double-stranded DNA products were transformed into *E. coli* JM109, and the plaques of mutant cDNAs were identified by sequencing the appropriate region. Once mutants were identified (20−30% of all plaques), the entire sequence between sites *Sal*I and *Eco*RI (924 bases, of which 678 are in the coding region) was sequenced (*30*). After digestion with *Sal*I/*Eco*RI, the mutant cDNAs were subcloned into a similarly digested pMV7 and transformed into the PEP carboxykinase-deficient *S. cerevisiae* strain PUK-3B (MATα *pck ura3*) by the lithium acetate method (*31*, *32*).

Expression and Purification of the Recombinant Wild-Type and Mutant Enzymes. The recombinant wild-type and mutant genes were expressed in *S. cerevisiae* PUK-3B cells grown under selective conditions on agar plates lacking uracil to prevent plasmid loss and using glucose as the carbon source. Isolated colonies of all three mutants were unable to grow in liquid cultures using pyruvate as the carbon source, thus indicating inadequate in vivo PEP carboxykinase activity. Therefore, 50 mL precultures were transferred to 1 L of rich medium containing 2% glucose and grown with shaking for 48 h at 30 °C in 2 L flasks. The cells were collected by centrifugation under sterile conditions and the combined pellets of four flasks were suspended in 1 L of minimal medium supplemented with 2% ethanol and 20 μg mL^{-1} adenine and incubated with shaking 24 h at 30 °C. Under these conditions expression of the PEP carboxykinase gene took place, as revealed by Western-blot analyses of cell-free extracts (not shown). The cells were collected, washed with 20 mM phosphate buffer (pH 7.2) containing 0.5 mM EDTA, and stored at −30 °C until used. The cells were broken with glass beads and the variant enzymes were purified by protamine sulfate treatment, ion-exchange chromatography on DEAE-Sephacel, size-exclusion chromatography on Sepharose S-200, and affinity chromatography on AMP−Sepharose as described for the wild-type *S. cerevisiae* PEP carboxykinase (*33*). Because of lack of activity, the presence of the enzyme in column fractions was followed by dot-blot analyses as described below. A yield of about 15−20 mg of pure enzyme/100 g of wet cells was consistently obtained for the variant PEP carboxykinases, which is about 30% of the yield for the wild-type recombinant enzyme. The concentration of PEP carboxykinase was

determined spectrophotometrically using the extinction coefficients (280 nm) $\epsilon^{1\%} = 12.3$ cm^{-1} (*34*) and $\epsilon = 7.5 \times 10^4$ M^{-1} cm^{-1} (*35*) and a MW of 60 983 for the enzyme subunit (*17*).

SDS−Polyacrylamide Gel Electrophoresis, Western Blots, and Dot Blots. SDS−polyacrylamide gel electrophoresis was performed according to ref *36* on a minigel apparatus. Proteins were visualized by staining with Coomassie Brilliant Blue.

Column fractions containing PEP carboxykinase mutants were identified by dot blots because their activities were too low to be measured spectrophotometrically. Dot blots and Western blots (both performed on nitrocellulose transfer membranes) were analyzed with horseradish peroxidase-conjugated goat anti-rabbit IgG from Sigma Immunochemicals (1:5000 dilution). Dot blots were prepared by placing 2 μL of the collected fraction on an activated nitrocellulose transfer membrane. Excess protein binding sites were blocked for 20 min with 1% lowfat milk in PBS buffer [10 mM sodium phosphate (pH 7.4), 140 mM NaCl, and 3 mM KCl]. Both the rabbit anti-*S. cerevisiae* PEP carboxykinase primary antibody and the horseradish peroxidase-conjugated secondary antibody were incubated for 30 min followed by four 3 min washes of the membrane with PBS buffer. The staining solution was 11 mM H_2O_2 and 7 mM 4-chloro-1-naphthol.

