Allosteric Regulation of Pyruvate Kinase M2 Isozyme Involves a Cysteine Residue in the Intersubunit Contact

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Pyruvate kinase M2 isozyme mutants with amino acid substitutions in the subunit interface were prepared and characterized. The substitutions were made in the allosteric M2 isozyme by the corresponding residues of the nonallosteric M1 isozyme to identify the residue involved in the allosteric effects. The replacement of Cys-423 by Leu led to substantial loss of both homotropic and heterotropic allosteric effects while the substitutions at Phe-389, Arg-398, Ala-401, Pro-402, Thr-408, and Ile-427 did not. The altered kinetic properties of the Cys-423-substituted mutant resulted from the shift of the allosteric transition toward the active R-state since the mutant exhibits the allosteric properties in the presence of an allosteric inhibitor, L-phenylalanine. The inverse correlation between the hydrophobicity of residue 423 and the extent of stabilization of the R-state was found by analysis of mutants with un-ionizable amino acids at position 423. Furthermore, the modification of Cys-423 with methyl methanethiosulfonate led to a shift of the allosteric transition toward the R-state, probably the result of increased hydrophobicity of the residue. These results suggest that Cys-423 is involved in the allosteric regulation of the enzyme through hydrophobic interactions.

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‡ The abbreviations used are: PK, pyruvate kinase; PEP, phosphoenolpyruvate; FBP, fructose-1,6-bisphosphate; S_c, substrate concentration giving one-half of V_max; PAGE, polyacrylamide gel electrophoresis; MMTS, methyl methanethiosulfonate; kb, kilobase(s).

Materials—Restriction endonuclease and DNA modifying enzymes were purchased from Takara and New England Biolabs. Fructose-1,6-bisphosphate, trisodium salt, was obtained from Sigma. Phosphoenolpyruvate and ADP were obtained from Wako Pure Chemicals and Oriental Yeast, respectively. Lactate dehydrogenase and NADH were products of Boehringer-Mannheim. Oligonucleotide primers were synthesized by Greiner Japan. Other reagents were from Wako Pure Chemicals or Nacalai Tesque.

Construction of the Transfer Plasmid—A 5′ SalI-PstI 0.65-kb fragment of rat PK-M2 cDNA (5) was subcloned into a pSVK3 vector (Amer sham Pharmacia Biotech). Subsequently, a BanHI-PstI 0.67-kb fragment was excised from the resulting plasmid and then ligated into a plBluescript SK+. A 3′ BglII-EcoRI 1.3-kb cDNA fragment was ligated downstream of the 5′ cDNA fragment contained in that plasmid, following digestion by these restriction endonucleases. The BanHI-EcoRI 1.9-kb fragment, which contains the entire coding sequence, was excised from the resultant plasmid and inserted into a transfer vector, pVL1393 (Invitrogen).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out according to Kunkel (16), as described previously (17). Prior to mutagenesis, the SphiI-KpnI 0.2-kb fragment of rat PK-M2 cDNA, which contains the amino acid sequences different between the M1 and M2 isozymes, was subcloned into a pMTV19N vector (Takara). A HindIII-KpnI fragment was excised from the resulting plasmid and then ligated to pBluescript SK+. The uracil-substituted single-stranded template was prepared from E. coli CJ236 transformed by the plasmid. The uracil-template was used with synthetic oligonucleotide primers to replace residues of the rat M2 isozyme with those of the rat M1. The
oligonucleotides primers used in this study are as follows: 5'-GAGAG-GCTGGGATCTTTTACCTGGC-3' for replacement of Tyr-389 by Phe (designated as Y389F), 5'-CGAGAAGATCCGGGCGGCTGGGGCC-3' for Arg-398 by Ala (R398A), 5'-CCGCCCCGTAGCCATCTTCCAGACAGCC-3' for Thr-408 by Leu (T408L), 5'-CCTCAACAGGCTTTAGGGCATTATCG-3' for Cys-423 by Leu (C423L), 5'-CAGTGGGCCCAGTGCTGCTACCC-3' for Ile-427 by Leu (I427L), 5'-TTCAATGGCGGTCAGGGGGCC-3' for Cys-423 by Ala (C423A), and 5'-TTCAATGGCGGTCAGGGGGCC-3' for Cys-423 by Ser (C423S). The resulting mutations were introduced by in vitro mutagenesis using M. hokkaidensis KOD-Plus Mutazyme (Toyobo, Japan) and confirmed by sequencing. For the determination of the optimal reaction temperature, the recombinant enzymes were incubated at different temperatures, and the reaction rate was measured using the lactate dehydrogenase-NADH coupled assay, as described above. 

