Conversion of Non-allosteric Pyruvate Kinase Isozyme into an Allosteric Enzyme by a Single Amino Acid Substitution*

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Pyruvate kinase M₁, a non-allesteric isozyme, was converted into an allosteric enzyme by replacement of an amino acid in the intersubunit contact. The substitution of Ala-398 with Arg resulted in the pronounced allosteric enzyme. The Hill coefficient and the substrate concentration giving one-half of Vₘₐₓ for the mutant with respect to phosphoenolpyruvate were 2.7 and 0.41 mM, respectively, whereas those values for the wild type were 1.0 and 0.049 mM. This mutation, however, gave rise to only minor effects on the apparent values of KM for ADP and on Vₘₐₓ. Furthermore, in contrast to the wild-type enzyme, the mutant was activated by fructose 1,6-bisphosphate. The Hill coefficient of the mutant was no longer increased by the allosteric inhibitor, l-phenylalanine, indicating that the equilibrium for the unligated enzyme is largely shifted toward the T-state. These results suggest that Ala-398 is one of the most critical residues allowing the enzyme to prefer the R-state and that allosteric regulation of pyruvate kinase involves amino acid residues in the intersubunit contact.

Pyruvate kinase (PK, 1 ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40) catalyzes the transfer of a phosphoryl group of phosphoenolpyruvate (PEP) to ADP and plays a key role as a regulatory enzyme in the glycolytic pathway (1–3). In general, the enzyme displays homotropic cooperativity with respect to PEP. In mammalian allosteric PKs, fructose 1,6-bisphosphate (FBP), an intermediate metabolite of glycolysis, functions as an allosteric effector to activate the enzyme heterotropically (1, 4, 5). Four distinct isozymes, L, R, M₁, and M₂, occur in mammalian tissues and differ in regulatory properties. All these isozymes are known to be tetramers and are allosteric enzymes with the exception of M₁(1, 5–8).

The M₁ and M₂ isozymes are produced from a single gene locus by mutually exclusive alternative splicing (8–10); the M₁ isozyme is expressed predominantly in the kidney, and M₃ is predominant in skeletal muscle, heart, and brain (1). In addition, it is also known that expression of these isozymes shifts of M₂ to M₁ during development of some fetal tissues (2). In contrast to the other PK isozymes, M₁ isozyme usually exhibits neither homotropic nor heterotropic allosteric effects (1, 5, 6). This isozyme, however, displays cooperative behavior under certain experimental conditions such as the presence of l-phenylalanine, an allosteric inhibitor (11–13). Furthermore, heterotropic activation of the M₁ isozyme by FBP is observed only as a reversal of such an inhibited enzyme (14). Nevertheless, the biological significance of the lack of allosteric effects on the M₁ isozyme is not fully understood.

In the rat PK-M₁ and -M₂ isozymes, the exon that is exchanged due to the alternative splicing encodes 56 amino acids, in which a total of 21 amino acid residues differ within a length of 35 residues (8, 9). Thus, it was proposed that the distinguishable kinetic properties of M₁ and M₂ isozymes could be attributed to these amino acid substitutions. Moreover, it has been shown by x-ray crystallographic analyses and computer modeling that the corresponding regions of their polypeptides participate directly in the intersubunit contact, which may be primarily responsible for the intersubunit communication required for allosteric cooperativity (15, 16). Therefore, it has been proposed that the amino acid residue(s) in the intersubunit contact of the allosteric isozymes, particularly in the two α helices (Fig. 1, CaI and Ca2), play a central role in the allosteric interaction of the subunits (17, 18).

The M₁ isozymes of vertebrates seem to result from amino acid substitutions in ancestral allosteric isozymes since non-allesteric PKs such as the M₁ isozyme have been found in vertebrates but never in microorganisms (19–22). The M₁ isozyme is thought to be a specialized isozyme that arose in vertebrates for energy metabolism in particular tissues such as muscle and brain. The structure of the intersubunit contact of the PK-M₁ isozyme appears to prefer the active conformation, as the isozyme has been regarded as a model of the R-state (14). Many studies have focused on the structures prerequisite to allosteric properties in PKs (17, 18, 23), while the structural basis of the maintenance of the R-state in the M₁ isozyme still remains to be elucidated.