Assays and Kinetic Studies. The standard assay of the enzyme in the PEP carboxylation direction was employed at 30 °C, with a 1 mL final reaction volume containing 100 mM imidazole buffer (pH 6.6), 0.24 mM NADH, 1.22 mM ADP, 2.5 mM PEP, 50 mM $NaHCO_3$, 2 mM $MnCl_2$, and 20 units of malate dehydrogenase, as described (*37*). One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of oxaloacetate min^{-1}. To obtain the kinetic parameters, enzyme activity was measured as the concentration of either substrate was varied in the range 0.006−0.303 mM (ADP) or 0.133−1.05 mM (PEP). Maximal velocity and the apparent K_m were determined by fitting the data to the Michaelis−Menten equation with the EZ-FIT program of Perella (*38*), which provides the standard deviation of the values. Because of the low activities of the variant PEP carboxykinases, 1 mg of protein/essay was used, which was more than 5000 times the amount employed for the essay of the wild-type enzyme.

The OAA decarboxylase activity was determined in a coupled assay with lactate dehydrogenase, which measured pyruvate formation. The assay mixture contained 100 mM Tris-HCl buffer (pH 7.8), 0.5 mM OAA, 0.24 mM NADH, 0.2 mM $MnCl_2$, and 20 units of 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. One unit of enzyme activity is defined as the amount of enzyme that decarboxylates 1 μmol of oxaloacetate min^{-1}. For the determination of K_m for OAA, the substrate was varied from $0.3K_m$ to $18K_m$ for the wild-type enzyme, from $0.1K_m$ to $3.3K_m$ for the Lys256Ala mutant, from $0.4K_m$ to $2.4K_m$ for the Lys256Gln mutant, and from $0.17K_m$ to $1.4K_m$ for the Lys256Arg mutant. The upper concentration of OAA employed was limited for the spontaneous metal-dependent decarboxylation of the substrate, to pyruvate + CO_2 (*7*), so that the maximum OAA concentration in these experiments was about 2.5 mM. In all cases this spontaneous decarboxylation rate was less than 40% of the rate of the enzymic reaction.

The pyruvate kinase-like activity of *S. cerevisiae* PEP carboxykinase was measured at 30 °C in 1 mL final volume containing 100 mM imidazole buffer (pH 6.6), 0.24 mM NADH, 2 mM $MnCl_2$, 1.25 mM ADP, 2.5 mM PEP, and 20 units of lactate dehydrogenase. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of pyruvate min^{-1}.

Equilibrium Dialysis Experiments. Equilibrium dialysis was performed using the microdialysis capsule kit provided by Spectrum Medical Industries. In the binding experiments, one compartment contained 120 μL of 50 mM Hepes buffer (pH 7.5) containing 3.8 μM wild-type, Lys256Arg, or Lys256Gln carboxykinase or 15.2 μM Lys256Ala *S. cerevisiae* PEP carboxykinase tetramer, plus 2 mM $MnCl_2$ and varying [8-^{14}C]ATP concentrations (specific radioactivity 1.1×10^4 dpm/nmol for the Lys256Ala variant or 2.2×10^4 dpm/nmol for the other enzymes) in the range 4−100 μM for wild-type, Lys256Arg, and Lys256Gln or 20−280 μM for the Lys256Ala carboxykinases. The other compartment contained 120 μL of Hepes buffer. The capsules were rotated at 6−8 rpm at 4 °C for 16 h, and the radioactivity was measured by scintillation counting in 40 μL aliquots withdrawn from each compartment. The enzyme retained about 75% activity after this treatment. The data are represented as Scatchard plots, and the equations used for curve-fitting were the two- or three-parameter models described by Rodbard and Feldman (*39*). The two-parameter equation (eq 2) considers the presence of one type of binding sites, while the three-parameters equation (eq 3) assumes the presence of two kinds of binding sites, one of them present with unlimited, nonsaturable binding capacity:

$$B/F = (n - B)/K_d \quad (2)$$

$$B/F = {}^1/_2[(n - B)/K_d - K + \{(B/K_d - n/K_d - K)^2 + 4KB/K_d\}^{1/2}] \quad (3)$$

in which B is the number of moles of ligand bound per mole of enzyme tetramer, F is the concentration of free ligand, n is the number of saturable binding sites per enzyme molecule, K_d is the dissociation constant for the saturable binding sites, and K is a term related to the unlimited, nonsaturable binding sites.