RESULTS

Expression of Recombinant Rat PK-M2 in Insect Cells—2 × 10⁶ SF21 cells were infected with the recombinant viruses carrying either wild-type or mutant PK-M2 at a multiplicity of infection of more than 8. The infected cells were harvested at about 90 h postinfection to purify the expressed proteins.

Analysis of the Recombinant Enzymes—SF21 cells producing the recombinant enzymes were pelleted by centrifugation at 2,500 × g for 10 min. The cells were homogenized in 20 mM Tris-HCl, 5 mM MgSO₄, 0.5 mM FBP, 1 mM EDTA, and 10 mM 2-mercaptoethanol (pH 7.5) with a Dounce homogenizer and then centrifuged at 10,000 × g to obtain clarified extracts. The supernatants were then reacted at 4 °C with 50 μM methyl methanethiosulfonate (MMTS) in 50 mM buffer, 0.1 M KCl, 5 mM MgSO₄, Tris-HCl, 5 mM MgSO₄, and 10 mM 2-mercaptoethanol (pH 7.5) prior to the kinetic analyses. Twelve different concentrations of PEP between 5 μM and 2.0 mM were used to obtain kinetic parameters for the substrate. When the parameters for ADP were determined, seven concentrations of the substrate from 31.3 μM to 2.0 mM were employed. Release of pyruvate was monitored using a Beckman DU-640 spectrophotometer by coupling the above reaction with the lactate dehydrogenase-NADH system. Kinetic parameters were obtained by fitting data for various concentrations of PEP to the Hill equation. The Michaelis-Menten equation was used to determine parameters for ADP. These calculations were carried out using nonlinear regression analysis based on the Marquardt algorithm. When the effects of L-phenylalanine on kinetic parameters were investigated, kinetic experiments were performed in a similar manner in the presence of these allosteric effectors.

Treatment with Methyl Methanethiosulfonate—The wild-type and C243S mutant of PK-M2 were reacted at 4 °C with 50 μM methyl methanethiosulfonate (MMTS) in 50 mM buffer, 0.1 M KCl, 5 mM MgSO₄, Tris-HCl, and sodium acetate buffer were used as the buffer components for the reactions at pH 7.5 and pH 5.5, respectively. An aliquot of the mixture was subjected to enzyme activity assay at several intervals. The assay was carried out using 100 μM PEP and 2.0 mM ADP in the absence or presence of 0.5 mM FBP. The other conditions were the same as described for the standard activity assay.

Electrophoresis—The purified enzymes were subjected to SDS-PAGE analysis on 10% gels, according to Laemmli (22) and visualized by Coomassie Brilliant Blue R-250.

Enzyme Activity Assay—A standard assay for PK activity was performed at 37 °C using 2 mM of both PEP and ADP in 50 mM Tris-HCl buffer, 0.1 M KCl, 5 mM MgSO₄, and 0.5 mM FBP (pH 7.5) as described earlier (1). This substrate mixture also contained 17 units/ml of lactate dehydrogenase and 0.17 mM NADH to monitor the release of pyruvate by the change of absorbance at 340 nm. One unit of activity was defined as the quantity of the enzyme that releases 1 μmol of pyruvate per min.

Kinetic Analyses—Enzymatic activity was assayed at 37 °C using various concentrations of PEP and ADP. The conditions used for kinetic analyses were the same as above except for the substrates and cofactor. In the assessment of parameters for one substrate, the concentration of the other was fixed at 2 mM. The purified enzymes were subjected to gel filtration (Sephadex G-50) and equilibrated with 0.1 M KCl, 50 mM Tris-HCl, 5 mM MgSO₄, 10 mM 2-mercaptoethanol (pH 7.5) prior to the kinetic analyses. Twelve different concentrations of PEP between 5 μM and 2.0 mM were used to obtain kinetic parameters for the substrate. When the parameters for ADP were determined, seven concentrations of the substrate from 31.3 μM to 2.0 mM were employed. Release of pyruvate was monitored using a Beckman DU-640 spectrophotometer by coupling the above reaction with the lactate dehydrogenase-NADH system. Kinetic parameters were obtained by fitting data for various concentrations of PEP to the Hill equation. The Michaelis-Menten equation was used to determine parameters for ADP. These calculations were carried out using nonlinear regression analysis based on the Marquardt algorithm. When the effects of L-phenylalanine on kinetic parameters were investigated, kinetic experiments were performed in a similar manner in the presence of these allosteric effectors.