Since the primary structures of the M₁ and M₂ isozymes are identical except the region forming the intersubunit contact, the structure of the corresponding region of the M₂ isozyme permits allosteric interactions whereas that of the M₁ isozyme appears to prevent the enzyme from changing from its active conformation. To identify an amino acid residue required to maintain the active conformation as the R-state in the PK-M₁ isozyme, our approach was to substitute the amino acid residues specific to the allosteric isozymes of PK so that we would convert the rat M₁ isozyme into an allosteric enzyme. In the current study, we prepared a series of PK-M₁ mutants in which the residues in the intersubunit contact were replaced and characterized them to examine whether the substitutions induce allosteric properties. This functional analysis provides insight into the contribution of a single amino acid residue to the maintenance of the R-state. In addition, since PK-M₁ is the

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1 The abbreviations used are: PK, pyruvate kinase; PEP, phosphoenolpyruvate; FBP, fructose 1,6-bisphosphate; Sₚₜ, substrate concentration giving one-half of Vₘₐₓ; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); Vₘₐₓ, apparent Vₘₐₓ; Kₘₐₓ, apparent Kₘₐₓ;
most stable isozyme in mammalian PKs, this allosteric mutant of PK-M1 might be available as a T-state model for further structural analyses such as x-ray crystallography.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonuclease and DNA-modifying enzymes were purchased from Takara. Fructose 1,6-bisphosphate trisodium salt was 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 common chemicals were from Wako Pure Chemicals or Nacalai Tesque.

**Construction of the Transfer Plasmid**—A 5′-SalI-PstI 0.65-kb fragment of rat PK-M1 cDNA, which we cloned previously (8), was subcloned into a pBluescript SK+ vector (Stratagene). Subsequently, a BamHI-PstI 0.67-kb fragment was excised from the resulting plasmid and then ligated into a pBluescript SK+ vector. A 3′-BglII-EcoRI 1.3-kb cDNA fragment was ligated downstream of the 5′-SalI fragment (8) encoding the wild-type PK-M1 cDNA, which contains the amino acid sequence different between the M1 and M2 isozymes, was subcloned into a pTV119N vector (Takara). Subsequently, a HindIII-EcoRI 1.9-kb fragment, which contains the entire coding sequence, was excised from the resultant plasmid and inserted into a transfer vector, pVL1989 (Invitrogen).

**Site-directed Mutagenesis**—Site-directed mutagenesis was carried out according to Kunkel (24), as described previously (25). Prior to mutagenesis, the SphI-KpnI 0.2-kb fragment of rat PK-M1 cDNA, which contains the amino acid sequences different between the M1 and M2 isozymes, was subcloned into a pTV119N vector (Takara). Subsequently, a HindIII-KpnI fragment excised from the resultant plasmid was then ligated to pBluescript SK+.

The uracil-template was used with synthetic oligonucleotide primers to replace residues of the M1 isozyme with those of mammalian allosteric PKs. The oligonucleotide primers used in this study are follows: sense 5′-GGACACCCGTTGACCCCGCTG-3′ for replacement of Phe-389 by Tyr (designated as F389Y), 5′-GAAGACGTTCGGC- CGAGCCCTCC-3′ for Ala-398 by Arg (A389R), 5′-GAGCCTTCCGGC- AGCCGGCCCTCCATCCAGACCCC-3′ for a double mutation of Ser-401 by Ala and Ser-402 by Pro (S401A/S402P), 5′-TCACACAGTTCGCCAGAGGCATG-3′ for Leu-408 by Thr (L408T), 5′-TATACAAAGTTT-GCGCAGACGC-3′ for Leu-423 by Cys (L423C), and 5′-GGACACCCG- CATCAGTCTTCTC-3′ for Leu-427 by Ile (L427I). The resulting mutations were verified by dideox sequencing using a DNA sequencer (Applied Biosystems, model 373A), as were the entire sequences subjected to mutagenesis. The corresponding region of the wild-type PK-M1 cDNA was replaced by each mutant sequence. The transfer plasmids for the mutant enzymes were constructed similarly to those of the wild-type enzyme and used for transfection.

**Cell Culture and General Manipulation of Viruses**—Spodoptera frugiperda (Sf) 21 cells were maintained at 27 °C in Grace’s insect media (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 3.33 g/liter yeastolate, 3.33 g/liter lactalbumin hydrolysate, and 100 μg/liter kanamycin. Recombinant viruses were manipulated as described (26).

