Pyruvate kinase (PK, EC 2.7.1.40) catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, producing pyruvate and ATP, and is a key enzyme in the glycolytic pathway (1–3). Four different isozymes are known to occur in mammalian tissues, namely the L, R, M₁, and M₂ isozymes (1, 3–6). The PK-L gene locus encodes for the L and R isozymes, whereas the M₁ and M₂ isozymes arise from a common gene, the PK-M gene locus (5–7). With the exception of the M₁ isozyme, these enzymes are allosteric enzymes and show positive homotropic cooperativity with respect to the substrate, PEP. The allosteric PKs undergo efficient activation in a heterotropic manner by fructose-1,6-bisphosphate (FBP), an intermediate metabolite in the glycolytic pathway, whereas the M₁ isozyme is devoid of allosteric properties (1, 2, 8). For the case of the allosteric PKs, the conformation of the enzyme is converted from the inactive T-state to the active R-state on the binding of ligands such as PEP and FBP. On the other hand, it is known that in the nonallosteric M₁ isozyme the equilibrium for the distribution between the T- and R-states is in favor of the R-state even in the absence of the ligands (1, 4).

Although allosteric M₂ and nonallosteric M₁ arise from a common gene, mutually exclusive alternative splicing in the transcript produces the structurally different enzymes (6, 7). The primary structures of these two isoforms differ in the regions encoded by single exons (exon 9 for M₁ and exon 10 for M₂). Each exon encodes a stretch of 56 amino acids, which comprises approximately 10% of the total polypeptide sequences of these PKs. Because only 23 amino acids are different in this region, the distinct kinetic properties of the M₁ and M₂ isoforms must be because of this structural difference (6, 9). Because the region forms the intersubunit contact, it has been suggested that it is involved in cooperative interactions between the subunits (10–13), as shown in our previous study (14, 15). Mutations in this region in the M₁ isozyme stabilize the inactive conformation and thereby shifts the allosteric transition toward the T-state, leading to the development of homotropic cooperativity with respect to PEP. This indicates that the allosteric transition is greatly shifted toward the R-state for the case of the M₁ isozyme.

The unregulated kinetic behavior of the M₁ isozymes is due, not only to the consequence of the shifted allosteric transition, but also to insensitivity to FBP-induced activation. Because the allosteric transition of the M₁ isozyme is intrinsically shifted toward the active conformation, the isozyme no longer undergoes activation by FBP. However, even under conditions where the transition is shifted to the T-state by either mutations or l-phenylalanine inhibition, the isozyme is not activated by FBP as efficiently as the M₂ isozyme. It has been found that an apparent activation constant (Kₐ(app)) for FBP, which is the concentration of FBP required for one-half activation under defined conditions, is less than 1 μM in the M₂ isozyme whereas the Kₐ(app) value for the M₁ isozyme is more than a thousand times higher, as shown by the activation of an allosteric mutant of the rat M₁ isozyme and the l-phenylalanine-inhibited rabbit M₁ isozyme by FBP (8, 9, 15). Thus, it has been suggested that the amino acid sequence of the M₁ isozyme, which differs from that of M₂, also includes a structural factor that causes a lowering in affinity for FBP. The M₁ isozyme is believed to be the result of the evolution of a prototypic allosteric PK for energy metabolism in specialized energy-consuming tissues, such as brain and heart. Therefore, the unregulated kinetic behavior, which is based on the shifted allosteric transition, and an insensit-

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¹ The abbreviations used are: PK, pyruvate kinase; PEP, phosphoenolpyruvate; FBP, fructose-1,6-bisphosphate; HPLC, high performance liquid chromatography.
The FBP-binding site in PKs. The entire structure of subunit (A) and the FBP-binding pocket (B) when complexed with FBP and phosphoglycerol, a PEP analog, in yeast PK are illustrated. The loop between Cβ1 and Cα3 (nonallosteric) and the FBP-binding pocket (active site) are shown. The side chain of Arg-459 involved in the interaction with the 1'-phosphate of FBP is indicated along with Asp-455, which interacts with the arginine. Thr-403 is also shown, to indicate the position of the glutamic acid residue. Amino acid sequences of nonallosteric and allosteric PKs from several species in the region participating in the FBP-binding site (5-7, 10, 29-32) are aligned. Glutamic acid residues, which are conserved only in the M1 isozymes are highlighted by shaded boxes.

