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Mutagenesis of the Active Site Lysine 221 of the Pyruvate Kinase from *Bacillus stearothermophilus*

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Lysine 221 of the pyruvate kinase from *Bacillus stearothermophilus* was mutated to arginine, leucine, asparatic acid and cysteine. All the mutated enzymes were 10^4 to 10^5 times less active than the wild-type enzyme. The cysteine-free enzyme C9S/C268S, and the enzyme C9S/C268S/K221C, which possessed a unique sulfhydryl group at position 221, were prepared. The former had comparable activity to the wild-type enzyme and the latter was 10^4 times less active. These enzymes were denatured and renatured after aminoethylation. The C9S/C268S/K221C enzyme failed to regain its activity when renatured without aminoethylation; but when it was renatured after aminoethylation, it regained 4.5% of the activity of the C9S/C268S enzyme. This evidence suggests the importance of the Lys221 for the pyruvate kinase activity. The kinetic parameters of the *S*-aminoethylated C9S/C268S/K221C enzyme suggest that it has decreased affinity for phosphoenolpyruvate.

Key words: aminoethylation, *S*-aminoethylcysteine, mutagenesis, reaction mechanism, site-specific modification.

Abbreviations: aminoethyl-8, *N*-(β-iodoethyl)trifluoroacetamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PEP, phosphoenolpyruvate; SH, sulfhydryl.

Pyruvate kinase catalyzes phosphoryl transfer from phosphoenolpyruvate (PEP) to ADP to form pyruvate and ATP. This reaction is one of the ATP-generating reactions in glycolysis. As the reaction is essentially irreversible, the enzyme is subject to various types of regulation, such as tissue-specific isozyme distribution, phosphorylation-dephosphorylation, and allosteric regulation (*1*). The reaction proceeds in two steps: first, phosphoryl transfer from PEP to ADP to give ATP and the enol form of pyruvate; and second, addition of a proton to the enolate to give the keto form of pyruvate. The crystal structure of a muscle enzyme complexed with a substrate analogue revealed some of the residues that are involved in substrate binding and catalysis (*2*). Among them, Lys269 of the muscle enzyme lies at the cleft between domains A and B and is conserved in all the pyruvate kinases whose sequences are known and seems to be essential for the activity (*1*). Bollenbach *et al.* mutated Lys240 in yeast enzyme (Lys269 in muscle enzyme) to methionine. Although its activity was greatly decreased, kinetic and fluorescent titration studies of the K240M enzyme suggested that the lysine residue interacts with a phosphoryl group (*3*).

To show that a particular residue is essential for a certain activity, site-directed mutagnetic study is a potent tool. Alone, however, it is not enough. To say a residue is essential for an enzymatic activity, we should show two lines of evidence. One is great loss of the enzymatic activity when the residue is mutated. Studies on this line have been widely done. Secondly, we should show the recovery of the enzymatic activity when the residue is restored. Studies on this line are very rare, because the restoration of a mutated residue at the protein level is very difficult.

Aminoethylation of a cysteinyl residue followed by trypsin digestion has been used to cleave a protein at its cysteinyl residue (*4*). Therefore, an aminoethylated cysteinyl residue may be expected to behave as a lysyl residue. If the essential residue is a lysine, replacement of the residue with cysteine will result in great loss of the enzymatic activity, and the activity is expected to be restored by aminoethylation of the cysteine. In this study, the enzymatic activity of the pyruvate kinase from *Bacillus stearothermophilus* was found to decrease greatly when Lys221 (corresponding to Lys269 in muscle enzyme) was mutated and to be restored by aminoethylation of the K221C mutated enzyme. The pyruvate kinase from *B. stearothermophilus*, a moderate thermophile, is stable and has only two cysteinyl residues (*5*, *6*). These properties make it favorable for such mutagenetic and chemical modificational studies.

