# Site-directed mutagenesis of the amino acid residues in β-strand III [Val$^{30}$–Val$^{36}$] of D-amino acid aminotransferase of *Bacillus* sp. YM-1

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**Abstract** The β-strand III formed by amino acid residues Val$^{30}$–Val$^{36}$ is located across the active site of the thermostable D-amino acid aminotransferase (D-AAT) from thermophilic *Bacillus* sp. YM-1, and the odd-numbered amino acids (Tyr$^{31}$, Val$^{33}$, Lys$^{35}$) in the strand are revealed to be directed toward the active site. Interestingly, Glu$^{32}$ is also directed toward the active site. We first investigated the involvement of these amino acid residues in catalysis by alanine scanning mutagenesis. The Y31A and E32A mutant enzymes showed a marked decrease in $k_{cat}$ value, retaining less than 1% of the wild-type enzyme activity. The $k_{cat}$ values of V33A and K35A were changed slightly, but the $K_m$ of K35A for α-ketoglutarate was increased to 35.6 mM, compared to the $K_m$ value of 2.5 mM for the wild-type enzyme. These results suggested that the positive charge at Lys$^{35}$ interacted electrostatically with the negative charge at the side chain of α-ketoglutarate. Site-directed mutagenesis of the Glu$^{32}$ residue was conducted to demonstrate the role of this residue in detail. From the kinetic and spectral characteristics of the Glu$^{32}$-substituted enzymes, the Glu$^{32}$ residue seemed to interact with the positive charge at the Schiff base formed between the aldehyde group of pyridoxal 5′-phosphate (PLP) and the ε-amino group of the Lys$^{145}$ residue.

*Key words:* D-Amino acid aminotransferase; Site-directed mutagenesis; Substituted aldamine; pH titration; Pyridoxal 5′-phosphate

## 1. Introduction

D-Amino acid aminotransferase catalyzes the transamination reaction between D-amino acid and α-keto acid and is known to play a key role in the synthesis of D-glutamic acid in *Bacillus* sp. [1]. In this case, D-alanine acts as an amino group donor and α-ketoglutarate as an amino group acceptor for the transamination reaction. During the last decade, much attention has been paid to the reaction mechanism of the enzyme, and D-AAT was found to catalyze the transamination reaction through the same mechanism as the L-specific aminotransferases [2].

Tanizawa et al. [3,4] isolated and characterized a thermostable D-AAT from the thermophilic *Bacillus* sp. YM-1. This enzyme was observed to have a broad substrate specificity, and thus a variety of D-amino acids and α-keto acids could be used as an amino group donor or acceptor. Recently, the three-dimensional structure of this enzyme was determined [5], but detailed assignment of the amino acid residues constituting the substrate binding domain has yet to be carried out.

We have been focusing on random mutagenesis of the enzyme to improve the catalytic properties and observed that substitution of alanine for the Val$^{33}$ residue resulted in a significant change in the catalytic properties of the enzyme (unpublished result). The Tyr$^{31}$ residue is known to interact with the 3′-oxygen of pyridoxal 5′-phosphate (PLP), a cofactor required by the mutant [5,6]. These results led us to speculate that these amino acid residues might play an important role in enzyme catalysis. The three-dimensional structure of D-AAT revealed that these amino acid residues were observed to be included in the β-strand III formed by amino acid residues 30–36. This β-strand is located across the active site and the side chains of odd-numbered amino acid residues face the active site. Contrary to other even-numbered amino acid residues, the side chain of Glu$^{32}$ residue is also directed toward the active site.

In this study, we demonstrate the role of each amino acid residue involved in the formation of β-strand III based on the kinetic characteristics of the mutant enzymes by alanine scanning mutagenesis. The Glu$^{32}$ residue which was observed to be critical to enzyme catalysis was replaced with various amino acids by site-directed mutagenesis. The resulting mutant enzymes were studied in terms of kinetic and spectral properties.

## 2. Materials and methods

### 2.1. Plasmids, bacterial strain, and culture conditions

*Escherichia coli* XL1 Blue was used as the host strain. Plasmid p*Trc*99A was purchased from Pharmacia. Cells were grown in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) containing 1 mM IPTG and 100 μg/ml ampicillin at 37°C. Restriction enzymes were obtained from Boehringer. T4 DNA ligase was from Gibco BRL. DNA sequencing was conducted with a Sequenase ver. 2.0 kit (USB). DNA polymerase used for the site-directed mutagenesis was Vent polymerase from NEB. Oligonucleotides were synthesized at Korea Biotech. All other chemicals were of analytical grade.

