Lysine 213 and Histidine 233 Participate in Mn(II) Binding and Catalysis in *Saccharomyces cerevisiae* Phosphoenolpyruvate Carboxykinase[†]

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ABSTRACT: *Saccharomyces cerevisiae* phosphoenolpyruvate (PEP) carboxykinase catalyses the reversible metal-dependent formation of oxaloacetate and ATP from PEP, ADP, and CO_2 and plays a key role in gluconeogenesis. This enzyme also has oxaloacetate decarboxylase and pyruvate kinase-like activities. Mutations of PEP carboxykinase have been constructed where the residues Lys^{213} and His^{233}, two residues of the putative Mn^{2+} binding site of the enzyme, were altered. Replacement of these residues by Arg and by Gln, respectively, generated enzymes with 1.9 and 2.8 kcal/mol lower Mn^{2+} binding affinity. Lower PEP binding affinity was inferred for the mutated enzymes from the protection effect of PEP against urea denaturation. Kinetic studies of the altered enzymes show at least a 5000-fold reduction in V_{max} for the primary reaction relative to that for the wild-type enzyme. V_{max} values for the oxaloacetate decarboxylase and pyruvate kinase-like activities of PEP carboxykinase were affected to a much lesser extent in the mutated enzymes. The mutated enzymes show a decreased steady-state affinity for Mn^{2+} and PEP. The results are consistent with Lys^{213} and His^{233} being at the Mn^{2+} binding site of *S. cerevisiae* PEP carboxykinase and the Mn^{2+} affecting the PEP interaction. The different effects of mutations in V_{max} for the main reaction and the secondary activities suggest different rate-limiting steps for these reactions.

Phospho*enol*pyruvate (PEP)[1] carboxykinases (GTP/ATP; oxaloacetate carboxylyase (transphosphorylating), EC 4.1.1.32/49) catalyze the nucleoside triphosphate-dependent reversible decarboxylation of oxaloacetate to yield PEP, CO_2, and the corresponding nucleoside diphosphate. This enzyme is found in all groups of organisms (*1*). ATP-dependent enzymes are monomers or oligomers of identical subunits found in microorganisms and plants, while GTP-dependent carboxykinases are only monomers and are found in animals and in certain bacteria (*2*−*4*). The ATP- and GTP-dependent PEP carboxykinases share no primary structure identity to each other (*4*, *5*). In addition to the physiologically important reaction, the decarboxylation of OAA to pyruvate and CO_2 (OAA decarboxylase activity) and the phosphorylation of the nucleoside diphosphate by PEP to yield the corresponding nucleoside triphosphate and pyruvate (pyruvate kinase-like activity) have been described for some PEP carboxykinases (*6*−*8*). *Saccharomyces cerevisiae* decarboxylase is one such enzyme that has been described (*6*). It is assumed that the pyruvate kinase-like and the OAA decarboxylase reactions reflect the phosphoryl transfer and decarboxylation steps of the main reaction, respectively, linked through the formation of enolpyruvate, a putative reaction intermediate (*4*, *8*), as depicted in Scheme 1. Catalysis of exchange of the methyl protons of pyruvate into solvent by *S. cerevisiae* PEP carboxykinase R336K supports the involvement of enolpyruvate as a reaction intermediate (*9*). Evidence for an essential role of Lys^{256} of *S. cerevisiae* PEP carboxykinase in the phosphoryl transfer step has been provided (*10*). Data of Hou et al. (*11*) indicate that the Asp268Asn mutation in *Escherichia coli* PEP carboxykinase suppresses the PEP carboxylating activity while it increases the OAA decarboxylase activity, as measured in cell-free extracts. These data suggest that these different activities can be decoupled.

PEP carboxykinases have an absolute requirement for divalent cations for activity. Mixed-metal studies showed a dual role for cations in these enzymes (*12*, *13*). One cation, preferentially a transition metal, interacts with the enzyme at metal binding site 1 to elicit activation, while the second cation, at metal binding site 2, interacts with the nucleotide to serve as the metal-nucleotide substrate. *E. coli* and *Trypanosoma cruzi* PEP carboxykinases are the only ATP-utilizing PEP carboxykinase for which X-ray diffraction data are available (*14*, *15*). The X-ray crystal structure of the *E. coli* enzyme at 1.9 Å resolution shows that the polypeptide consists of a 275 residue N-terminal domain and a more compact 265-residue C-terminal, with the active site located

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[1] Abbreviations: CD, circular dichroism; EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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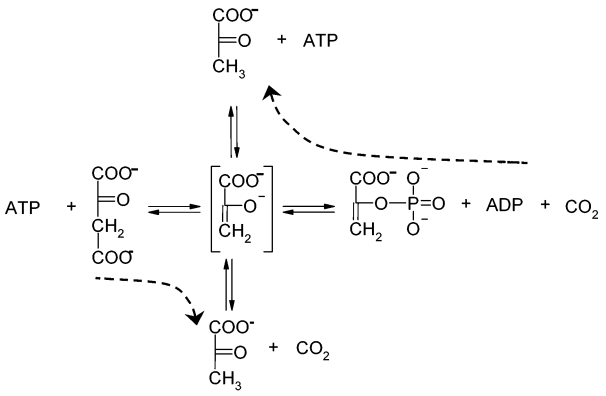
Scheme 1: Phosphoenolpyruvate Carboxykinase Catalytic Activities[a]