MATERIALS AND METHODS

Materials—Pig muscle lactate dehydrogenase, tricyclohexylammonium PEP, ribose 5-phosphate and ADP were purchased from Roche Molecular Biochemicals. NADH was a product of Oriental Yeast Co. Ltd. (Japan). DTNB was a product of Sigma. *N*-(β-Iodoethyl)trifluoroacetamide (Aminoethyl-8 reagent) was purchased from Pierce Biotechnology Inc. (IL, USA). Butyl-Toyopearl 650S was obtained from Toyo-Soda (Japan). Mono-Q HR10/10 column was a product of Amersham Biosciences Inc. *Escherichia coli* strains used in this study were MV1184, CJ236, and XL-1 Blue.

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DNA Manipulation and Site-Directed Mutagenesis—Recombinant DNA techniques are essentially based on the methods described by Sambrook *et al*. (*7*). Site-directed mutagenesis was done by Kunkel's method (*8*). Uracil-containing single-strand DNA was prepared using *E. coli* CJ236 and helper phage M13KO7. Introduction of the mutation was confirmed by DNA sequence analysis. The expression vector was constructed as follows: a 1.98-kb of *Nhe*I–*Cpo*I fragment of pKH320 (containing entire structural gene of the pyruvate kinase, Ref. *6*) was blunted and ligated to a blunted *Xba*I–*Hinc*II large fragment of pBluescript II SK+. This plasmid was designated as pKH501. To facilitate further manipulation, *Bam*HI, *Hin*dIII, *Pst*I, *Xba*I, and *Eco*RI sites which do not alter the amino acid sequence were introduced in the coding region of the enzyme at positions 262, 576, 950, 1225, and 1529 bases from the initial ATG codon using the following oligonucleotides: 5′-AATGACGAGCTTGGATCCTTCCTTCAGC-3′, 5′-CCCGGCGCACAAAGCTTGCGGCGATCAAAT-3′, 5′-CGGATACTGGCCTGCAGCCGTTTCCCCG-3′, 5′-CAAGCGCCAGCCGTCTAGATACCGCTTCAT-3′, and 5′-TGACAGTGACTAGAATTCCGCCGTCGACC-3′, respectively. The underlined sequences indicate the restriction sites introduced. This plasmid was designated as pKH510 and used as a template for the mutagenesis in this study. The following oligonuleotides were used to prepare the mutated enzymes: K221R, 5′-TTCATTTTCAATCCGGGCGATAATTTG-3′; K221D, 5′-TTCATTTTCGATATCGGCGATAATTTG-3′; K221L, 5′′-TTCATTTTCGATCAGGGCGATAATTTG-3′; K221C, 5′-TTCATTTTCAATGCATGCGATAATTTG-3′; C9S, 5′-GGCCCGATCGTCGAGACGATTTTC-3′; C268S, 5′-GCCGAGCATGTTCGACTTTTTAATGA-3′. The mutated codons are underlined.

Expression and Purification of the Pyruvate Kinases—*E. coli* XL-1 Blue cells harboring various plasmids derived from pKH510 were cultured in Luria-Bertani broth containing 50 μg/ml of ampicillin with a 1% inoculum of overnight culture. Isopropyl-1-thio-β-D-galactopyranoside was added after 3 h to 0.5 mM. The cultivation was continued for 16–18 h. Cells were collected by centrifugation and suspended in 3 volumes of 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 0.05 mM DTT, DNase (20 μg/ml), and 0.1 M NaCl. Cells were disrupted using a Sonifier ultrasonic disruptor (Branson, Connecticut). Debris was removed by centrifugation, and solid ammonium sulfate was added to the supernatant to bring to 0.35 saturation. The solution was centrifuged to remove insoluble materials and applied on a Butyl-Toyopearl 650S column equilibrated with 25 mM Tris-HCl buffer pH 7.5, containing 1 mM EDTA, 0.05 mM DTT, and 0.35 saturated ammonium sulfate. The enzyme was eluted with a decreasing linear gradient of ammonium sulfate. The fractions containing the enzyme were collected and dialyzed against 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 0.05 mM DTT, then applied on a MonoQ HR10/10 column, which was equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 0.05 mM DTT. The enzyme was eluted with a linear gradient of NaCl (0–0.5 M). The eluted enzyme was concentrated, and the buffer was changed to 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 0.05 mM DTT by use of a Centricon-30 (Amicon, Danvers, MA). Proteins were analyzed by SDS-PAGE (*9*).