**Preparation of Recombinant Viruses**—The purified transfer plasmids containing the wild-type or mutant PK-M1 cDNA (1 μg) were co-transfected into 5 × 10⁷ SF21 cells with 10 ng of BaculoGold DNA (PharMingen), which was used as Autographa californica nuclear polyhedrosis viral genome. Transfection experiments were carried out by the Lipo- fectin (Life Technologies, Inc.) method (27), as described previously (28, 29). Media containing the recombinant viruses generated by homologous recombinations were collected 6 days after transfection. The recombinant viruses were further amplified to more than 5 × 10⁹ plaque-forming units/ml prior to use.

**Expression of Recombinant Rat PK-M1 in Insect Cells**—2 × 10⁷ SF21 cells were infected with the recombinant viruses carrying either wild-type or mutant PK-M1 at multiplicity of infection of more than 8. The infected cells were harvested after 90 h postinfection to purify the expressed proteins.

**Purification of the Recombinant Enzymes**—Expressed recombinant PKs were purified basically according to Imamura and Tanaka (1). 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, 1 mM EDTA, and 10 mM 2-mercaptoethanol (pH 7.5) with a Dounce homogenizer and were centrifuged at 10,000 × g to obtain clarified extracts. Subsequently, the supernatants were treated with polyethyleneimine to remove contaminating nucleic acids and then subjected to fractionation by 45–80% saturation of ammonium sulfate. After dialysis against 10 mM sodium phosphate, 2 mM MgSO₄, and 10 mM 2-mercaptoethanol (pH 8.0), the enzymes were subjected to a phosphocellulose column (Whatman P-11) pre-equilibrated with the same buffer used in dialysis. After washing intensively with the buffer containing 80 mM KCl, the bound enzymes were eluted with an addition of 0.5 mM PEP to the wash buffer.

**Electrophoresis**—The purified enzymes were subjected to SDS-PAGE analysis on 10% gels, according to Laemmli (30). The proteins were visualized by Coomassie Brilliant Blue R-250.

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

**Kinetic Analyses**—Enzymatic activity was assayed at 37 °C using various concentrations of PEP or ADP. The condition used for kinetics was the same as above except the substrates and effector. In assessment of parameters for one substrate, the concentration of the other was fixed at 2 mM. Ten different concentrations of PEP between 10 μM and 2.0 mM were used to obtain kinetic parameters for the substrate. When parameters for ADP were determined, six concentrations of the substrate from 62.5 mM to 2.0 mM were employed with the constant concentration of MgSO₄ (5 mM). Therefore, the parameters obtained for ADP are apparent ones. Release of pyruvate was monitored using a Beckman DU-640 spectrophotometer by coupling with the lactate dehydrogenase-NADH system. Kinetic parameters were obtained by fit-
**Allosteric Mutants of M<sub>t</sub>-type Pyruvate Kinase**

**Table I**

| Enzyme               | Phosphoenolpyruvate<sup>a</sup> | FBP<sup>b</sup> | Hill coefficient | ADP<sup>c</sup> |
|----------------------|---------------------------------|----------------|-----------------|-----------------|
|                      | V<sub>max(app)</sub> | S<sub>0.5</sub> |                  | V<sub>max(app)</sub> | K<sub>m(app)</sub> |
| Wild type            | µmol/min/mg        | µM            |                  | µmol/min/mg        | µM            |
| F389Y                | 440 ± 17           | 0.044 ± 0.005 | 1.0 ± 0.12      | 450 ± 17           | 0.043 ± 0.006  |
| A398R                | 490 ± 7            | 0.061 ± 0.003 | 1.5 ± 0.08      | 500 ± 21           | 0.10 ± 0.003  |
| L427I                | 510 ± 6            | 0.10 ± 0.003  | 1.2 ± 0.08      | 520 ± 21           | 0.12 ± 0.003  |

<sup>a</sup> Assayed in the presence of 2 mM ADP and 5 mM MgSO<sub>4</sub>.  
<sup>b</sup> Determined with variable ADP in the presence of 2 mM PEP and 5 mM MgSO<sub>4</sub>.  
<sup>c</sup> Activated with 1 mM FBP.  
<sup>d</sup> Equivalent to K<sub>m</sub> for the wild type.