Molecular Weight Determination. The molecular weights of the wild-type and mutant enzymes (2 mg/mL) were determined in a Merck-Hitachi HPLC system using a Superose-12 column eluted with 50 mM sodium phosphate (pH 7.4) containing 150 mM NaCl at a flow rate of 0.6 mL min^{-1}. The molecular mass markers used, obtained from Sigma, were rabbit muscle lactate dehydrogenase (144 kDa), aldolase (160 kDa), sweet potato β-amylase (200 kDa), rabbit muscle pyruvate kinase (234 kDa), and bovine thyroglobulin (669 kDa).

Circular Dichroism and Fluorescence Spectroscopies. CD spectra were recorded in a Jasco J-720 spectropolarimeter in 10 mM sodium phosphate buffer (pH 7.0) at 25 °C using 0.10 cm cells, as described previously (*20*). Fluorescence spectra of the native and mutant PEP carboxykinases were obtained in 50 mM Hepes buffer (pH 7.5) at 21 °C in a Spex

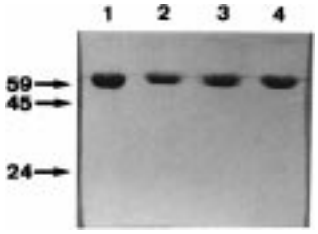

FIGURE 2: SDS−polyacrylamide gel electrophoresis of wild-type and mutant *S. cerevisiae* PEP carboxykinases. Lane 1, wild type; lane 2, K256A; lane 3, K256R, lane 4, K256Q. The migration of MW standards from a separate gel is indicated. Approximately 6 μg of protein was loaded in each lane.

1681 Spectrolog fluorometer with 1.25 nm slits for excitation and emission. The protein concentration of all samples was 0.10 mg/mL.

RESULTS

Expression, Purification, and Structural Characterization of Lys^{256} Mutant Enzymes. Expression of the variant PEP carboxykinases was confirmed by resolving the crude extract proteins with SDS−polyacrylamide gel electrophoresis. *S. cerevisiae* PEP carboxykinase was identified by immunoblotting with antibody prepared against the wild-type enzyme (not shown). All the variant enzymes were expressed at a level similar to the recombinant wild-type enzyme, based on the results of immunoblotting, and the apparent size of the enzyme subunit of the variants was the same as that of the wild-type protein. The variant enzymes were purified to greater than 95% homogeneity according to SDS−polyacrylamide gel electrophoresis, as shown in Figure 2. Throughout the purification process, the three mutant proteins behaved similarly to wild-type PEP carboxykinase.

The apparent sizes of the variant enzymes were the same as that of the wild-type enzyme, as determined by gel exclusion in a calibrated ($R = 0.995$) Superose-12 column (results not shown). In this column, the calculated molecular weight of wild-type PEP carboxykinase was 267×10^3, and the molecular weight of the mutated enzymes ranged from 259×10^3 to 267×10^3. These values agree well with the expected molecular weight of 244×10^3 for the enzyme tetramer (*17*) and are in the range of those determined by other authors for this same protein (*40*, *41*).

To examine whether the mutations at position 256 disrupted the enzyme's secondary structure, the far-UV circular dichroism spectra of Lys^{256} mutants were compared to the wild-type spectrum. Wild-type *S. cerevisiae* PEP carboxykinase has 20% α-helix (*20*), and the CD spectrum of the wild-type enzyme exhibits a negative peak at 208 nm and a negative shoulder at 222 nm. The CD spectra of all three *S. cerevisiae* PEP carboxykinases mutants are very similar to that of the wild-type enzyme (Figure 3), thus indicating that the secondary structure was not greatly altered by the mutations at residue 256. The intrinsic fluorescence spectra ($\lambda_{exc} = 287$ nm) of all mutants were superimposable with that of the native enzyme, with an emission maximum at 334 nm (not shown), indicating similar tryptophan microenvironments within the protein structures.