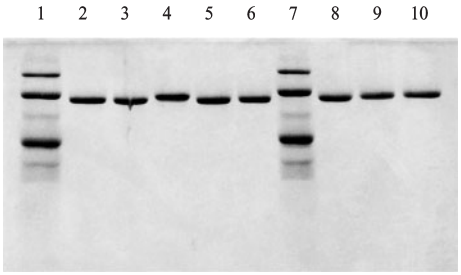

Fig. 1. **SDS-PAGE of the purified enzymes.** Lanes 1 and 7: molecular mass markers, from top to bottom: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor. Lanes 2 to 6: wild-type enzyme, K221C, K221D, K221L, and K221R enzyme, respectively. Lanes 8 to 10: wild-type enzyme, C9S/C268S, and C9S/C268S/K221C mutant, respectively. Two micrograms of the purified enzymes were applied on a 12% acrylamide gel and stained with Coomassie Brilliant Blue R-250.

Enzymatic Assay, CD Measurement, and Protein Determination—The activity of the enzyme was measured by a coupling assay method using lactate dehydrogenase at 30°C (*5*). The reaction mixture contained 50 mM imidazole-HCl buffer, pH 7.2, 50 mM KCl, 7 mM $MgCl_2$, 0.12 mM NADH, 20 μg/ml lactate dehydrogenase, 2 mM PEP, and 4 mM commercial ADP. In the kinetic measurements, the enzymatic activity was assayed in the presence of 0.1 mM ribose 5-phosphate, which is an allosteric activator of the enzyme, and 4 mM commercial ADP with varying concentrations of PEP. The kinetic data were analyzed using KaleidaGraph (Synergy Software, Reading, PA). CD spectra were taken with a J-720 circular dichroism spectropolarimeter (Jasco, Japan) at a protein concentration 50 μg/ml in 10 mM phosphate buffer, pH 7.0. Protein concentrations were determined from the absorbance at 280 nm using the value 0.231 for 1 mg/ml protein solution (*5*).

Titration of Free SH Group—The enzyme was placed in a cuvette containing 0.2 M Tris-HCl buffer, pH 8.6, containing 1 mM EDTA and 0.053 mM DTNB. The absorbance at 412 nm was followed continuously. After 30 min, an equal volume of 8 M guanidine hydrochloride was added, and the change in absorbance was followed for an additional 30 min. The absorbance was extrapolated to the time of the addition of the guanidine hydrochloride, and the reagent blank was subtracted. The amount of SH group was calculated after correcting the volume change using an extinction coefficient of 13,600 M^{-1} cm^{-1} (*10*).

Aminoethylation—The enzyme (18 mg/ml) in 0.2 M Tris-HCl buffer, pH 8.6, containing 1 mM EDTA, 0.05 mM DTT, and 4 M guanidine hydrochloride was divided to two portions. Aminoethyl-8 (*11*) was added to 5 mM to one portion, and both portions were incubated at 50°C for 2 h. The same amount of Aminoethyl-8 was added again to the former, and both portions were further incubated for 2 h. The solution was dialyzed at room temperature against 0.2 M Tris-HCl, pH 8.6, containing 1 mM EDTA and 0.05 mM DTT for 2 h, then further dialyzed exten-