**Fig. 3.** Sequence alignment of amino acid residues in the intersubunit contacts of PKs from various vertebrates. Shaded boxes indicate residues identical to rat M<sub>1</sub> isozyme. The regions corresponding to Ca1 and Ca2 are boxed. Arrowheads show the candidate residues to be examined in rat PK-M<sub>1</sub>. These are located at the positions where residues are conserved among allosteric PKs but not in M<sub>1</sub> isozymes.

**Fig. 4.** SDS-PAGE analysis of purified PK-M<sub>1</sub> mutants and wild type. One µg of the purified enzymes were subjected to SDS-PAGE. The gel includes a molecular mass marker in the left lane.

**Results**

We used the baculovirus-insect cell system, which is known as one of the most powerful expression systems (32), to produce a sufficient amount of active recombinant rat PK-M<sub>1</sub>. When insect cells were infected with the recombinant virus carrying the PK-M<sub>1</sub> cDNA, the PK activity increased to about 100 units/mg of cell protein 90 h after infection. The activity was more than 100 times greater than that in uninfected cells. As shown in Fig. 2, the expression level of the PK-M<sub>1</sub> protein was apparently as high as 20% of the total soluble proteins in the infected cells. Thus, a large amount of the active enzyme of rat PK-M<sub>1</sub> can be produced by the baculovirus system.

We obtained more than 10 mg of the purified enzyme from 2 × 10<sup>8</sup> cells infected with the recombinant virus. SDS-PAGE analysis demonstrates a single band of about 57 kDa, which corresponds to a single subunit of the M<sub>1</sub> isozyme (Fig. 2). When the purified enzyme was subjected to a gel permeation chromatography on a fast protein liquid chromatography system equipped with Superose 12 (Pharmacia), the elution time of the recombinant enzyme was identical to that of the authentic M<sub>1</sub> isozyme purified from rat muscle (data not shown), suggesting that the recombinant enzyme is a tetramer similar to the authentic isozyme (1, 2). In addition, kinetic properties of the recombinant M<sub>1</sub> given in Table I were indistinguishable from those of the authentic rat M<sub>1</sub> reported (1).

Amino acid sequences of the regions forming the intersubunit contacts of various PKs (7–10, 15, 37) were aligned to find candidate amino acid residues to be replaced for the conversion of PK-M<sub>1</sub> into an allosteric enzyme (Fig. 3). Mutagenesis was used to introduce changes into the corresponding positions in the mutant M<sub>1</sub> isozymes. The residues indicated by arrowheads in rat PK-M<sub>1</sub> were substituted by the corresponding amino acids that are conserved in allosteric PKs but not in M<sub>1</sub> isozymes.

**Experimental Procedures.** These purified mutants also exhibited a single protein band with apparent molecular mass of 57 kDa on SDS-PAGE (Fig. 4).

Kinetic behavior of the purified mutant M<sub>1</sub> enzymes with respect to PEP are shown in Fig. 5, and Table I compares kinetic parameters of the mutants with those of the wild type. Consistent with earlier work (1, 6), the wild-type M<sub>1</sub> demon-
strated a hyperbolic response to the increasing concentrations of the substrate and essentially no activation by FBP. On the other hand, all of the substituted PK-M₁ mutants exhibited a sigmoidal response to PEP to some extent and had greater \( S_{0.5} \) values. The \( S_{0.5} \) is analogous to the \( K_m \) in non-allosteric enzymes such as the wild-type M₁. Furthermore, the \( S_{0.5} \) values were sensitive to FBP, as their Hill coefficients and \( S_{0.5} \) values were decreased in the presence of 1 mM effector. Thus, every substitution resulted in appearance of both homotropic and heterotropic allosteric effects. The A398R mutant had the most pronounced homotropic cooperativity among the mutants, as indicated by its Hill coefficient of 2.7. This value was larger than the coefficient of 1.4–1.8 for the rat M₂ isozyme (1, 14, 38). The \( S_{0.5} \) value of 0.41 mM for the A398R mutant was 8.4 times greater than that of the wild-type M₁ (Table I) and was comparable to the value of 0.26–0.4 mM reported for the rat allosteric M₂ isozyme (1, 38). Thus, a single substitution by arginine at position 398 is sufficient to convert the non-allosteric PK-M₁ into a typical allosteric enzyme comparable to the PK-L, -R, and -M₂ isozymes.