Kinetic Characterization of Lys^{256} Mutant Enzymes. The recombinant wild-type enzyme elicited a specific activity of 55 μmol min^{-1} mg^{-1} in the standard assay in the PEP carboxylation direction (eq 1), similar to that previously reported (*17*). Under normal assay conditions, mutated enzymes appeared inactive. All variants were concentrated to 4−8 mg mL^{-1} and then an amount of enzyme over 5000 times higher than that usually used for the standard assay was added to the assay mixture in order to increase the sensitivity of the test. In this way, we were able to measure enzyme activity and determined the apparent kinetic parameters shown in Table 1. From the data in Table 1 it is clear that all mutant enzymes show unaltered K_m values for MnADP, whereas V_{max} values are lowered about 20 000 times below the value for the wild-type enzyme.

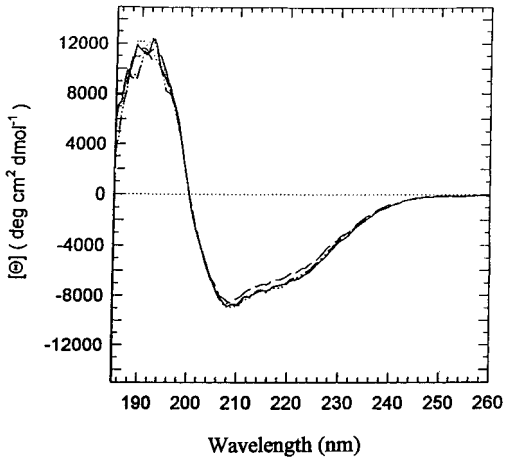

FIGURE 3: Far-UV circular dichroism spectra of wild-type and mutant *S. cerevisiae* PEP carboxykinases. Spectra were run five times and data were averaged for the native (—), Lys256Arg (- - -), Lys256Ala (···), and Lys256Gln(- ·· -) PEP carboxykinases. Protein concentration was 0.18 mg/mL. All other conditions were as described in Materials and Methods.

Table 1: Kinetic Parameters for Wild-Type, Lys256Ala, Lys256Arg, and Lys256Gln *S. cerevisiae* PEP Carboxykinases in the PEP Carboxylation Reaction[a]

	apparent K_m (μM)		
enzyme	MnADP	PEP	V_{max} (μmol min^{-1} mg^{-1})
native	67 ± 10	244 ± 51	72 ± 6
Lys256Ala	35 ± 7	157 ± 72	0.0032 ± 0.0004
Lys256Arg	31 ± 7	157 ± 103	0.0052 ± 0.0018
Lys256Gln	21 ± 4	304 ± 102	0.0031 ± 0.0002

[a] Kinetic constants were determined as indicated in Materials and Methods. Values given are the mean ± SD. The values given for the native enzyme are from ref *17*.

The proposed reaction mechanism for phosphoenolpyruvate carboxykinases involves the transient formation of enolpyruvate and its phosphorylation to PEP (*4*, *12*, *42*). According to this mechanism, it is conceivable that Lys^{256} could play a role in the decarboxylation reaction, in the phosphorylation reaction, or in both. As a way to test the effect of the Lys^{256} substitution in the decarboxylation reaction, the OAA decarboxylase activity (OAA → pyruvate + CO_2) of *S. cerevisiae* PEP carboxykinase was determined for both the wild-type and variant enzymes. A previous paper had reported the absence of OAA decarboxylating activity of *S. cerevisiae* PEP carboxykinase (*7*); however, we have now found that this activity is present in the wild-type enzyme, although at a low level. The low OAA decarboxylase activity precluded its detection under the assay conditions previously employed. A comparison of the kinetic