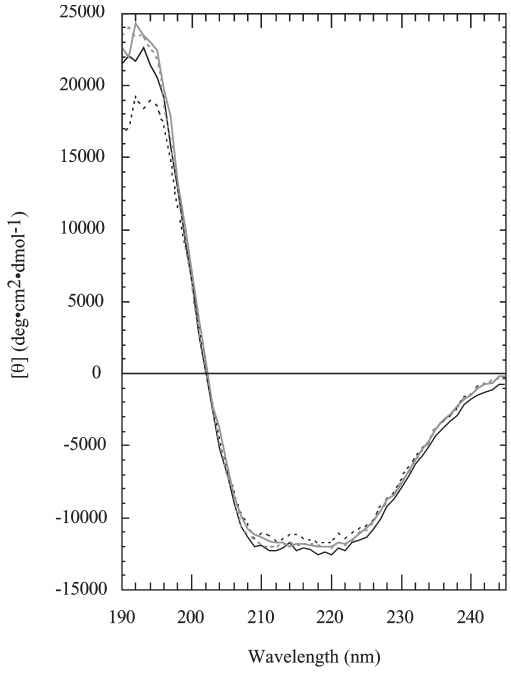

Fig. 2. **CD spectra of the wild-type and mutated enzymes.** CD spectra were measured in 10 mM phosphate buffer, pH 7.0, at the protein concentrations of 50 μg/ml in a 1 mm light path cell. Solid black line, wild-type enzyme; dotted black line, K221L mutant; solid gray line, C9S/C268S mutant; dotted gray line, C9S/C268S/K221C mutant.

sively against 0.2 M Tris-HCl, pH 8.6, containing 1 mM EDTA.

RESULTS

K221 Mutants—Lys221 of the *B. stearothermophilus* pyruvate kinase was mutated to arginine, leucine, asparatic acid and cysteine. The mutated enzymes were purified to homogeneity as shown in Fig. 1, and their specific activities are shown in Table 1. All the mutated enzymes showed great loss of the enzymatic activity (10^{-4} to 10^{-5} times the wild-type enzyme). Although the enzymes were highly purified, the possibility remains that these low activities may be due to traces of contaminating endogenous *E. coli* enzymes. The CD spectra of the wild-type enzyme and K221L enzyme are shown in Fig. 2. The other mutated enzymes had essentially identical spectra, indicating there was no large structural disintegration in the mutated enzymes.

Preparation of a Cysteine-Free Enzyme—To assess the role of Lys221 in the enzymatic activity, two other mutated enzymes were prepared. One is a cysteine-free enzyme. The pyruvate kinase has two cysteine residues (C9 and C268), which were titratable by DTNB only when the enzyme was denatured (*5*). These two residues were mutated to serine, and the resultant mutated enzyme was designated C9S/C268S. The second mutant prepared is the triple mutant C9S/C268S/K221C, which had only one cysteinyl residue, at position 221. The SH groups of these enzymes were titrated using DTNB. No SH groups were detected in the enzymes in the native state, but 1.66, 0.88, and 0.16 SH groups were titrated in the presence of 4 M guanidine hydrochloride in wild-type enzyme, C9S/C268S/K221C enzyme, and C9S/C268S enzyme, respectively. The specific activities of these enzymes are presented in Table 2. The C9S/C268S enzyme has an enzymatic activity of about 75% of the wild-type enzyme, indicating that the two cysteine residues do not have a critical role in the enzymatic activity. The C9S/C268S/K221C mutant showed greatly decreased activity, comparable to that of the K221C mutant. The CD spectra of C9S/C268S and C9S/C268S/K221C mutants are also presented in Fig. 2. They were essentially identical to that of the wild-type enzyme.

Restoration of the Enzymatic Activity after Aminoethylation—The wild-type and two cysteine mutant enzymes were denatured in 4 M guanidine hydrochloride and the SH groups were aminoethylated using Aminoethyl-8. The modified enzymes were renatured by dialysis. Their activities are shown in Table 2. The wild-type enzyme regained about 6% of its original activity after the denaturation-renaturation step. The value decreased to about half when the enzyme was aminoethylated in the denatured state. The modification of the interior cysteine residues may prevent proper refolding of the enzyme. On the other hand, the C9S/C268S mutant regained about half of its original activity with or without aminoethylation. The C9S/C268S/K221C mutant showed very low activity before denaturation and also after renaturation without aminoethylation. When the enzyme was renatured after aminoethylation, it developed relatively high activity (10.1 U/mg). This specific activity is comparable to that of renatured wild-type enzyme. This result suggests that K221 is essential for the activity of the *B. stearother-*