To examine whether amino acid replacements caused concomitant effects on other kinetic parameters, apparent parameters of \( V_{\text{max}} \) and \( K_m \) for ADP were assessed by varying the concentrations of ADP in the presence of the fixed concentrations of PEP (2 mM) and MgSO₄ (5 mM). Hyperbolic responses were observed in all of the mutants, similar to the wild-type PK-M₁ (data not shown). Furthermore, none of the amino acid substitutions including the A398R caused any substantial changes of the apparent parameters for ADP (Table I). These results indicate that the substitutions affect the binding of PEP without any effect on the binding of ADP or on the catalysis.

Effects of FBP and L-phenylalanine on the A398R mutant were evaluated to characterize the nature of the allosteric properties of this mutant. It is known that these effectors act on the activity of PK through the shift of equilibrium of the allosteric transition between the active R-state and the inactive

![Kinetic behaviors of the wild-type and mutants of rat PK-M₁ isozyme with respect to PEP. Assays were carried out with various concentrations of PEP and 2 mM ADP in the absence (closed circles) and presence of 1 mM FBP (open circles). The condition used is described in detail under “Experimental Procedures.” Velocities are expressed as the values normalized by \( V_{\text{max}} \). Curves are drawn by fitting data sets to the Hill equation with non-linear regression.](http://www.jbc.org/entry/20498)

**Phosphoenolpyruvate (mM)**
Allosteric Mutants of M_1-type Pyruvate Kinase

The Hill coefficient of the mutant was not increased compared to the wild type (13, 14, 33). While FBP had little effect on the properties of the wild type (Fig. 6), the profile of the kinetic behavior of A398R changed remarkably from a sigmoidal curve to a nearly hyperbolic one as the concentration of FBP increased (Fig. 6B). As shown in Fig. 6, C and D, the \( S_{0.5} \) and Hill coefficient for A398R decreased in a simple saturating manner with the increase of FBP concentration. FBP caused little change in \( V_{\text{max}} \) of the mutant. The FBP activation profiles observed for A398R seem to be similar to those reported by Consler et al. (14), in which rabbit muscle PK was activated by FBP in the presence of L-phenylalanine. Activation of the A398R mutant by FBP allowed this enzyme to behave similar to the wild type, indicating that the effects of the substitution of Ala-398 by Arg on the kinetic properties are reversal by FBP. These results suggest that the consequence of the substitution is a reversible change that shifts the allosteric transition from the R-state to the T-state.

The other allosteric effector, L-phenylalanine, exhibits inhibitory effects by shifting the allosteric transition toward the T-state (14). \( S_{0.5} \) values increase with L-phenylalanine concentration, revealing that the T-state became predominant relative to the R-state in both the wild type and A398R mutant (Fig. 7, A, B, and C). In the wild type, the Hill coefficient increased from 1.0 up to about 2.5 in the range of the L-phenylalanine concentrations examined (Fig. 7, A and D). In contrast, the Hill coefficient of the mutant was not increased further by L-phenylalanine, but rather decreased at higher concentrations of the effector (Fig. 7D). Thus, these results show that the homotropic cooperativity of this mutant for PEP is nearly at its maximum and that the state of the allosteric transition in the unligated enzyme is largely shifted toward the T-state.

**DISCUSSION**

In the present study, we examined the effects of amino acid substitutions on the kinetic properties of the rat PK-M_1 isozyme to identify the residues responsible for the enzyme to favor the R-state. Our results suggest that Ala-398 is a critical residue for the stabilization of the R-state in the PK-M_1 isozyme. The requirement of Ala-398 for maintenance of the R-state was revealed by a major disturbance of the allosteric properties of the A398R mutant (Fig. 5). The other residues examined in this study appear to participate, at least in part, in exhibition of the unique kinetic properties of the PK-M_1 isozyme distinct from allosteric isozymes since replacements of these residues also affected the properties of the PK-M_1 isozyme, albeit to a lesser extent. It seems that the subtle interactions of residues in the intersubunit contact allow the PK-M_1 isozyme to barely maintain its active conformation regarded as the R-state.