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Treatment with Methyl Methanethiosulfonate—The wild-type and C243S mutant of PK-M2 were reacted at 4 °C with 50 μM methyl methanethiosulfonate (MMTS) in 50 mM buffer, 0.1 M KCl, 5 mM MgSO₄, Tris-HCl, and sodium acetate buffer were used as the buffer components for the reactions at pH 7.5 and pH 5.5, respectively. An aliquot of the mixture was subjected to enzyme activity assay at several intervals. The assay was carried out using 100 μM PEP and 2.0 mM ADP in the absence or presence of 0.5 mM FBP. The other conditions were the same as described for the standard activity assay.

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binant baculoviruses carrying the cDNAs. SDS-PAGE analysis of these purified enzymes showed single bands of about 57 kDa, which correspond to the molecular mass of the subunit, for all enzymes (data not shown).

In order to evaluate the effects of the substitutions on allosteric properties, the mutants were subjected to kinetic analysis with variable concentrations of PEP and fixed concentrations of ADP (2 mM) and MgSO4 (5 mM). As shown in Fig. 2 and Table I, the substitution of Cys-423 resulted in substantial loss of homotropic allosteric effect while the other mutant and the wild type exhibited sigmoidal responses to some extent. Furthermore, the C423L mutant did not exhibit a heterotropic allosteric effect for FBP and was no longer activated by the effector. In addition, this mutant as well as the others showed essentially no differences in $K_m$ for ADP nor apparent $V_{max}$ (Table I), suggesting that the mutation at Cys-423 has an effect on the interaction of the enzyme with PEP but not on the binding of ADP nor on catalysis.

While the $S_{0.5}$ and the Hill coefficient of the wild-type M2 isozyme were 180 $\mu$M and 2.2 in the absence of FBP, those of the C423L mutant were 50 $\mu$M and 1.3 (Table I). These kinetic parameters of the mutant in the absence of FBP were nearly the same as those of the FBP-activated wild-type M2 and were also very similar to the nonallosteric PK, M1-isozyme (13). Thus, the substitution of Cys-423 with Leu converted the allosteric M2 isozyme into a nearly nonallosteric enzyme. These results suggest that the mutant remains in the active conformation as the R-state in the absence of an allosteric effector.

However, the C423L mutant exhibited cooperative properties for PEP in the presence of the allosteric inhibitor l-phenylalanine, as evidenced by the sigmoidal curvature, resulting in an increase of $S_{0.5}$ and Hill coefficient (Fig. 3). L-Phenylalanine inhibits PKs by stabilizing the T-state (8, 24), as shown for the wild-type M1 in Fig. 3. This indicates that the mutant subunits have the potential to cooperatively interact, which is involved in homotropic allosteric effect. When the C423L mutant was inhibited by 0.4 mM l-phenylalanine, the kinetic properties of the mutant were indistinguishable from those of the wild-type M2, as revealed by an $S_{0.5}$ of 180 $\mu$M and a Hill coefficient of 2.4.

**Fig. 2.** Kinetic properties of the wild-type and mutant PK-M2 with respect to PEP. Activities were determined with the various concentrations of PEP, 2 mM ADP, and 5 mM MgSO4 in the presence (closed circles) or absence (open circles) of 0.5 mM FBP. Details are given under "Experimental Procedures." The velocities are indicated with the values normalized by $V_{max}$. The recombinant rat wild-type PK-M1 (13) was also subjected to kinetic analysis for comparison. Curves are drawn by fitting the data to the Hill equation with nonlinear regression analysis.
for the mutant (Fig. 3). Since the profile of activation of the l-phenylalanine-inhibited mutant by FBP was essentially identical to that of the wild-type, the amino acid substitution would have no effect on the binding of FBP and FBP-induced activation (Fig. 4). These results are consistent with the suggestion that replacement of Cys-423 by Leu affects kinetic properties only through the shift of the allosteric transition toward the R-state.