[a] The possible involvement of enolpyruvate as an intermediate in the primary reaction (horizontal lane), the pyruvate kinase-like activity (upper dashed line), and the oxaloacetate decarboxylase activity (lower dashed line) of PEP carboxykinase is represented

within a cleft between the two domains. Upon binding ATP−Mg^{2+}−oxalate, the *E. coli* enzyme undergoes a domain closure through a 20° rotation of the N- and the C-terminal domains toward each other (*14*). Two divalent metal cation-binding sites are associated with the active site with a 5.2 Å separation between the two ions. The metal ions are coordinated in an octahedral geometry at both sites. Mn^{2+}, at site 1, is ligated by one imidazole (the $N_{\epsilon-2}$ atom of His^{232}), one carboxylate (Asp^{269}), two water molecules, an oxygen from P_γ of ATP, and N_ϵ from Lys^{213}. Mg^{2+}, at site 2, is coordinated with three water molecules, two oxygen atoms from the β- and γ- phosphoryl groups of ATP, and O_γ of Thr^{255}. Two of the water molecules coordinating Mg^{2+} are also hydrogen bonded to the carboxylate groups of Asp^{268} and Asp^{269} (*14*). All residues found to coordinate Mg^{2+} and Mn^{2+} in various ATP- and GTP-dependent PEP carboxykinases are highly conserved based on sequence alignments (*4*). Nuclear Overhauser effect (NOE) experiments with the mitochondrial chicken liver PEP carboxykinase suggest an anti conformation for bound GTP (*16*), in contrast to the syn conformation detected in the crystal structure of ATP complexes of *E. coli* PEP carboxykinase (*14*). The NOE data are in agreement with the recently published crystal structure of the β-γ-methylene GTP complex of the human carboxykinase (*17*) that also shows that the residues that coordinate the Mg^{2+} and Mn^{2+} cations are identical to those observed for the *E. coli* enzyme (*14*). Tetrameric *S. cerevisiae* PEP carboxykinase is 48% identical to the monomeric *E. coli* enzyme, suggesting important structural similarities between the two carboxykinases.

In this work we evaluate the roles of Lys^{213} and His^{233} in the *S. cerevisiae* enzyme in Mn^{2+} binding and in catalysis. These residues are equivalent to Lys^{213} and His^{232} in *E. coli* PEP carboxykinase, where they are part of metal binding site 1. The analysis of Lys213Arg and His233Gln mutants of *S. cerevisiae* PEP carboxykinase indicates that Lys^{213} and His^{233} are required for proper Mn^{2+} binding and their mutation critically affects catalysis. The mutation of Lys^{213} or His^{233} does not critically affect catalysis of the secondary reactions of the enzyme.

MATERIALS AND METHODS

Materials. Malate dehydrogenase, pyruvate kinase, lactate dehydrogenase, ADP, NADH, PEP, $MnCl_2$, Chelex-100, and MOPS were purchased from Sigma Chemical Co. BioGel P-6 was from Bio-Rad. All other reagents were of the highest purity commercially available. All nonmetal solutions were passed through a Chelex-100 column to remove contaminating metal ions.

Site-Directed Mutagenesis and Enzyme Purification. Specific substitutions were introduced at the histidine and lysine triplets 233 and 213, respectively, in the cloned *S. cerevisiae* PEP carboxykinase gene (pMV7 plasmid) by the method of Künkel (*18*) for His233Gln or by using recombinant PCR for Lys213Arg (*9*). To obtain the His233Gln substitution, synthetic oligonucleotide 5′-GCGGAAGAGT**T**CAAAGTTA-3′ (base substitution is shown in bold and underlined) was used, and all procedures were as previously described (*10*, *18*). To obtain the Lys213Arg substitution, 10 ng of template DNA was amplified with 10 pmol of the mutagenic oligonucleotide, oligo M1: 5′-CTGTGAAAATACCT**C**TTTTCATT-TCACCGG-3′ (base substitution is shown in bold and underlined) and 10 pmol of the nonmutagenic oligonucleotide, oligo A: 5′-CCGGAAGATCTCCAAAGGAC-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 nonmutagenic oligonucleotide B: 5′-GTTCAGGTTCAGTGACAC-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 nonmutagenic primers described above. The purified product was digested with restriction enzymes *Bam*HI and *Sal*I, 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 (*10*, *18*). 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, Lys213Arg, and His233Gln PEP carboxykinases were purified as described (*10*), except that the last step in AMP−Sepharose was omitted. Pooled enzyme fractions from the Sephacryl S-200 chromatographic step were concentrated and exchanged into 50 mM MOPS buffer (pH 7.0) by passage through a Bio Gel P10 column or by repeated concentration and dilution in an Amicon ultrafiltration unit. The enzyme that was 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 having a 2-cm layer of Chelex-100 on the top. Without this preincubation, a tight binding, contaminating cation is not totally removed to form apoenzyme. The column was preequilibrated with 50 mM MOPS buffer (pH 7.0). PEP carboxykinase concentration was determined spectrophotometrically at 280 nm using the extinction coefficients $\epsilon^{1\%} = 12.3\ cm^{-1}$ and $\epsilon = 7.5 \times 10^4\ M^{-1}cm^{-1}$ and a MW of 60 983 for the enzyme subunit (*18*). Through this work, all enzyme concentrations are expressed in terms of the enzyme subunit and not tetrameric protein.

HPLC Analysis of Wild-Type and Mutant PEP Carboxykinases. The molecular masses of wild-type and mutant

enzymes (0.8 mg/mL) were estimated in a Merck-Hitachi HPLC system using a Superose-12 column eluted with 50 mM HEPES buffer (pH 7.5) containing 100 mM KCl at a flow rate of 0.2 mL/min. The molecular markers were aldolase (166 kDa), sweet potato β-amylase (200 kDa), rabbit muscle pyruvate kinase (237 kDa), apoferritin (443 kDa), bovine thyroglobulin (669 kDa), and *E. coli* PEP carboxykinase (61 kDa). All molecular markers were obtained from Sigma except *E. coli* PEP carboxykinase, which was a gift from Dr. Hughes Goldie.