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 mM KCl, 5 mM MgSO4, and 0.5 mM FBP (pH 7.5) as described previously (14). The resulting mutations were verified by dideoxy sequencing using a DNA sequencer (Applied Biosystems, model 373A), as were the entire sequences, which were subjected to mutagenesis. After replacing the corresponding sequence of the wild-type M1 with the mutated sequences, the transfer plasmids for the mutant enzymes were constructed in a manner similar to that of the wild-type enzyme and were used for transfection.

Electrophoresis—The purified enzymes were subjected to SDS-polyacrylamide gel electrophoresis analysis on 15% gels, according to Laemmli (21, 22). The resulting proteins were subjected to autoradiography. The positions of the PK isozymes were determined by staining with 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 mM KCl, 5 mM MgSO4, and 0.5 mM FBP (pH 7.5) as described previously (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 in absorbance at 340 nm. One unit of activity was defined as the amount of enzyme required to release 1 μmol of pyruvate/min.

Kinetic Analyses—Enzymatic activity was assayed at 37°C using...
various concentrations of PEP and ADP. The condition used for kinetics was the same as above except for the substrates and effector. In the assessment of parameters for one substrate, the concentration of the other was fixed at 2 mM. Twelve different concentrations of PEP between 5 μM and 2.0 mM were used to obtain kinetic parameters for the substrate. Parameters for ADP were determined with seven concentrations of the substrate from 31.3 μM to 2.0 mM. The release of pyruvate was monitored using a Beckman DU-640 spectrophotometer by coupling with the lactate dehydrogenase-NADH system. Kinetic parameters were obtained by fitting the data for varying 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.

Effects of the Allosteric Activator and Inhibitor on Enzyme Activity—When the value for \( K_{\text{act(app)}} \) for FBP was estimated, the activity was determined using various concentrations of FBP with 100 μM PEP and 2 mM ADP in the absence of l-phenylalanine for the A398R and the double mutants or in the presence of 2 mM l-phenylalanine for the wild type, E432A, E432K, and E432D. On the other hand, 1 mM PEP and 2 mM ADP were used with various concentrations of l-phenylalanine for the inhibition. Other assay conditions were the same as the standard assay of PK.

Fluorescence Change Induced by FBP—Intrinsically tryptophan fluorescence quenching induced by FBP was measured with a fluorescence spectrophotometer (Shimazu RF5000). The wavelengths used were 280 nm for excitation and 350 nm for emission. Fluorescence was measured at 37 °C in 0.1 M KCl, 50 mM Tris-HCl buffer, 5 mM MgSO\(_4\) (pH 7.5), and various concentrations of FBP. Protein concentration was 0.5 mg/ml. Fluorescence quenching (\( Q \)) was calculated according to Equation 1,

\[
Q = \frac{F_0 - F}{F_0} \quad \text{(Eq. 1)}
\]

where \( F_0 \) and \( F \) are fluorescence intensities in the absence and presence of FBP, respectively. In the increasing phase of fluorescence quenching, the data were fitted to Equation 2 to determine \( K_d \) for FBP,

\[
Q = Q_{\text{max}} \times \left( \frac{[\text{FBP}]}{[\text{FBP}] + K_d} \right) \quad \text{(Eq. 2)}
\]

where \( Q_{\text{max}} \) represents the maximal quenching, \( K_d \) the dissociation constant, and \( n \) the Hill coefficient.

Size Exclusion Chromatography—Subunit assembly of the PK-M1 mutants was examined by size exclusion chromatography using an HPLC system equipped with a TSKgel G3000SWXL (7.8 × 300, Tosoh). The solvent used was 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M KCl and 5 mM MgSO\(_4\), and the flow rate was 1.0 ml/min. Twenty μl of a solution containing 0.5 mg/ml enzymes were injected into the column. The eluted proteins were monitored by absorbance at 210 nm.