### 2.2. Site-directed mutagenesis

Mutagenesis was carried out by the megaprimer method using a two-stage polymerase chain reaction [7]. The mutagenic primers were designed depending on the amino acid residue to be replaced as follows: Y31A, 5′-CTT TCA CAA CTT CAG CTA CGC CAT C-3′; E32A, 5′-AC TTT CAC AAC TGC ATA TAC GCC-3′; E32D, 5′-AC TTT CAC AAC ATC ATA TAC GCC-3′; E32Q, 5′-AC TTT CAC AAC TTG ATA TAC GCC-3′; V33A, 5′-TAT ACT TTC ACA GCT TCA TAT AC-3′; K35A, 5′-TTT CAC CGT TAT ATA CTG CCA CAA CTT C-3′. The underlined bases are mismatched ones. The primers for each terminus were as follows: N-terminal primer, 5′-GCC ATG GGA TAC ACT TTA TGG AA-3′; C-terminal primer, 5′-CCT GCA GGA TCC AAG CTT ATT ATA TAT GAA

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GCG GTT TTG-3′. The N-terminal primer contained *Nco*I site and C-terminal primer had *Pst*I, *Bam*HI, and *Hin*dIII sites.

The megaprimer was made in the first stage PCR using N-terminal primer as the upstream primer, a mutagenic primer as the downstream primer, and pICT113P [2] as the template DNA. The second stage PCR was performed to make a full-length D-AAT gene with the megaprimer as upstream primer and the C-terminal primer as downstream primer. The PCR fragment was digested with *Nco*I and *Bam*HI and inserted into the p*Trc*99A plasmid. The sequence of each mutant was confirmed by DNA sequencing.

*2.3. Enzyme purification*

Cells were suspended in 30 mM Tris-HCl (pH 7.3) containing 0.01% β-mercaptoethanol and 20 mM PLP and then broken by sonication. After centrifugation, the supernatant was heated at 50°C for 30 min and centrifuged to remove aggregated proteins. The supernatant was applied to a Resource Q anion exchange column (Pharmacia) which had been equilibrated with 30 mM Tris-HCl (pH 7.3). Proteins were eluted with a linear gradient from 0 to 1 M NaCl. Fractions showing D-AAT activity were collected and concentrated by ultrafiltration. The concentrated protein solution was loaded on a Phenyl Superose column (Pharmacia) which had been equilibrated in 30 mM Tris-HCl (pH 7.3) containing 25% ammonium sulfate. Proteins were eluted with a linear gradient of 25–0% ammonium sulfate. Fractions showing D-AAT activity were collected and dialyzed against 30 mM Tris-HCl (pH 7.3) containing 20 mM PLP, 0.01% β-mercaptoethanol, 0.2 mM EDTA, 0.01 mM PMSF, and 0.01 mM TPCK. All column procedures were carried out with an FPLC system (Pharmacia).

*2.4. Spectrophotometric analysis*

Absorption spectra were obtained at 25°C by using a spectrophotometer (Shimadzu UV2100, Japan). Fluorescence intensities of the wild-type and mutant enzymes were measured at 25°C by using a fluorescence spectrophotometer (Shimadzu RF-5301PC).

*2.5. Enzyme activity assay*

The enzyme activity was assayed by coupling with lactate dehydrogenase (LDH). The reaction mixture contained 100 mM Tris-HCl (pH 8.5), 0.5 mM NADH, 10 μg/ml LDH, and various concentrations of D-alanine and α-ketoglutarate. When necessary, α-ketoisovalerate was used instead of α-ketoglutarate. The reaction was started by adding enzyme solution. Decrease in the absorbance at 340 nm was measured at 50°C. The rate of the enzyme reaction was calculated using a molar extinction coefficient of 6220 $M^{-1}$ $cm^{-1}$ for NADH.