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 initial velocities to the Michaelis−Menten equation with the Microcal Origin program:

$$v = V_{max}[S]/(K_m + [S]) \quad (1)$$

The standard assay in the PEP carboxylation direction was in 100 mM MOPS buffer (pH 6.6), containing 0.20 mM NADH, 1.25 mM ADP, 50 mM $KHCO_3$, 2.5 mM PEP, 2 mM $MnCl_2$, and 4 units of malate dehydrogenase in 1 mL, essentially as described before (*19*). The final pH of the assay medium was 6.9. To obtain the kinetic parameters, enzyme activity was measured as a function of concentration of PEP, ADP, or bicarbonate while keeping the concentration of the other substrates at fixed, standard saturating levels. $KHCO_3$ was kept at 50 mM since higher concentrations caused alterations in the pH of the assay medium. For the wild-type enzyme, substrates were varied in the range 0.08−0.36 mM (ADP), 0.09−1.35 mM (PEP), 5−50 mM (HCO_3^-), and 0.01−1.45 mM (Mn^{2+}). For the Lys213Arg mutant, substrates were varied in the range 0.08−0.36 mM (ADP), 0.35−4.5 mM (PEP), 5−50 mM (HCO_3^-), and 0.2−8.20 mM (Mn^{2+}), while the fixed concentrations of PEP and Mn^{2+} were 8 and 15 mM, respectively. For the His233Gln mutant, substrates were varied in the range 0.005−0.10 mM (ADP), 0.1−3 mM (PEP), 5−50 mM (HCO_3^-), and 0.5−15 mM (Mn^{2+}), while fixed concentrations of ADP, PEP, and Mn^{2+} were 1.25, 2.5, and 10 mM, respectively. Because of the low activity of mutant PEP carboxykinases, 0.3 mg of protein/assay was used, more than 500 times the amount employed for the assay of the wild-type enzyme. The values for free Mn^{2+} concentration were calculated with the GEOCHEM-PC V.2.0 program (*20*), using K_d values of 8.1×10^{-5} M for the $MnADP^-$ complex and 1.8×10^{-3} M for the $MnPEP^-$ complex (*21*) and taking into account the total concentrations of each species in the assay.

The standard assay in the PK-like activity was in 100 mM MOPS buffer (pH 6.6), containing 0.20 mM NADH, 1.25 mM ADP, 2.5 mM PEP, 2 mM $MnCl_2$, and 4 units of lactate dehydrogenase in 1 mL. Enzyme activity was measured as a function of substrate concentration with the concentration of the second substrate at fixed, saturating levels. For the wild-type enzyme, substrates were varied in the range 0.04−0.36 mM (ADP) and 0.09−1.35 mM (PEP) while saturating concentrations of PEP, ADP, and Mn^{2+} were 2.5, 1.25, and 2 mM, respectively. For the Lys213Arg mutant, substrates were varied in the range 0.08−0.36 (ADP) and 0.35−4.5 mM (PEP), while standard concentrations of PEP, ADP, and Mn^{2+} were 8, 1.25, and 10 mM, respectively. For the His233Gln mutant, substrates were varied in the range 0.010−0.15 (ADP) and 0.1−1.8 mM (PEP), while saturating concentrations of PEP, ADP, and Mn^{2+} were 8, 1.25, and 10 mM, respectively.

The OAA decarboxylase activity of PEP carboxykinase was determined in 100 mM MOPS buffer (pH 7.8), containing 0.5 mM OAA, 0.20 mM $MnCl_2$, 0.20 mM NADH, and 4 units of lactate dehydrogenase. The rate of the spontaneous decarboxylation of OAA was measured prior to the addition of PEP carboxykinase. The rate of the spontaneous reaction was subtracted from the enzyme-catalyzed reaction. For the determination of K_m for OAA, the substrate was varied from 0.068 to 0.68 mM for the wild-type enzyme and from 0.003 to 0.05 mM for the His233Gln and Lys213Arg PEP carboxykinases. The upper concentration of OAA employed for the assay of the wild-type enzyme was limited by the rate of the spontaneous metal-dependent decarboxylation of OAA to pyruvate + CO_2.

Circular Dichroism, Fluorescence, and Electron Paramagnetic Resonance Spectroscopy. CD spectra were recorded with an Aviv 26A DS circular dichroism spectrometer with an enzyme concentration of about 2.5 μM in subunits in 20 mM potassium phosphate buffer (pH 6.8) at 25 °C using a 0.1 cm path cell. Fluorescence measurements were made at 22 °C in a Perkin-Elmer LS50B luminescence spectrometer with 3 nm slits for excitation and emission. The excitation wavelength to follow the tryptophan fluorescence was 290 nm, and the emission was scanned from 300 to 400 nm. Urea denaturation experiments were carried out by incubating the enzyme (1.7 μM in subunits) in 50 mM MOPS buffer (pH 7.0) for 16 h at 16 °C at different urea concentrations, as previously described (*22*). To compare the transitions detected, the unfolding curves were expressed as the fraction of the folded form, Y_F, using the relation

$$Y_F = \frac{I_{F_{obs}} - I_{F_U}}{I_{F_N} - I_{F_U}} \quad (2)$$

where $I_{F_{obs}}$ is the measured fluorescence intensity (340 nm) at a given urea concentration and I_{F_N} and I_{F_U} are the respective values for the native and unfolded state. Mn^{2+} binding was measured by EPR spectroscopy on a Bruker EMX X-band EPR spectrometer at a frequency of 9.8 GHz. Enzyme solutions (40, 60, and 100 μM of enzyme subunits for wild-type, Lys213Arg, and His233Gln PEP carboxykinases, respectively) were prepared in 50 mM KCl and 100 mM MOPS buffer (pH 7.0). The total concentration of Mn^{2+} was varied in each experiment over a range of 40−240 μM. Each sample was prepared in a 50 μL volume, and $[Mn^{2+}]_{free}$ was measured. The limit of detection of $[Mn^{2+}]_{free}$ is approximately 1 μM. The binding data were calculated and analyzed using the Scatchard equation:

$$v/[L]_{free} = n/K_{diss} - v/K_{diss} \quad (3)$$

in which v is the number of moles of ligand bound per mole of enzyme monomer, $[L]_{free}$ is the concentration of free ligand, n is the number of saturable binding sites per enzyme monomer, and K_{diss} is the dissociation constant for the saturable binding sites.