Protein Determination—Protein contents were determined according to the method of Bradford using bovine serum albumin as a standard (24).

RESULTS

The recombinant baculoviruses that carried the cDNAs for the PK-M1 mutants in which replacement of Glu-432 by Ala, Lys, and Asp had been made in the wild type and the A398R mutant were prepared and used for expression of the recombinant proteins. The mutant enzymes were successfully expressed in yields of 10–20% of the total soluble proteins, as described previously for the wild-type and other mutants of the rat M1 isozyme (14). The recombinant proteins were purified from the baculovirus-infected cells, and SDS-polyacrylamide gel electrophoresis analysis of the purified proteins indicated single bands of 57 kDa corresponding to the single subunits in all mutants (data not shown).

The single mutants in which Glu-432 was replaced by Ala, Lys, and Asp were subjected to kinetic analysis to examine the effects of these replacements on the kinetic properties of the protein. As shown in Fig. 2 and Table I, these replacements did not significantly alter the kinetic properties of the PK-M1, either in the presence or absence of 1 mM FBP, suggesting that these mutations at Glu-432 have little effect on the intrinsically shifted equilibrium of the allosteric transition. In addition, these amino acid replacements appeared to lead to no alteration of catalysis or the binding of substrates, PEP and ADP, as shown by the \( V_{\text{max}} \) and \( K_m \) values, which were similar to those of the wild type. When the wild type and these single mutants were inhibited by various concentrations of l-phenylalanine, which is known to inhibit PK via shifting the equilibrium toward the inactive T-state (9, 26–28) in the presence of 1 mM PEP and 2 mM ADP, no marked difference was observed in the susceptibility to the inhibitor among the enzymes (data not shown). As shown in Fig. 3, however, it was found that the E432A and E432K mutants are activated by FBP much more efficiently than the wild type and the E432D mutant, when the susceptibility of the wild type and the single mutants to FBP was examined by evaluating the activation of the enzymes inhibited by 2 mM l-phenylalanine. This suggests that the existence of a negatively charged side chain of the residue 432 specifically precludes the activation of the enzyme by FBP but that it has no other effects on the kinetic properties.

For a more detailed examination of the inhibitory role of the Glu-432 in the activation by FBP, the double mutants in which Glu-432 had been replaced by Ala, Lys, and Asp in the allosteric PK-M1, mutant, A398R, were characterized by kinetic analysis. These double mutants would be useful in investigating the function of Glu-432 in the heterotropic allosteric effect by FBP, because the A398R single mutant has been found to
A398R/E432D mutants continued to show significant sigmoidal
concentration of FBP in the presence of 2 mM L-phenylalanine, 100
of Glu-432 by FBP. Activities were assayed with the various concen-
tration. The presence of 1 mM FBP in the assay resulted in left-
shifted plots, as shown in Fig. 2, and thus it was found that these double
mutants retain cooperative properties with respect to PEP in a
manner similar to the single mutant, A398R. The $K_{d\text{act}}$(app) for ADP in the double mutants and $V_{\text{max(app)}}$ were essentially the same as those for the wild type and the A398R mutant (Table 1). The additional mutations introduced into Glu-432 appeared to lead to a slight modification in the homotropic allosteric effect, as compared with the A398R. However, the $S_{0.5}$ values and the Hill coefficients of the double mutants were still much higher than those of their corresponding single mutants, which lacked the Ala-398 mutation. These suggest that the replacements of Glu-432 have only modest effects on the equilibrium of the allosteric transition.

The presence of 1 mM FBP in the assay resulted in left-
Figure 3. Activation of L-phenylalanine inhibited single mutants
of Glu-432 by FBP. Activities were assayed with the various concen-
tration. The presence of 1 mM L-phenylalanine, 100 mM PEP, and 2 mM ADP. Circles denote the wild type, triangles the E432K mutant, squares the E432A mutant, and diamonds the E432D mutant.