## 3. Results and discussion

*3.1. Kinetic parameters of the β-strand III mutant enzymes*

As mentioned earlier, some amino acid residues in β-strand III were expected to make a critical contribution to the enzyme catalysis. In an effort to test this hypothesis, these amino acids were replaced with alanine and the kinetic parameters of the resulting mutant enzymes were investigated. As shown in Table 1, each mutant showed a significant change in kinetic constant compared to the wild-type enzyme. The phenolic oxygen of $Tyr^{31}$ residue was postulated to form hydrogen bond with the 3′-oxygen of PLP [5]. Thus, substitution of this residue with alanine resulted in a marked decrease in $k_{cat}$, showing results similar to those for the Y31Q mutant [6]. Increased $K_m$ for α-ketoglutarate may be attributed to the enhanced mobility of the cofactor in the active site as in the Y31Q mutant. The negatively charged side chain of $Glu^{32}$ residue seemed to be very important in the enzyme catalysis, since the E32A mutant demonstrated only 1% of the original activity even though the binding affinity for α-ketoglutarate remained almost the same. In the case of V33A, the $k_{cat}$ and $K_m$ values for α-ketoglutarate were not altered significantly but a considerable increase in the affinity was observed when α-ketoisovalerate was used as an amino group acceptor. It seems that the branched side chain of $Val^{33}$ residue may hinder access of the substrate containing branched side chain to the substrate binding region. Therefore, replacement of the valine residue with alanine results in an increase in the affinity toward branched substrate by reducing the steric hindrance. The $K_m$ values for α-ketoisovalerate of Y31A and E32A were difficult to obtain because the activities of these mutants toward α-ketoisovalerate were too low. The ε-amino group of the $Lys^{35}$ residue is located about 14 Å from the ε-amino group of the $Lys^{145}$ residue which plays a catalytic role and forms the internal aldimine with PLP (Fig. 1). When D-glutamate as a substrate forms the external aldimine with PLP, the negatively charged side chain of this substrate can interact electrostatically with the positive charge of the ε-amino group of the $Lys^{35}$ residue because the side chain of lysine is very flexible. When the $Lys^{35}$ residue was changed to alanine, the $K_m$ value for α-ketoglutarate increased to 35.6 mM, compared to the $K_m$ value of 2.5 mM of the wild-type enzyme. The $k_{cat}$ of the K35A mutant was similar to that of wild enzyme, and the $K_m$ value for α-ketoisovalerate was not significantly changed. From these results, it is suggested that the $Lys^{35}$

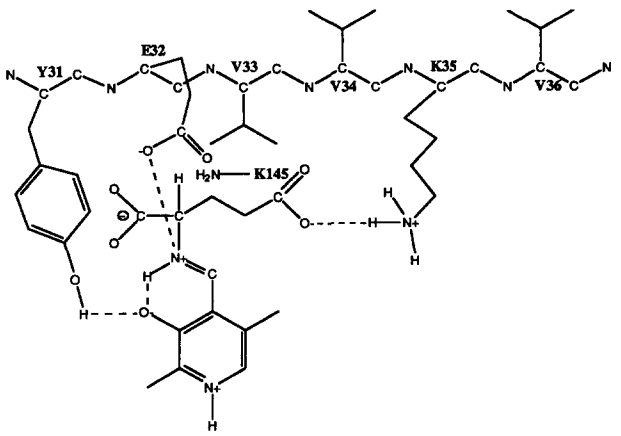


Fig. 1. Proposed interaction of the side chain of amino acid residues in the β-strand III of D-AAT with D-glutamate.

Table 1
Kinetic parameters for the wild-type (WT) and β-strand III mutant enzymes

| | $k_{cat}$ ($s^{-1}$) | $K_m^{\alpha-KG}$ (mM) | $k_{cat}/K_m^{\alpha-KG}$ ($M^{-1}$ $s^{-1}$) | $K_m^{\alpha-KIV}$ (mM) |
|---|---|---|---|---|
| WT | 131.2 | 2.5 | $5.2\times10^4$ | 28.8 |
| Y31A | 0.5 | 10.1 | $4.8\times10^1$ | ND |
| E32A | 1.3 | 1.8 | $7.2\times10^2$ | ND |
| V33A | 145.1 | 2.4 | $5.2\times10^4$ | 15.5 |
| K35A | 84.3 | 35.6 | $1.2\times10^3$ | 32.2 |

$K_m^{\alpha-KG}$, $K_m$ for α-ketoglutarate; $K_m^{\alpha-KIV}$, $K_m$ for α-ketoisovalerate; ND, not determined.

Table 2
Kinetic parameters for $Glu^{32}$ mutant enzymes

| | $k_{cat}$ ($s^{-1}$) | $K_m^{D-ala}$ (mM) | $K_m^{\alpha-KG}$ (mM) | $k_{cat}/K_m^{D-ala}$ ($M^{-1}$ $s^{-1}$) | $k_{cat}/K_m^{\alpha-KG}$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|---|
| WT | 131.2 | 2.1 | 2.5 | $6.2\times10^4$ | $5.2\times10^4$ |
| E32A | 1.3 | 0.1 | 1.8 | $8.6\times10^3$ | $7.2\times10^2$ |
| E32Q | 13.4 | 2.1 | 1.4 | $6.2\times10^3$ | $9.3\times10^3$ |
| E32D | 85.6 | 1.2 | 2.1 | $6.8\times10^4$ | $4.1\times10^4$ |