Table 2: Kinetic Constants for the OAA Decarboxylase Activity of Wild-Type and Mutant *S. cerevisiae* PEP Carboxykinases[a]

enzyme	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})
native	0.12 ± 0.02	0.26 ± 0.02
Lys256Ala	0.63 ± 0.08	0.82 ± 0.04
Lys256Arg	1.89 ± 0.20	0.06 ± 0.01
Lys256Gln	1.43 ± 0.35	0.11 ± 0.01

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

parameters of this reaction for the wild-type and variant PEP carboxykinases is shown in Table 2. It is seen that all mutated enzymes show increased K_m values, while V_{max} is 3-fold increased in the Lys256Ala carboxykinase and 2.4- and 4-fold decreased in the Lys256Gln and Lys256Arg mutants, respectively.

The relevance of Lys^{256} for the pyruvate kinase-like reaction 2 of wild-type and mutated *S. cerevisiae* PEP carboxykinase was also addressed in this work. Under the assay conditions described in Experimental Procedures, the wild-type enzyme displayed a specific activity of 0.68 μmol min^{-1} mg^{-1} for this reaction.[2] However, this activity could not be detected in the Lys256Ala, Lys256Gln, and Lys256 Argcarboxykinases, even when assayed at concentrations of 2.4, 2.5, and 6.0 mg mL^{-1}, respectively. These concentrations are 53−130-fold higher than that employed for the wild-type enzyme in this assay.

ATP Binding by Native and Mutant S. cerevisiae PEP Carboxykinases. The crystal structure of the ATP−Mg^{2+}−oxalate complex bound to the homologous *E. coli* PEP carboxykinase places the ϵ-amino group of the equivalent Lys^{254} residue in a position between the β- and γ-phosphates of ATP, establishing hydrogen bonds with nonbridge oxygen atoms of the corresponding phosphate groups (*19*). Hence, the possibility of a role for Lys^{256} of *S. cerevisiae* PEP carboxykinase in MnATP binding was specifically addressed. Determination of the K_m for MnATP or other kinetic parameters in the OAA to PEP direction was not possible because of the very low activities of the mutated enzymes, and also because the spontaneous decarboxylation of OAA to pyruvate interfered with the coupled assay employed. Under our assay conditions, this nonenzymic Mn^{2+}-catalyzed decarboxylation rate of OAA to pyruvate has been calculated to be 0.21 s^{-1} (*7*). Consequently, equilibrium binding experiments were performed to measure [8-^{14}C]ATP binding in the presence of Mn^{2+} to native and mutated PEP carboxykinases. Considering that under the conditions employed most ATP exists as MnATP (*43*) and that the true substrate for the enzyme has been shown to be the MnATP complex (*44*), we assume that the equilibrium binding experiments here described represent a measure of the binding of this metal complex to the enzyme. Some results are shown in Figure 4 in the form of a Scatchard plot, where it is clearly seen that MnATP binding is severely diminished

[2] This pyruvate kinase-like activity of *S. cerevisiae* PEP carboxykinase is not due to a trace of contaminating pyruvate kinase in the enzyme preparation. Additional experiments indicate that the pyruvate kinase-like activity shown by the carboxykinase differs from *S. cerevisiae* pyruvate kinase both in kinetics for substrate saturation and response to fructose 1,6-bisphosphate activation (A. M. Jabalquinto, M. Laivenieks, J. G. Zeikus, and E. Cardemil, manuscript in preparation).