In order to assess which character of the side chain of residue 423 leads to the aforementioned altered allosteric properties, Cys-423 of the M2 isozyme was further replaced by structurally related but un-ionizable amino acids, serine and alanine. Fig. 5A shows the responses of the Cys-423-substituted mutants for variable concentrations of PEP. The \( S_{0.5} \) values for the mutants were 130, 87, and 50 \( \mu M \) for replacements by Ser, Ala, and Leu, respectively. Plots of these \( S_{0.5} \) values as a function of the hydrophobicity (25) of the side chains (Fig. 5B) suggest that hydrophobicity of residue 423 is an important factor in shifting the equilibrium of the allosteric transition toward the active R-state because the \( S_{0.5} \) value is inversely correlated with the hydrophobicity of residue 423.

To further test the significance of the hydrophobicity of the residue 423 in stabilizing the R-state, the modification of cysteine by methyl methanethiosulfonate (26) was carried out. The modified wild-type M2 isozyme at pH 7.5 displayed a biphasic reaction consisting of rapid activation followed by inactivation when the activity was assessed in the absence of FBP (Fig. 6A). The activity measured in the presence of FBP was decreased in a single exponential manner. In addition, only inactivation without prior activation was observed for the modified C423S mutant (Fig. 6B). Therefore, the MMTS-induced activation appears to be associated with the formation of a mixed disulfide, S-methylthio-cysteine, at Cys-423, and the modification of the other cysteine residue(s) would result in loss of activity. It was also found that Cys-423 is much more highly reactive toward MMTS than the other cysteine residue(s) whose modification leads to inactivation.

### Table I

| Enzyme          | PEP<sup>a</sup> | 0.5 mM FBP<sup>d</sup> | ADP<sup>b</sup> |
|-----------------|-----------------|------------------------|-----------------|
|                 | \( V_{\text{max}} \) | \( S_{0.5} \) | Hill coefficient | \( V_{\text{max}} \) | \( S_{0.5} \) | Hill coefficient | \( V_{\text{max}} \) | \( K_m \) |
|                 | \( \mu M \) / min / mg | \( \mu M \) |              | \( \mu M \) / min / mg | \( \mu M \) |              | \( \mu M \) / min / mg | \( \mu M \) |
| Wild type       | 530 ± 4.6       | 180 ± 2.6              | 2.3 ± 0.067     | 560 ± 5.4       | 52 ± 1.5    | 1.2 ± 0.045     | 600 ± 7.5     | 0.32 ± 0.010 |
| Y390F           | 500 ± 1.4       | 370 ± 19               | 1.7 ± 0.12      | 520 ± 6.2       | 60 ± 2.5    | 1.2 ± 0.060     | 530 ± 12      | 0.33 ± 0.020 |
| R398A           | 630 ± 2.7       | 320 ± 2.4              | 3.0 ± 0.056     | 640 ± 5.4       | 60 ± 1.5    | 1.2 ± 0.036     | 650 ± 12      | 0.31 ± 0.015 |
| A401S/P402S     | 500 ± 3.6       | 190 ± 2.7              | 2.6 ± 0.087     | 510 ± 4.6       | 47 ± 1.3    | 1.2 ± 0.042     | 570 ± 8.2     | 0.36 ± 0.014 |
| T408L           | 550 ± 5.8       | 120 ± 3.0              | 2.0 ± 0.088     | 580 ± 3.8       | 51 ± 1.0    | 1.2 ± 0.029     | 680 ± 6.6     | 0.36 ± 0.009 |
| C423L           | 500 ± 4.4       | 47 ± 1.1               | 1.3 ± 0.040     | 530 ± 6.8       | 43 ± 1.6    | 1.1 ± 0.057     | 610 ± 10      | 0.30 ± 0.013 |
| H427L           | 510 ± 5.7       | 180 ± 4.4              | 2.4 ± 0.13      | 570 ± 7.0       | 51 ± 1.7    | 1.3 ± 0.052     | 640 ± 24      | 0.39 ± 0.035 |

<sup>a</sup> Assayed with 2.0 mM ADP in 50 mM Tris-HCl, 0.1 mM KCl, and 5.0 mM MgSO\(_4\) (pH 7.5).