Table I
Kinetic parameters for the wild-type and mutants of rat PK-M1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>FBP $-10^{-5}$</th>
<th>1 mM FBP</th>
<th>ADP $-10^{-5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max(app)}}$</td>
<td>$S_{0.5}$</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>Wild type</td>
<td>530</td>
<td>58</td>
<td>1.2</td>
</tr>
<tr>
<td>E432A</td>
<td>520</td>
<td>66</td>
<td>1.3</td>
</tr>
<tr>
<td>E432K</td>
<td>550</td>
<td>70</td>
<td>1.2</td>
</tr>
<tr>
<td>E432D</td>
<td>470</td>
<td>47</td>
<td>1.0</td>
</tr>
<tr>
<td>A398R</td>
<td>560</td>
<td>390</td>
<td>2.7</td>
</tr>
<tr>
<td>A398R/E432A</td>
<td>560</td>
<td>530</td>
<td>1.9</td>
</tr>
<tr>
<td>A398R/E432K</td>
<td>550</td>
<td>510</td>
<td>2.5</td>
</tr>
<tr>
<td>A398R/E432D</td>
<td>520</td>
<td>370</td>
<td>2.1</td>
</tr>
</tbody>
</table>

$^a$ Assayed with 2.0 mM ADP in 50 mM Tris-HCl, 0.1 M KCl, and 5.0 mM MgSO4 (pH 7.5).
$^b$ Standard errors were within 5% in all parameters.
$^c$ Assayed with 2.0 mM PEP in 50 mM Tris-HCl, 0.1 M KCl, and 5.0 mM MgSO4 (pH 7.5).
$^d$ In the absence of FBP.

To compare the susceptibility of the A398R and the double mutants to FBP by estimating the $K_{d\text{act}}$(app) for FBP, the enzyme activities were determined in the presence of various concentrations of FBP with the fixed concentrations of 100 mM PEP and 2 mM ADP (Fig. 4). The $K_{d\text{act}}$(app) values for the A398R and A398R/E432D mutants were as high as 900 and 1700 mM, respectively, whereas $K_{d\text{act}}$(app) for A398R/E432A and for A398R/E432K was much lower, 77 and 33 mM, respectively. It was found that the FBP-induced activation of the A398R and A398R/E432D mutants, both of which have negatively charged side chains at position 432, is much less efficient than the other double mutants which lack a negative charge at position 432. Furthermore, because both replacements by a nonpolar amino acid and one which contained a positively charged side chain decreased the $K_{d\text{act}}$(app) to a similar extent, it seems unlikely that a positive-charged side chain plays an important role in the efficiency of activation by FBP. Slight decreases in the activities observed at extremely high concentration of FBP could be because of the interaction of the effector with the active site for ADP and/or PEP because the effector has two phosphoryl groups common to both the substrates. These findings strongly indicate that the mutations leading to a loss of the negative charge at the residue 432 cause a dramatic enhancement in activation by FBP. These results suggest that the negatively charged $\gamma$-carboxyl group of Glu-432 serves to hinder the heterotropic allosteric effect involving FBP in the M1 isozyme and are consistent with the results obtained from the analysis of the single mutants.

It is well known that allosteric PKs undergo conformational changes, which reflect the allosteric transition from the inactive T-state to the active R-state, on the binding of FBP, and this conformational change can be detected as a change of the intrinsic fluorescence of tryptophan (25, 26). Because the enzyme conformation in the FBP-bound state displays a fluorescent intensity that is different from that without the effector, the binding of FBP can be titrated by monitoring the change of fluorescence intensity (25). To investigate the effects of the replacements of Glu-432 by various amino acids on the binding of FBP, the apparent dissociation constant ($K_{d\text{act}}$(app)) for the effector was estimated by monitoring the intrinsic tryptophan fluorescence quenching that results from the binding of FBP for a series of the allosteric PK-M1 mutants. As shown in Fig. 5, the increase in fluorescence quenching was observed in the concentration range of FBP where each enzyme was activated. When $K_{d\text{act}}$(app) for FBP was calculated from the fluorescence quenching data, the values for A398R/E432A and A398R/E432D mutate...
E432K were 79 and 48 \( \mu M \), respectively, whereas the values for the A398R single mutant and A398R/E432D were 490 and 1400 \( \mu M \), respectively. These \( K_d^{(app)} \) values are in good agreement with the \( K_{act}^{(app)} \) values determined on the basis of the activation.