Homology Modeling. The programs InsightII, Homology, and Discover 972 (MSI) were used on an O2 SGI workstation to build an homology-based three-dimensional model of *S. cerevisiae* PEP carboxykinase−ADP−PEP−Mn^{2+}, as previ-

Table 1: Kinetic Parameters For Wild-Type, Lys213Arg, and His233Gln *S. cerevisiae* PEP Carboxykinases in the PEP Carboxylation Reaction[a]

	K_m				
enzyme	Mn^{2+} (μM)	MnADP (μM)	PEP (μM)	HCO_3^- (mM)	V_{max} (μM)
wild type	23 ± 7	34 ± 4	307 ± 40	18 ± 2	62 ± 9
Lys213Arg	263 ± 54	68 ± 10	930 ± 130	23 ± 2	0.010 ± 0.001
His233Gln	438 ± 57	27 ± 7	330 ± 56	19 ± 2	0.012 ± 0.001

[a] Kinetic constants were determined as indicated in Experimental Procedures. Data for wild-type enzyme are from ref *9*. Values given are the mean $\pm$ SD.

ously described (*22*). In brief, the reference protein used was the *E. coli* PEP carboxykinase−ATP−pyruvate−Mg^{2+}−Mn^{2+}complex (1AQ2) (*14*). Sequence conservation between *E. coli* and *S. cerevisiae* PEP carboxykinases shows 48% identity and 57% similarity. To build the PEP structure, bonds were reorganized between the phosphate groups of ATP and the pyruvate molecule. The PEP molecule was let free in the active site, and the structure relaxed using cycles of molecular dynamics and minimization calculations. Then, molecular dynamics simulation was run for 300 ps in a zone of 20 Å around the metal at site 1. All solvation water molecules of the reference structure were considered for energy minimization and molecular dynamics calculations. The force field ESFF (MSI) was employed and nonbonding interactions were calculated using the cell multipole method for van der Waals and Coulombic interactions.

RESULTS

Cell Growth, Gene Expression, Enzyme Purification, and Structural Characteristics of Mutated Enzymes. The Lys213Arg and His233Gln PEP carboxykinase plasmids were constructed and identified by restriction digest. The entire PEP carboxykinase gene was sequenced to confirm the absence of any spurious mutations in the coding region outside the area of the mutation. Expression of the wild-type, Lys213Arg, and His233Gln PEP carboxykinase was achieved in the PEP carboxykinase-deficient *S. cerevisiae* strain, PUK-3B, containing the pMV7 plasmid. The cells containing the Lys213Arg or His233Gln mutations were unable to grow on medium containing ethanol as the primary carbon source. 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 approximately 60 g of cells. Each enzyme was purified using the procedure previously reported (*10*). The final yield of pure Lys213Arg and His233Gln PEP carboxykinases was approximately 25 and 15 mg, respectively from about 60 g of cells. The enzymes were judged to be $\geq$95% pure, as determined by SDS−PAGE.

The apparent mass of the variant enzymes was the same as that of the wild-type enzyme, as determined in a calibrated ($R = 0.97$) Superose-12 column (results not shown). In this column, the calculated molecular mass of wild-type PEP carboxykinase was 251 kDa, and the molecular masses of Lys213Arg and His233Gln PEP carboxykinases were 251 and 262 kDa, respectively. These values agree with the expected molecular mass of 244 kDa for the wild-type enzyme tetramer (*18*) and are in the range of those determined by other authors for this same protein (*23*, *24*). To examine if these mutations alter the enzyme's secondary structure, the far-UV circular dichroism spectra of Lys213Arg and His233Gln mutant enzymes were compared with the wild-type spectrum. The CD spectra for wild-type and mutant PEP carboxykinases (not shown) exhibit double minima at 209 and 222 nm. The ratio of the mean residue ellipticity at 209 and 222 nm was 0.78, 0.77, and 0.84 for wild type, Lys213Arg, and His233Gln, respectively, indicating that the secondary structure of the enzyme was not significantly altered by mutations at residues 213 or 233. 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 (18). No alteration in the λ_{max} of emission was detected. The His233Gln mutant enzyme showed a 15% decrease 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. No fluorescence alteration was seen for the Lys213Arg PEP carboxykinase.

Steady-State Kinetics. Steady-state kinetic studies for the primary reaction (PEP carboxylation direction) and for the pyruvate kinase-like and OAA decarboxylase activities were performed for wild-type and mutant enzymes. The kinetic responses of the enzymes to the substrates or to free Mn^{2+} were fit to eq 1. A summary of the resulting steady-state parameters for the PEP carboxylation reaction is listed in Table 1. V_{max} decreased by 6200- and 5200-fold for Lys213Arg and His233Gln, respectively, compared to that for the wild-type enzyme. The K_m values for ADP and HCO_3^- are not significantly altered in the mutant enzymes. There is a 3-fold increase in $K_{m,PEP}$ for Lys213Arg compared to that for the wild-type enzyme. The K_m for Mn^{2+} is significantly altered for the two mutants, increasing 11- and 19-fold for Lys213Arg and His233Gln, respectively. These data indicate that mutation of Lys^{213} or His^{233} primarily alters the interaction of Mn^{2+} with the enzyme and decreases transition state stabilization. The data also show a decreased steady-state affinity for PEP with Lys213Arg PEP carboxykinase.

When the kinetic parameters for the pyruvate kinase-like activity were determined, alteration of Lys^{213} or His^{233} caused a 7- and 44-fold decrease in V_{max}, respectively (Table 2). This effect is significantly less than that observed for the primary reaction of the enzyme (Table 1), where more than a 5000-fold decrease in V_{max} is produced. These results indicate that the residues Lys^{213} or His^{233} are not critical for the pyruvate kinase-like activity of *S. cerevisiae* PEP carboxykinase, in contrast to their importance in the carboxylation reaction. Any concern about contamination of wild-type or mutant *S. cerevisiae* PEP carboxykinases with *S. cerevisiae* pyruvate kinase can be eliminated because of

Table 2: Kinetic Parameters for Wild-Type, Lys213Arg, and His233Gln *S. cerevisiae* PEP Carboxykinases in the Pyruvate Kinase-Like Reaction[a]

enzyme	K_m (μM) MnADP	K_m (μM) PEP	V_{max} (μmol min^{-1} mg^{-1})
wild type	151 ± 20	135 ± 20	0.80 ± 0.01
Lys213Arg	70 ± 20	2200 ± 300	0.11 ± 0.01
His233Gln	28 ± 5	750 ± 170	0.018 ± 0.002

[a] Kinetic constants were determined as indicated in the text. Data for the wild-type enzyme are from ref *9*. Values given are the mean ± SD.