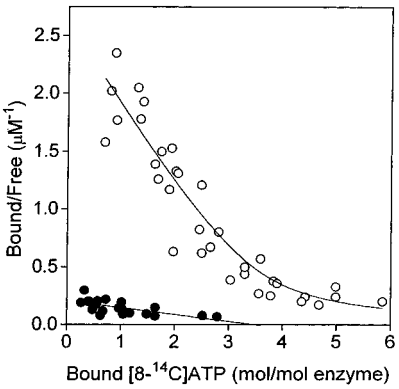

FIGURE 4: Scatchard plot for [8-^{14}C]ATP binding to wild-type and Lys256Gln mutant *S. cerevisiae* PEP carboxykinases. Binding was measured at 4 °C in 50 mM Hepes buffer (pH 7.5) using microdialysis capsules as described under Materials and Methods. The solid line is fit to eq 3 for the wild-type enzyme (○) or to eq 2 for the Lys256Gln mutant (●).

Table 3: Dissociation Constants for MnATP Binding to Wild-Type and Lys^{256} Mutant *S. cerevisiae* PEP Carboxykinases[a]

enzyme	K_d (μM)	*n*
native	1.3 ± 0.3	3.5 ± 0.5
Lys256Arg	2.0 ± 0.6	3.7 ± 0.6
Lys256Gln	17 ± 4	3.6 ± 0.7
Lys256Ala	20 ± 6	3.3 ± 0.5

[a] Dissociation constants and number of saturable binding sites were determined as shown in Figure 4 using eq 2 (mutant enzymes) or 3 (wild type). Values given are the mean ± SD.

in the Lys256Gln carboxykinase. For the wild-type enzyme, the Scatchard plot of the data was clearly biphasic, suggesting that in *S. cerevisiae* PEP carboxykinase MnATP binds to two different sites. The experimental data were best fitted by a model that considers a saturable binding site and a second, nonsaturable binding site (*39*). The best fit of the data to eq 3 (wild-type) or 2 (mutated enzymes) provided the PEP carboxykinase−MnATP dissociation constants indicated in Table 3, showing that the Lys256Arg carboxykinase binds MnATP with an affinity similar to that for the wild-type enzyme. However, 13- and 15-fold decreases in binding affinity for MnATP were detected for the Lys256Gln and Lys256Ala carboxykinases, respectively. The nature of the unsaturable binding site seen in wild-type *S. cerevisiae* PEP carboxykinase has not been further investigated in this work.

The dissociation constant for MnATP from the wild-type enzyme reported in Table 3 in Hepes buffer (pH 7.5) is an order of magnitude lower than the value of K_m for MnATP in Tris-HCl buffer (pH 7.8) at 25 °C (*7*). The difference could be due to the intrinsic difference between K_d and K_m or to the use of different buffers. Inasmuch as the kinetic mechanism of *S. cerevisiae* is random sequential (*7*), it is possible that the affinity of the enzyme−OAA complex for MnATP is different from that of the free enzyme. The total number of saturable binding sites for MnATP (3.5 ± 0.5 for the wild-type enzyme) is the same within experimental error as the number of subunits. Additional experiments (not shown) indicated that this number decreased to 2.5 in enzyme samples stored at −30 °C in 50 mM Hepes buffer (pH 7.5) for several months and where the enzyme had lost 30−35% of its original specific activity.