However, it has been reported for an allosteric PK, the M\(_2\) isozyme, that this isozyme undergoes reversible subunit assembly, the equilibrium of which is affected by the binding of FBP (34, 35). In the allosteric M\(_2\) isozyme, the absence of FBP appears to induce the dissociation of the subunits, and the binding of the effector may possibly depend on the state of the subunit assembly. If the replacement of Ala-398 by Arg in PK-M\(_1\) shifts the equilibrium of the subunit assembly and then the second replacements of Glu-432 by Ala and Lys reverse this altered equilibrium, it is possible that the decreased \( K_{act}^{(app)} \) or \( K_d^{(app)} \) in the A398R/E432A and A398R/E432K mutants might reflect the modulated equilibrium of the subunit assembly, rather than the modification of the FBP-binding pocket. To test this possibility, the subunit assembly of the allosteric mutants was explored by analytical size exclusion chromatography at the same enzyme concentrations as were used for fluorescence quenching study. As shown in Fig. 6, all the allosteric mutants, the A398R single mutant and the double mutants, form tetrameric structures even in the absence of FBP in a manner similar to the wild type and an authentic rabbit muscle PK. The results indicate that the amino acid replacements of Ala-398 and Glu-432 do not affect the equilibrium of the subunit assembly, and thus suggests that the decreased \( K_{act}^{(app)} \) and \( K_d^{(app)} \) in the A398R/E432A and A398R/E432K mutants are because of the modulation of the FBP-binding pocket rather than the alteration of the subunit assembly. Therefore, as revealed by the fluorescence quenching study, it may be concluded that the negatively charged side chain of the residue-432 serve to directly prevent the binding of FBP and thereby impair the heterotropic allosteric effect involving FBP.

**DISCUSSION**

As reported previously, whereas the mutant M\(_1\) isozyme (A398R) in which Ala-398 has been replaced by Arg, the corresponding residue in the allosteric M\(_2\) isozyme, exhibits a homotropic allosteric effect with respect to PEP, this mutant is still not efficiently activated by FBP. The aim of the present study was to identify a residue that hinders the activation by FBP in the M\(_1\) isozyme of PK, and to elucidate the molecular basis for the lack of the efficient heterotropic allosteric effect involving FBP in this isozyme. It would be difficult to examine the involvement of the residue in terms of its insensitivity to FBP without this useful mutant, because the M\(_1\) isozyme is fully active even in the absence of FBP. The findings reported herein suggest that Glu-432, the conserved glutamic acid residue among the M\(_1\) isozymes from various species, serves to impair the heterotropic allosteric effect by preventing the binding of the allosteric effector, FBP. However, this inhibitory effect of the residue on the binding of FBP does not appear to be because of a loss of the FBP-binding residue, resulting from the replacement of a certain amino acid of importance in the binding by glutamic acid, but rather it is more likely that the conserved Glu-432 plays a dominant negative role in the binding of FBP. Furthermore, it also appears that a negative charge on this residue is required for inhibiting the binding of FBP.

Our results are, in part, consistent with suggestions made on the basis of crystallographic analysis (16). However, although the replacement of Glu-432 in the rat M\(_1\) isozyme by the corresponding residue in the rat M\(_2\) isozyme, Lys, substantially decreased the \( K_{act}^{(app)} \) for FBP, the value is still about 100 times higher than that of the M\(_2\) isozyme (15). We thus con-
clude that, not only a difference at residue 432 accounts for the extremely high affinity for FBP in M₂ isozymes, but other residues or structural factors may also be involved. Nevertheless, the present study clearly indicates that a negatively charged side chain of the residue 432, the γ-carboxyl group in glutamic acid, is of critical importance for the M₁ isozyme to lose its susceptibility to FBP.