Table 3: Kinetic Parameters for the OAA Decarboxylase Activity of Wild-Type, Lys213Arg, and His233Gln *S. cerevisiae* PEP Carboxykinases[a]

enzyme	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
wild type	140 ± 40	0.27 ± 0.09
Lys213Arg	12 ± 2	0.09 ± 0.01
His233Gln	27 ± 6	0.020 ± 0.002

[a] Kinetic constants were determined as indicated in the text. Data for the wild-type enzyme are from ref *9*. Values given are the mean ± SD.

the hyperbolic kinetic response of the activity of the former enzyme as a function of PEP concentration and the lack of response to fructose 1,6-bisphosphate. A significant alteration in $K_{m,PEP}$ is found in the Lys213Arg and His233Gln enzymes, which increase 16- and 6-fold, respectively. The $K_{m,ADP}$ was affected by mutation of Lys^{213} and His^{233} to a lesser degree, the values decreasing 2- and 5-fold for each mutant enzyme, respectively. The increased values of $K_{m,PEP}$ of the mutated enzymes suggest that Lys^{213} and His^{233} play a role in PEP interactions with the enzyme. This is most striking in the 16-fold increase with Lys213Gln. Relatively minor effects in V_{max} were detected for the OAA decarboxylase activity of *S. cerevisiae* PEP carboxykinase (Table 3), similar to effects observed for PK-line activity and in contrast to the effects measured for the primary reaction. The decrease in V_{max} for Lys213Arg and His233Gln are 3- and 14-fold, respectively, compared to the decrease by 3 orders of magnitude in V_{max} for these mutant enzymes for the primary reaction. The K_m values of the mutant enzymes for OAA are decreased 12- and 5-fold for the Lys213Arg and His233Gln PEP carboxykinases. The value of V_{max}/K_m is 4-fold greater for Lys213Arg and only 2.6-fold less for His233Gln than the V_{max}/K_m value for wild-type enzyme. The data obtained for the two secondary activities of mutant PEP carboxykinases indicate that the mutations at Lys213 or His233 elicit a modest affect on V_{max}, in contrast to the large effects on V_{max} for the primary reaction of the enzyme. These results agree with previous observations of this enzyme (*9*) and support the hypothesis that there are different rate-limiting steps for the primary reaction and the two secondary activities of PEP carboxykinase and the two "partial reactions" are not independent of each other.

Mn^{2+} Binding. The binding of Mn^{2+} to wild-type, Lys213Arg, and His233Gln enzymes was measured by EPR spectroscopy. The data for the wild-type and Lys213Arg PEP carboxykinase are shown in Figure 1 in the form of a Scatchard plot. In all cases, one binding site for Mn^{2+} per enzyme subunit was detected. The dissociation constant for

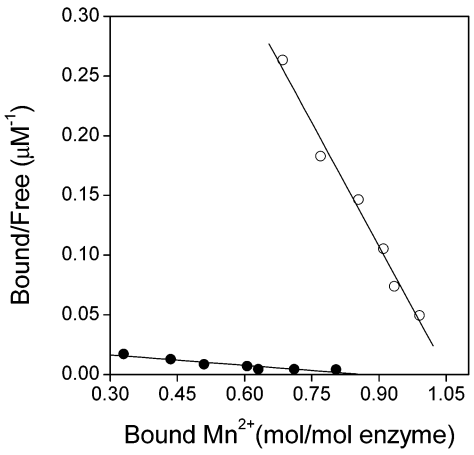


FIGURE 1: Scatchard plot for Mn^{2+} binding to wild-type and Lys213Arg PEP carboxykinases. The binding of Mn^{2+} was measured at 21 °C in the presence of 100 mM MOPS (pH 7.0) plus 50 mM KCl by EPR spectroscopy, as described under Materials and Methods. The solid lines are fits to the Scatchard equation, eq 3, for the wild-type enzyme (○) and for the Lys213Arg mutant (●).

Table 4: Dissociation Constants and Stoichiometry for Mn^{2+} Binding to Wild-Type, Lys213Arg, and His233Gln *S. cerevisiae* PEP Carboxykinases[a]

enzyme	K_d (μM)	*n*
wild type	1.5 ± 0.2	1.1 ± 0.1
Lys213Arg	35 ± 8	0.9 ± 0.2
His233Gln	160 ± 40	1.4 ± 0.4

[a] Dissociation constants and number of saturable binding sites were determined as shown in Figure 1. Values given are the mean ± SD.

the enzyme−Mn^{2+} complex is significantly altered for the mutant enzymes (Table 4), increasing 23- and 107-fold for Lys213Arg and His233Gln PEP carboxykinases, respectively. These results indicate that both Lys^{213} and His^{233} are important for Mn^{2+} binding to yeast PEP carboxykinase.