<sup>b</sup> Assayed with 2.0 mM PEP in 50 mM Tris-HCl, 0.1 mM KCl, and 5.0 mM MgSO\(_4\) (pH 7.5).

<sup>c</sup> In the absence of FDP.

<sup>d</sup> In the presence of 0.5 mM FDP.
In order to show the activation induced by MMTS more precisely, the enzymes were reacted with MMTS at pH 5.5. MMTS reacts only with thiolate anions, and the low pH decreases the reaction rates even more (27). As shown in Fig. 7, the inactivation phase became negligible under these conditions at pH 5.5, and only activation was observed in the wild type, albeit the rate was decreased. Because no activation was observed for the C423S mutant, the modification of Cys-423 would be expected to result in activation. MMTS had no effect on the activity when assayed in the presence of 0.5 mM FBP, suggesting that the modification by MMTS did not alter the kinetic properties of the R-state. To characterize the MMTS-induced activation, the wild-type enzyme was subjected to kinetic analysis after incubating at pH 5.5 with MMTS for 40 min. The reaction with MMTS decreased \( S_{0.5} \) and Hill coefficient to 120 \( \mu \text{M} \) and 1.9, respectively. As a result, the plots of the velocities as a function of PEP concentrations were significantly shifted to the left (data not shown). However, treatment with MMTS at pH 5.5 resulted in no significant change in \( V_{\text{max}} \). These results suggest that the formation of the mixed disulfide at residue 423 induces the shift of the allosteryic transition toward the R-state.

**DISCUSSION**

In the present study, we prepared a series of rat PK-M\(_2\) mutants and analyzed their properties to identify the amino acid residue involved in the allosteryic effects. This study reveals that Cys-423 is important in order for the enzyme to exhibit allosteryic properties. Replacement of the cysteine residue with several non-ionic amino acids significantly alters the properties of the protein and reveals that the extent of stabilization of the R-state inversely correlates with the hydrophobicity of the residues. This is also consistent with a chemical modification study using MMTS. These results suggest that the introduction of a more hydrophobic moiety into residue 423 relatively stabilizes the active R-state.

An x-ray crystallographic analysis of PK-M\(_1\) isozyme whose residue 423 is Leu has shown that Leu-423 is surrounded by hydrophobic amino acids, as indicated in Fig. 8, all of which are common in rat M\(_1\) and M\(_2\) isozymes. The corresponding residue in the M\(_2\) isozyme, Cys-423, also must be located in a hydrophobic environment because the gross steric structure of the M\(_2\) isozyme is believed to be essentially the same as that of the M\(_1\) isozyme. It has been reported that the conformation of a protein is destabilized when a thiol of a cysteine residue, buried in the hydrophobic environment of the protein, is deprotonated and negatively charged (28). Thus, it is most likely that the hydrophobic interactions involving the cysteine with the pro-
The side chain of the cysteine (–CH$_2$–S$^-$) appears to play a regulatory role in the allosteric effect observed in the rat M$_1$ isozyme. The M$_1$ mutant exhibited significant allosteric properties that were similar to the wild-type M$_2$ isozyme. These considerations suggest that Cys-423 regulates the allosteric transition by altering the relative stability of the R-state.

In addition, deprotonation of a thiol generates a negative charge, and the side chain of the cysteine (–CH$_2$–S$^-$) becomes less hydrophobic. This, in turn, would lead to destabilization of the active conformation (R-state) due to disruption of the hydrophobic interactions, and as a result, the inactive T-state may be relatively stabilized. Therefore, the stabilization of the R-state might depend on the protonation state of the side chain surrounding Cys-423. In fact, when the leucine was replaced with cysteine at Cys-423 via reaction with MMTS (26) appears to stabilize the R-state, and it has been reported that the rotatory movement of domains is likely to be responsible for intersubunit communication (29, 30). The mechanism of the allosteric transition on the binding of ligand(s) would be expected to initially trigger the conformational change of a single subunit and subsequently to induce changes in the other associated subunit(s). However, the initial event prior to the subunit interaction remains unknown, but would involve Cys-423 in mammalian M$_2$ isozymes. In addition, it is possible that SH group-containing metabolite(s) or other small thiol-containing molecules regulate the activity of PK-M$_2$ in vitro via altering the equilibrium of the allosteric transition through the formation of mixed disulfides with Cys-423.

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