The loop between the Cβ1 strand and the Cα3 helix and a portion of the Cα5 helix, all of which participate in the FBP-binding site, of the M₁ isozyme from rabbit muscle were compared with the corresponding structures of FBP-complexed yeast PK (Fig. 7). The structures of the mammalian M₁ isozyme were aligned to those of the yeast enzyme by fitting coordinate data for the α-carbons (16, 33). The FBP molecule is located according to the coordinate data for the effector from the crystallographic analysis of the FBP-complexed yeast enzyme (16). Side chains of Glu-432 and Arg-488 of the rabbit enzyme and Arg-459 of the yeast PK are shown, as well as the main chains, and the rabbit enzyme structure is highlighted. A, a schematic representation of possible interactions between the mammalian M₁ isozyme and FBP. Numbers beside the dashed lines indicate the distance between the atoms in Å. Numbers in parentheses are the distance estimated from the superimposition of FBP into the mammalian M₁ isozyme structure.

Glu-432 does not appear to prevent the binding of FBP via impairing the interactions between Arg-488 and the 1'-phosphate of FBP, as positioned analogous to that of the yeast PK, even though the γ-carboxylate of Glu-432 in mammalian may possibly interact with the guanidino group of an arginine residue, which is situated 4.7 Å from the carboxylate group. In addition, the Glu-432 causes no disorder in the loop involved in the interaction with the 6'-phosphate group of FBP, and it is also unlikely that the γ-carboxyl group occupies the binding pocket by mimicking the phosphate, as shown in this modeling.

Glu-432 does not appear to prevent the binding of FBP via impairing the interactions between Arg-488 and the 1'-phosphate of FBP because the mutant in which Glu-432 was replaced by Asp also exhibited the similar effect on the interaction with the effector, despite its shorter side chain. This replacement would be expected to lead to a longer distance between the carboxyl group and Arg-488, therefore weakening their interaction, if the carboxyl group at the residue-432 significantly interacted with the guanidine group of the arginine residue. On the other hand, the γ-carboxylate of Glu-432 appears to be about 4.1 Å away from the 1'-phosphate of FBP and more than 6.7 Å from the other phosphate, as estimated in the superimposition of FBP into the M₁ isozyme structure by alignment. This suggests that Glu-432 serves to hinder the binding of FBP through its repulsive electrostatic interaction with the 1'-phosphate, rather than the 6'-phosphate. The higher $K_{act,app}$ and $K_{d,app}$ values found in the case of the re-

![Amino Acid Substitution in the FBP-binding Site of PK-M₁](http://www.jbc.org/)
placement of Glu-432 by Asp in the A398R mutant suggest more enhanced repulsive interactions with FBP, and this could be because of an interaction with 6’-phosphate, in addition to that with the 1’-phosphate group.

The M1 isozymes have lost the regulatory properties in which glycolytic metabolites, such as PEP and FBP control the enzyme activity, and the substitution of glutamate for the residue 432 is one of the most critical substitutions to have accumulated during the molecular evolution of the PKs, because the substitution led to a loss of regulation by FBP. However, sole replacement of Glu-432 by another amino acid examined did not appear to significantly affect the persistent active conformation of the M1 isozyme, suggesting that the allosteric transition is intrinsically shifted toward the R-state in the M1 isozyme independently of the Glu-432 residue. This shifted allosteric transition toward the R-state would be sufficient for the loss of regulations by both PEP and FBP, and it is entirely possible that Glu-432 is not necessarily required for such unregulated properties. In this respect, it may be possible that the replacement by another residue at position 432, which had occurred in the allosteric prototype of the PK-M1, might be an excess step in the evolutionary process for the acquisition of the unregulated properties.

However, the absolute conservation of the glutamic acid residue in the M1 isozymes suggests that the residue may be required for the isozyme to avoid any unknown and/or more complicated regulating mechanism involving FBP. Therefore, the elucidation of the significance of the glutamic acid residue leads us to understand the regulating mechanism(s) of PKs in more detail and thereby to provide new insights into the regulation of the glycolytic pathway.

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Dominant Negative Role of the Glutamic Acid Residue Conserved in the Pyruvate Kinase M1 Isozyme in the Heterotropic Allosteric Effect Involving Fructose-1,6-bisphosphate
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