Although Ala-398 has been shown to play an important role for the rat PK-M_1 isozyme to exhibit non-allosteric properties, the alanine residue is not conserved among M_1 isozymes from other species. Amino acids with aliphatic side chains of various lengths are common to position 398 or its equivalent position for all of the M_1 isozymes (Fig. 3). Therefore, it seems likely that allosteric effects are induced in the rat PK-M_1 by introduction of a positive charge to position 398 rather than by that of a bulky side chain. Furthermore, Ala-398 is located at a position that is able to interact with Arg-391 of the symmetrically associated subunit (16), a residue that is also conserved in L and R isozymes but not in M_2 isozyme. This could account for a more pronounced cooperativity found in the A398R mutant than for the native M_2 isozyme. It is possible that an interaction of the arginine residue introduced into position 398 with Arg-391 of the facing subunit could contribute significantly to the occurrence of more cooperative behavior.

The reaction mechanism of PK is believed to involve the ternary complex that consists of PEP, ADP, and the enzyme (15). In this view, the transfer of a phosphoryl group occurs directly from PEP to ADP, and does not proceed via a phosphoenzyme species. The subsites for binding of these substrates must be very close to each other to carry out the direct transfer. Nonetheless, operations of the residues in the intersubunit contact induced both homotropic and heterotropic allosteric effects in the PK-M_1 isozyme without substantial changes of the apparent \( K_{\text{app}} \) for ADP and of the catalytic constant, indicating that the effects of the amino acid replacements are specific to the binding of PEP and do not affect arrangement of catalytic residues or the subsite for ADP binding. Therefore, it is unlikely that gross structural effects occurred in the mutants resulting in a distortion of the PEP-binding site. The mutants of the PK-M_1 isozymes seem to...

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**Fig. 6.** Activation of A398R mutant by FBP. A and B, effects of FBP on the responses of the wild type (A) and A398R mutant (B) to various concentrations of FBP. Enzyme activities were measured in the absence (●) or presence of 0.5 mM (○), 1.0 mM (▲), 2.0 mM (▼), 4.0 mM (●), and 8.0 mM (▲) FBP. Velocities are normalized by \( V_{\text{max}} \) values calculated from the data set at each FBP concentration. C and D, effects of the activator on \( S_{0.5} \) values (○) and Hill coefficients (▲) of the wild type (▲) and the mutant (●).
mimic the allosteric transition displayed by the native allosteric isozymes. These are consistent with the suggestion that the amino acid residues in the intersubunit contact region regulate the state of the allosteric transition of PKs.

In the FBP activation profile of A398R, data fitting by a simple saturation model reveals that the apparent activation constant for FBP is about 1 mM (Fig. 6, C and D). This value is much higher than micromolar or submicromolar levels for native allosteric PKs such as M₂ and L isozymes (1). In contrast to these allosteric isozymes, the low binding affinity for FBP is intrinsic to mammalian M₁ isozyme, as indicated by the apparent activation constant of millimolar range (14). Therefore, the amino acid substitution has no significant effect on the binding of FBP since it is likely that the replacement leads to no alteration in the intrinsic properties of the M₁ isozyme. This suggests that the mutation introduced into the intersubunit contact does not change the local environment at the FBP-binding site.

Vertebrate PK-M₁ isozymes are predominantly expressed in muscle, heart, and brain, organs that are always more energy-consuming than other organs and tend to demand more glucose. Those organs seem to prefer smooth consistent catabolism consuming than other organs and tend to demand more glucoses. Hence, this mutant is available as a stable model of PK in the T-state and might enable us to prepare a crystal of the T-state of mammalian PKs.

Recently, the unligated PK of E. coli was crystallized as the T-state conformation, and its crystal structure has been solved. The comparison between the structures of the E. coli PK (as the T-state) and mammalian M₁ isozyme (as the R-state) showed that the two structures are unstable without FBP. The stability of A398R, the most pronounced allosteric mutant, was found to be comparable to that of the wild-type PK-M₁ isozyme, as it could be purified and stored in the absence of a stabilizing agent such as FBP. Hence, this mutant is available as a stable model of PK in the T-state and might enable us to prepare a crystal of the T-state of mammalian PKs.

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