Urea Denaturation Experiments. The conformational stability of wild-type and mutant PEP carboxykinases was found to be quite similar as all enzymes lost 50% of their tertiary structure at about 2.1−2.2 M urea (Figure 2). The presence of PEP plus Mn^{2+} caused marked shifts in the urea concentration required for 50% loss of tertiary structure in wild-type, Lys213Arg, and His233Gln PEP carboxykinases, in agreement with previous data obtained for wild-type *S. cerevisiae* PEP carboxykinase under slightly different conditions (*22*). The amount of PEP needed to produce the enhanced stability was determined at 2.5 M urea (Figure 3). At low PEP concentrations, the maximum of the fluorescence emission corresponds to that of the unfolded protein (354−355 nm), and increasing the PEP concentration causes a gradual decrease of the fraction of unfolded protein, as shown by the spectral shift to shorter wavelengths. This effect saturates at lower PEP concentrations for the wild-type enzyme (Figure 3A) than those for mutant PEP carboxykinases (Figure 3B), with a maximum wavelength that corresponds to the tryptophan residues of the native protein. The midpoint between the two extremes reveals apparent dissociation constants for the enzyme−PEP−Mn^{2+} complexes of 3 μM, 0.45 mM, and 2 mM for wild-type, Lys213Arg, and His233Gln PEP carboxykinases, respectively, at 2.5 M urea. Although these K_D values are apparent,

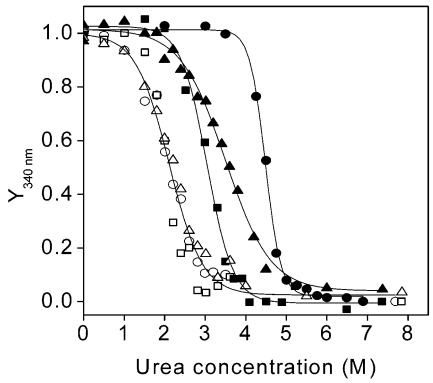


FIGURE 2: Unfolding by urea of wild-type, Lys213Arg, and His233Gln *S. cerevisiae* PEP carboxykinases measured by fluorescence spectroscopy. The enzymes (1.7 μM) were equilibrated 16 h at 16 °C in 50 mM MOPS (pH 7.0) with different urea concentrations, and the fluorescence spectra were recorded (wild type (○), Lys213Arg (△), His233Gln (□)). The effect of incubation in the presence of saturating concentrations of PEP and Mn^{2+} is also presented for wild-type (1 mM PEP plus 2 mM Mn^{2+} (●)), Lys213Arg (5 mM PEP plus 15 mM Mn^{2+} (▲)), and His233Gln (5 mM PEP plus 15 mM Mn^{2+} (■)) PEP carboxykinases. The data are represented as the fraction of the folded protein according to eq 2, calculated from the fluorescence intensity at 340 nm.

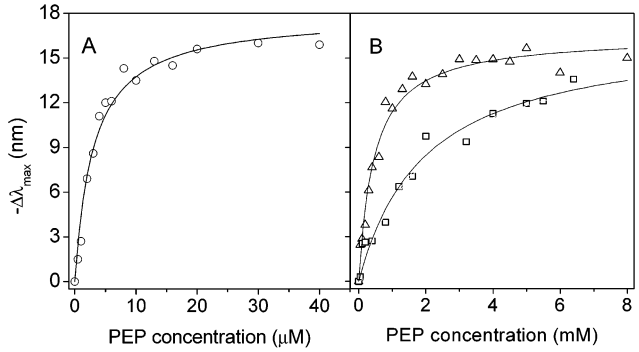


FIGURE 3: Effect of PEP concentration on unfolding by urea of (A) wild-type and (B) Lys213Arg and His233Gln *S. cerevisiae* PEP carboxykinases. The enzymes were incubated in 2.5 M urea with varying concentrations of PEP as indicated and saturating concentrations of Mn^{2+} (1 mM for wild type (○) and 15 mM for Lys213Arg (△) and His233Gln (□) PEP carboxykinases). The data are presented as the shift in the emission maximum from the maximum fluorescence of the unfolded protein in the absence of PEP (355−356 nm). Curves are best fits to the equation of a rectangular hyperbola for binding and yield apparent dissociation constants for PEP from the corresponding enzyme−Mn^{2+}−PEP complexes of 3.0 ± 0.4 μM (wild type), 0.45 ± 0.04 mM (Lys213Arg), and 1.98 ± 0.41 mM (His233Gln).

they suggest that the alteration of Lys^{213} and His^{233} significantly decrease the affinity of PEP carboxykinase for PEP.

DISCUSSION

Site-directed mutagenesis was used to evaluate the catalytic role of two of the three residues of metal binding site 1 of *S. cerevisiae* PEP carboxykinase. The residues chosen for analysis were Lys^{213} and His^{233} that interact with Mn^{2+} via their N atoms. His^{233} was replaced with glutamine, a residue similar in size, hydrophobicity, and hydrogen-bonding potential. Gln is, however, a poor ligand for Mn^{2+}, in contrast with His. Replacement of Lys^{213} with arginine was used to examine the effect of a positively charged residue that is similar to lysine.

Circular dichroism and fluorescence spectroscopy analyses as well as gel filtration and urea denaturation experiments showed no appreciable conformational changes of *S. cerevisiae* PEP carboxykinase or alterations in its oligomeric state upon formation of Lys213Arg or His233Gln. These results indicate that the mutation of Lys^{213} or His^{233} does not cause major structural changes in the protein. Kinetic studies with the Lys213Arg and His233Gln mutants showed 11- and 19-fold increased K_m values for Mn^{2+}, respectively, relative to that for the wild-type enzyme. These values are analogous to the increase in the dissociation constants for Mn^{2+} to the enzyme (Figure 1 and Table 4) that show a loss of 1.9 and 2.8 kcal/mol in binding affinity for Mn^{2+} to Lys213Arg and His233Gln PEP carboxykinases, respectively, relative to that for the wild-type enzyme. These results confirm that Lys^{213} and His^{233} are important for binding of Mn^{2+} to wild-type *S. cerevisiae* PEP carboxykinase. The imidazole ligand of His^{233} plays a more important role in Mn^{2+} binding and its kinetic interaction to the enzyme than does Lys^{213}. Mutation of Lys^{213} causes 3- and 16-fold increase in the K_m for PEP in the PEP carboxylation reaction and in the PK-like reaction, respectively, relative to that for the wild-type enzyme. The mutation of His^{233} shows no change in $K_{m,PEP}$ for the primary reaction but gives rise to a 5.5-fold increase in this value in the PK-like reaction. These results indicate that Lys^{213} and His^{233} are important in the interaction of PEP to PEP carboxykinase. The Lys213Arg or His233Gln mutations caused more than a 5000-fold decrease in V_{max} for the PEP carboxylation reaction with respect to that for the wild-type enzyme, clearly indicating important catalytic roles for Lys^{213} and His^{233}.