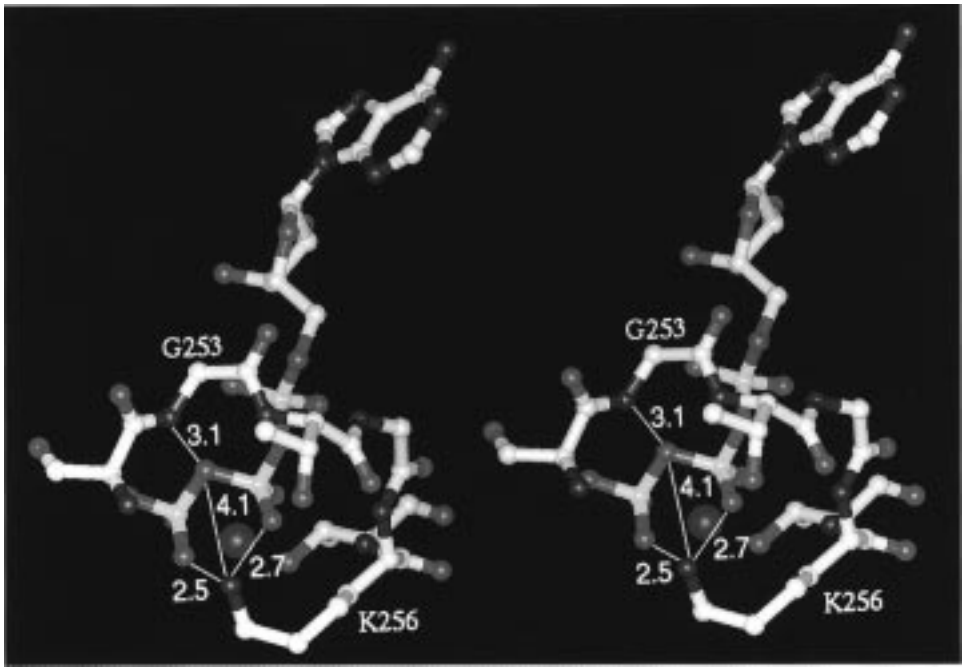

FIGURE 5: Model of the complex between *S. cerevisiae* PEP carboxykinase and MnATP, showing the distances between the ϵ-amino group of Lys^{256} and selected P_β- and P_γ-linked oxygens. The model is based on the published atomic coordinates for the ATP−Mg^{2+}−oxalate complex of the *E. coli* PEP carboxykinase (19). After coordinate transferance, Discover 950/MSI (*55*) was employed for energy minimization. Minimization was done for the whole protein molecule (rms = 0.828). Only enzyme groups of the kinase 1a sequence are shown. The distances between heteroatoms (red, oxygen; yellow, phosphorus; gray, carbon; blue, nitrogen) are shown in angstroms. The manganese ion is shown in green.

DISCUSSION

In this work we have prepared mutated *S. cerevisiae* PEP carboxykinases in which Lys^{256} was replaced by Ala, Arg, or Gln. The mutated enzymes behaved similarly to the recombinant wild-type enzyme throughout the purification procedures. In particular, their similar behavior in the AMP−Sepharose affinity chromatography step indicated that essential features of the nucleotide-binding site remained unaltered. Moreover, the mutations introduced did not alter the quaternary structures of the proteins, and the CD and fluorescence spectroscopy analyses showed no evidence of gross conformational changes. Hence, we conclude that replacement of Lys^{256} by Ala, Arg, or Gln in *S. cerevisiae* PEP carboxykinase does not perturb the general structure of the protein.

The 20 000-fold decrease in V_{max} for the PEP carboxylation reaction detected in the mutated enzymes clearly points to an essential catalytic role for the ϵ-amino group of Lys^{256}, a role that cannot be replaced by the positively charged guanidinio group of an arginyl residue. The catalytic mechanism of PEP carboxykinase is believed to involve the decarboxylation of OAA to enolpyruvate and the phosphorylation of this transient intermediate to PEP (*4*, *12*, *42*). This proposal is supported by the observations that several PEP carboxykinases display OAA decarboxylase (OAA → pyruvate + CO_2) and pyruvate kinase-like activities (*2*−*4*). The relatively minor changes in K_m for OAA and V_{max} for the mutated enzymes clearly indicate that Lys^{256} does not perform an essential function for the OAA decarboxylase activity of *S. cerevisiae* PEP carboxykinase. It is obvious, though, that changing Lys^{256} to Arg, Ala, or Gln led to active-site modifications that affect this activity, a situation similar to that reported for an Asp268Asn variant of *E. coli* PEP carboxykinase, which has increased OAA decarboxylase activity (*45*). Taking these results together with the absence of pyruvate kinase-like activity and substantial alteration of the normal PEP carboxykinase activity of the Lys^{256} mutants, we conclude that Lys^{256} plays an essential function in phosphoryl transfer in *S. cerevisiae* PEP carboxykinase.