Table 1. **Specific activities of the wild-type and mutated pyruvate kinases.**

Enzyme	Specific activity (U/mg)	Fold
Wild-type	555. ± 46	1.
K221R	0.0449 ± 0.0058	0.000081
K221L	0.0072 ± 0.0005	0.000013
K221D	0.0369 ± 0.0005	0.000066
K221C	0.0890 ± 0.0056	0.00016

Errors are presented as standard deviations of 3 measurements.

Table 2. **Specific activities of the enzymes with or without aminoethylation.**

Enzyme	Denatured and renatured		
	Native	Not modified	Aminoethylated
Wild-type	555 ± 46	32.07 ± 2.29	14.6 ± 0.18
C9S/C268S	412 ± 91	202.1 ± 2.7	222.7 ± 14.6
C9S/C268S/K221C	0.0796 ± 0.0089	0.0071 ± 0.001	10.1 ± 0.70

Activities are presented as U/mg protein. Errors are presented as standard deviations of 3 to 5 measurements.

Table 3. **k_{cat} and K_m values of the enzymes with respect to PEP in the presence of 0.1 mM ribose 5-phosphate.**

Enzyme	k_{cat} (s^{-1})	K_m (mM)
Wild-type	3,204 ± 74	0.186 ± 0.030
C9S/C268S	1,736 ± 37	0.113 ± 0.009
Aminoethylated C9S/C268S/K221C	82.8 ± 1.98	1.23 ± 0.107

Errors are presented as standard error.

mophilus pyruvate kinase. No SH group was titrated by DTNB in the aminoethylated C9S/C268S/K221C mutant.

The Kinetic Parameters of the Enzyme—The kinetic parameters with respect to PEP in the presence of an activator, ribose 5 phosphate, are presented in Table 3. The enzyme is an allosteric enzyme and is activated in the presence of ribose 5-phosphate. The C9S/C268S enzyme had a reduced k_{cat} value, but its K_m value for PEP was essentially unchanged. Although the aminoethylated C9S/C268S/K221C enzyme had significant activity, its k_{cat} value was reduced to 4.8% and K_m value was increased 11-fold relative to the C9S/C268S enzyme.

DISCUSSION

The evidence presented here strongly suggests the importance of Lys221 for the pyruvate kinase activity. The lysine residue is in the bottom of a cleft formed by the domain A and domain B interface (*2*) and has not been modified by chemical modification. A reactive lysine residue of the muscle (K366) or *E. coli* type I (K317) enzyme at the entrance of the cleft may prevent modification of this concealed residue (*6*). Bollenbach *et al.* converted this residue of yeast enzyme (K240) to methionine. The K240M enzyme was 1,000-fold less active than wild-type enzyme. They measured the K240M enzymatic activity and applied physicochemical analysis, and they concluded that the residue interacts with the phosphoryl group of PEP and helps to stabilize the pentavalent phosphate transition state during phosphoryl transfer (*3*). Whether this lysine residue participates in the phosphoryl group transfer step or the protonation of enol form step remains an open question. As schematically shown in Fig. 3, the restoration of the activity by the aminoethylation of C9S/C268S/K221C enzyme suggests the necessity of an amino group at the position of the ε-amino group of Lys221. As the K221R mutant failed to maintain the activity, a positively charged group may not be needed here. The property required for the group may be a nucleophilic character to stabilize the phosphoryl transfer transition state. Accordingly, it seems unlikely that K221 has a role in the protonation of the enol form pyruvate. Recently, Susan-Resiga and Nowak suggested that a water molecule near the T298 of yeast enzyme (T279 in the *B. stearothermophilus* enzyme) is a potential proton donor (*12*). Although significant activity was restored by the aminoethylation, the k_{cat} value of the modified enzyme was only 4.8% of that of the C9S/C268S enzyme (efficiency of renaturation is not considered) and the K_m value was increased 11-fold. Differences in the lengths and angles of C-C and C-S bonds should result in slight displacement of the position of the amino group of the aminoethylated enzyme. This may cause the large changes in the kinetic parameters.