Mutation of Lys^{213} or His^{233} in *S. cerevisiae* PEP carboxykinase caused only minor effects in V_{max} of the pyruvate kinase-like and OAA decarboxylase activities of the enzyme, as compared with the effects in V_{max} for the primary reaction. These observations are similar to previous findings reported with Arg^{336} mutants of this same enzyme and suggest the existence of different rate-limiting steps for the primary reaction and for the secondary activities of *S. cerevisiae* PEP carboxykinase (*9*). Hence, the mutation of Lys^{213}, His^{233}, or Arg^{336} of *S. cerevisiae* PEP carboxykinase appears to affect the rate of formation and the stabilization of enolpyruvate, the putative reaction intermediate of the PEP carboxykinase reaction (Scheme 1), thus affecting V_{max} of the main reaction. Our results imply that, for the secondary reactions, protonation of enolpyruvate would be rate-limiting. This suggests that the secondary reactions do not compete with catalysis of the primary reaction when all substrates are available for the enzyme. The available data suggest that, in *S. cerevisiae* PEP carboxykinase, protonation of enolpyruvate occurs upon departure from the enzyme active site (*9*), in contrast to pyruvate kinase, where protonation of enolpyruvate is a specific enzyme-catalyzed step in the net reaction. For PK, it has been proposed that a water molecule, interacting with the metal-bound water at the active site is the proton donor to the enolpyruvate intermediate in the net enzyme reaction (*25*). Figure 4 shows a model of the *S. cerevisiae* PEP carboxykinase−PEP−ADP−Mn^{2+} complex, based on the crystalline structure of the *E. coli* PEP carboxykinase−ATP−pyruvate−Mn^{2+}−Mg^{2+} complex (*14*). The model places the C3 of PEP in a region of the protein where no obvious proton donors exist in its immediate neighborhood. This eliminates

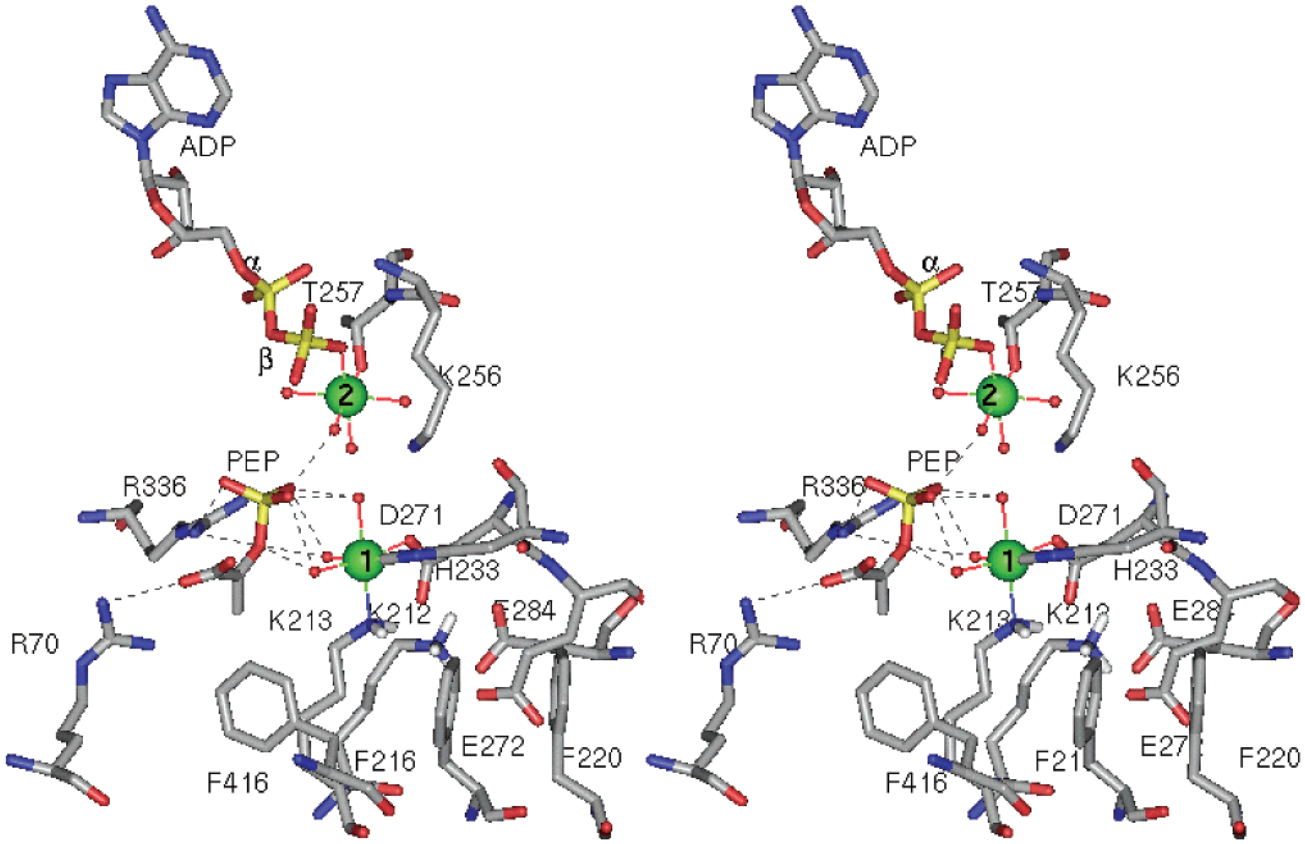


FIGURE 4: Stereoview of a homology model of *S. cerevisiae* PEP carboxykinase−ADP−PEP−Mn^{2+} complex. The model was obtained using as a structural reference the *E. coli* PEP carboxykinase−ATP−pyruvate−Mg^{2+}−Mn^{2+} complex (*14*). The average structure of a 300 ps molecular dynamics simulation is shown. Lys^{212} is shown in the protonated form and Lys^{213} in the neutral, unprotonated form. Hydrogen bonds are shown as dashed lines. Mn^{2+} ions at sites 1 and 2 are shown in green. The other atoms are as follows: carbon, gray; hydrogen, white; nitrogen, blue; oxygen, red; phosphorus, yellow.

the possibility of the protonation of the enolpyruvate intermediate at the catalytic site of PEP carboxykinase.