A possible role of Lys^{256} in catalysis can be derived from a consideration of the placement of Lys^{256} with respect to the β- and γ-phosphates of MnATP (Figure 5). The structure of the E−MnATP complex places Lys^{256} at 2.7 and 2.5 Å from nonbridging oxygens of the β- and γ-phosphates, respectively. These interactions might help catalysis, facilitating the nucleophilic attack of the reactive enolate intermediate (*4*, *12*, *42*). Similar functions for active-site lysines in enzymes catalyzing phosphoryl transfer reactions have been proposed before (*46*−*48*). In the model shown in Figure 5, the backbone amide of Gly^{253} is positioned to donate a hydrogen bond to the $\beta-\gamma$ bridging oxygen of MnATP (Figure 5). The N(H)···O heteroatom distance (3.1 Å) and N−H···O angle (151°) are similar to the values observed for hydrogen bonds donated by backbone amides in peptides and proteins of 2.9−3.1 Å and 150−160 ° (*49*). In the phosphoryl group transfer, negative charge accumulates on the bridging oxygen in the transition state for P−O cleavage (*50*−*52*). If the main-chain N−H of Gly^{253} remains hydrogen-bonded to the bridging oxygen, it will stabilize the transition state for P−O bond cleavage in phosphoryl transfer. In this respect, it is of relevance that Gly^{253} is conserved in its position in the kinase 1a sequence GXX***X***XGKT in all PEP carboxykinases so far described (*10*−*13*).

Table 3 shows that the affinity for MnATP was reduced by 1.6−1.7 kcal/mol in the Lys256Gln and Lys256Ala

mutants, thus pointing to a direct interaction of Lys^{256} with MnATP. These results are consistent with the observed role of comparable lysines in other mononucleotide-binding proteins. For example, Seefeldt et al. (*53*) have shown that altering Lys^{15} in the nucleotide binding site of *Azotobacter vinelandii* nitrogenase did not alter its ability to bind ADP, whereas the affinity for ATP was reduced, leading the authors to propose that this lysyl residue interacted with the γ-phosphate of ATP. A similar result was reported for the F_1-ATPase from *E. coli* when its nucleotide binding site lysine was replaced with glutamate or glutamine (*54*). With respect to the *S. cerevisiae* PEP carboxykinase, the fact that the Lys256Arg mutant shows an affinity for MnATP comparable to that of the native enzyme indicate that electrostatic interactions play an important role in binding of this nucleotide.

In conclusion, the replacement of Lys^{256} of *S. cerevisiae* PEP carboxykinase by Ala, Arg, or Gln allowed us to show that this lysyl residue is catalyticaly essential for phosphoryl transfer between MnATP and OAA. The fact that K_d for the enzyme−MnATP complex is little affected in the Lys256Arg mutant but increases at least by 1 order of magnitude in the Lys256Gln and Lys256Ala mutants indicate that a positive charge is a major requirement for nucleotide triphosphate binding. The substantial OAA decarboxylase activity of the mutant enzymes indicates that Lys^{256} is not required for this reaction, in agreement with the view (*4*) that decarboxylation and phosphorylation are separate steps of the catalytic reaction mechanism of PEP carboxykinases.

ACKNOWLEDGMENT

We thank Dr. José Manuel Andreu and Mr. Juan Evangelio (Instituto de Investigaciones Biológicas, CSIC, Spain) for kindly performing the CD spectroscopy and Dr. Leonor Contreras (Universidad de Santiago de Chile) for providing advice and allowing us to use computer facilities of her laboratory. We also thank Mr. Renato Chávez for help in the gel-exclusion analyses and Mr. Claudio Gómez for preparing the rabbit anti *S. cerevisiae* PEP carboxykinase antibodies. Valuable discusions with Dr. María Victoria Encinas are gratefully acknowledged.

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BI971515E