Planas and Kirsch mutated Lys258 of *E. coli* aspartate aminotransferase to cysteine (*13*). The mutant retained less than 10^{-6} of the activity of the wild-type enzyme. As the enzyme contained five additional SH groups, they protected nontarget SH groups at different stages of unfolding. The resultant K258C aminoethylated enzyme had a k_{cat} value of about 7% of that of wild-type enzyme. This may be the first report of a functional lysyl residue mimicked by cysteine aminoethylation. Kim *et al.* also mutated Lys239 of aspartate aminotransferase from *Bacillus* sp. to cysteine. The mutation of this pyridoxal 5′-phosphate-binding residue resulted in loss of the activity, but modifications of the SH groups by aminoethylation, aminopropylation, and aminoethylthiolation restored the k_{cat} value to 19.6–0.065% of that of the wild-type enzyme, depending on the modification (*14*). Salvucci *et al.* mutated Lys247 of tobacco leaf Rubisco activase and aminoethylated but failed to improve the catalytic performance of the mutant enzyme (*15*). In the case of Rubisco activase, the position of the amino group can be strictly defined. The restoration of the activity may depend on

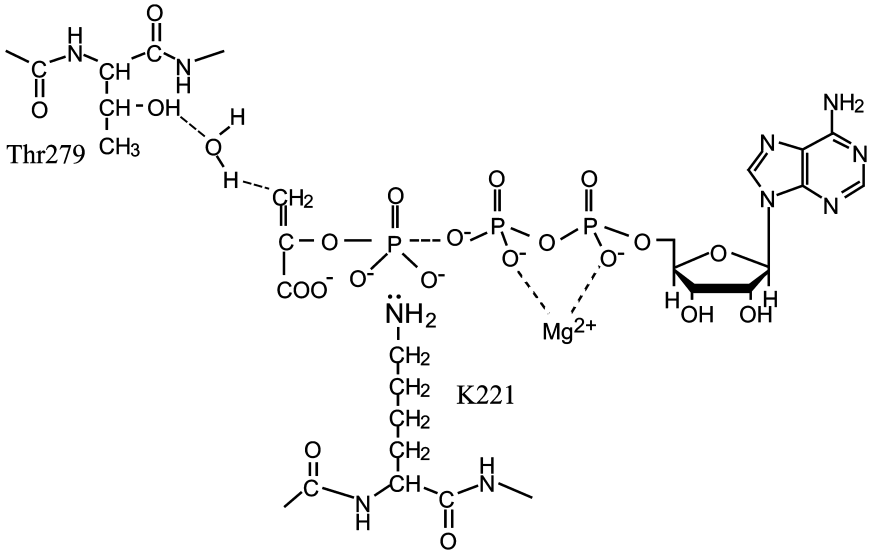

Fig. 3. Possible functional role of the K221 in the pyruvate kinase reaction. In the phosphoryl transfer, a pentavalent phosphate transition state is formed. The ε-amino group of Lys221 may be situated near the phosphoryl group of phosphoenolpyruvate and stabilize the transition state. The ionization of the amino group may not be necessary. The proton necessary to form a keto-form pyruvate may be supplied by another molecule, possibly a water molecule tightly bound to Thr279.

the tolerance for the position of the nucleophilic group participating in the enzymatic reaction mechanism.

The present study has clearly shown the importance of lysine 221 in the pyruvate kinase activity by the combination of site-directed mutagenesis and group-specific chemical modification. As the SH group of the cysteinyl residue is reactive and various types of modification reagents are available, the mutation of a residue to cysteine followed by the modification of the residue by a group-specific reagent can be a potent tool to study the structure and function of a protein. Recently, the combination of mutagenesis to cysteine and chemical modification by a fluorescent or spin-labeled reagent has been used to study membrane protein topology (*16*, *17*).

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