The model of the *S. cerevisiae* PEP carboxykinase−PEP−ADP−Mn^{2+} complex (Figure 4) shows that Mn^{2+} at site 1 is directly coordinated to Lys^{213}, His^{233}, Asp^{271}, and three water molecules. Coordination of Mn^{2+} to His or Asp is relatively common in proteins. Coordination to Lys is less frequent, and one of the few examples is the binding of Mn^{2+} to a deprotonated Lys residue in chloroplast H^+-ATPase (*26*). In the catalytically homologous enzyme PK from *S. cerevisiae*, Lys^{240} is close to the Mn^{2+} at the catalytic site (*27*) and is probably the group responsible for the observation of ^{14}N superhyperfine coupling to bound Mn^{2+} seen by pulsed spin−echo EPR (*28*). Such coupling only occurs in cases where the amino group and the Mn^{2+} are in sufficient proximity for electron delocalization although Lys^{240} is protonated and interacts with the phosphoryl group of the substrate (*29*). The coordination of Mn^{2+} with N_ϵ of Lys^{213} of PEP carboxykinase is interesting and implies that the residue is in the neutral form, suggesting a low pK_a for the ϵ-NH_2 group. A low pK_a for a Lys residue could be explained by the presence of a hydrophobic and/or a positively charged microenvironment. The proximity of Lys^{212} (Figure 4) that has a positive charge must contribute to keep Lys^{213} in the neutral, unprotonated form. Phe^{216} and Phe^{416}, positioned at a short distance from the N_ϵ of Lys^{213}, add a partial hydrophobic microenvironment around this residue. The higher K_m for PEP in the mutant PEP carboxykinases could be related to an alteration in PEP binding affinity because of the lower Mn^{2+} binding affinity of the altered enzymes. This agrees with the model shown in Figure 4 that shows a network of hydrogen bonds between PEP and the three water molecules coordinated to Mn^{2+}. This model agrees with the structure of the *E. coli* PEP carboxykinase−ATP−pyruvate−Mg^{2+}−Mn^{2+} complex, where Mn^{2+} interacts with pyruvate through two water molecules (*14*). This also agrees with the structure of the human PEP carboxykinase−PEP−Mn^{2+} complex (*17*), where the PEP molecule also interacts with the metal ion through two water molecules. The decreased PEP binding affinity can result from a perturbation in the structure of the metal ion-coordinated water molecules from an altered mode of Mn^{2+} binding in the mutant enzymes. Alteration in PEP binding at the catalytic site can result in changes in the rates of phosphoryl transfer and in the carboxylation of enolpyruvate.

A comparative evaluation of the interaction of PEP with the Mn^{2+} complexes of wild-type and mutant PEP carboxykinases was obtained through the urea-denaturation experiments shown in Figure 3. The fact that much higher concentrations of PEP are required to protect the enzyme against urea denaturation in the mutant enzymes supports the proposal that the mutations Lys213Arg and His233Gln in *S. cerevisiae* PEP carboxykinase affect both Mn^{2+} and PEP binding. Data obtained with both GTP- and ATP-dependent PEP carboxykinases indicate that Mn^{2+} facilitates PEP binding (*22*, *30*, *31*), supporting the observation of synergism between Mn^{2+} and PEP interactions with this enzyme. Recent data on the crystal structure of human PEP

carboxykinase (*17*) reveal that in the enzyme−Mn^{2+}−PEP complex, two of the oxygen atoms from the phosphate moiety of PEP interact with water molecules that are in the first coordination sphere of the Mn^{2+} ion. Previous ^{31}P NMR data obtained with chicken liver PEP carboxykinase (GTP) indicate that the distance between Mn^{2+} at site 1 and ^{31}P of PEP in the enzyme-PEP−Mn^{2+} complex is 7.38 ± 0.48 Å, indicating that with this family of enzymes, PEP forms second sphere complexes with the Mn^{2+} at site 1 (*32*). The model of the *S. cerevisiae* PEP carboxykinase-ADP−PEP−Mn^{2+} complex shown in Figure 4 also reveals a cluster of conserved negatively charged residues (Asp^{271}, Glu^{272}, and Glu^{284}**)** at a short distance (3−4 Å) from N_ϵ of Lys^{212}. While Asp^{271} is involved in Mn^{2+} binding, no function has been ascribed to Glu^{272} and Glu^{284}. It is possible that these negatively charged residues could be important for maintaining Lys^{212} in a protonated form (*33*).

In conclusion, the results presented in this paper indicate that Lys^{213} and His^{233} of *S. cerevisiae* PEP carboxykinase are required for proper Mn^{2+} binding. Kinetic data obtained with Lys213Arg and His233Gln mutant enzymes indicate that proper Mn^{2+} binding is required to stabilize the transition state of the reaction. The removal of either residue has a detrimental effect on the rate determining step, in essential agreement with the proposal put forward by Tari et al. (*14*). Mutation of Lys^{213} or His^{233} did not greatly affect the secondary reactions of the enzyme, as the k_{cat} for Lys213Arg is decreased by an order of magnitude and for His233Gln by about 1.5 orders of magnitude. These results, and the previous observations carried out in Arg^{336} mutants of PEP carboxykinase by Llanos et al. (*9*), suggest that the rate-limiting steps for the main reaction and for the two secondary activities are different.

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