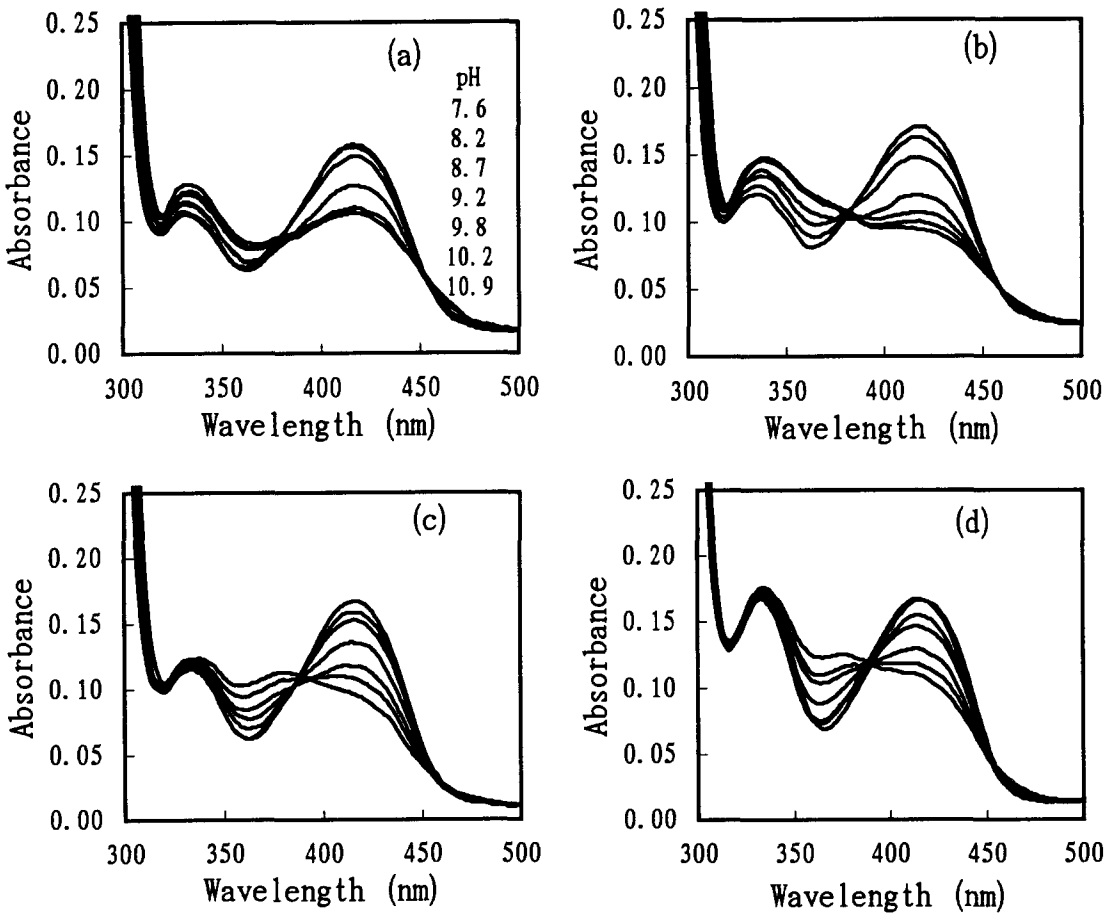


Fig. 2. Absorption spectra of the wild-type and mutant enzymes at various pH values. (a) Wild-type, (b) E32D, (c) E32Q, (d) E32A. The enzyme concentration was about 3.5 mg/ml in 0.1 M buffer. Buffers used were MES for pH 6–7, HEPES for pH 7–8, Bicine for pH 8–9, and CAPS for pH 9–11. The spectrum was obtained at 25°C.

residue is closely linked with binding of the negatively charged side chain of substrate, playing a role similar to that of $Arg^{292}$ of aspartate aminotransferase [8].

### 3.2. Catalytic properties of $Glu^{32}$ mutant enzymes

As described above, the Y31A and E32A mutant enzymes showed the most significant change in catalytic properties as compared to the wild-type enzyme. The functional role of the $Tyr^{31}$ residue has been studied by other researchers [6]. Therefore, we tried to investigate the characteristics of $Glu^{32}$-substituted enzymes as an approach to demonstration of the role of the $Glu^{32}$ residue. The glutamine mutant (E32Q) retained 10% of the wild-type activity as seen in Table 2. Replacement of the negatively charged side chain with a neutral one also resulted in a marked reduction in the $k_{cat}$ value. On the other hand, when the $Glu^{32}$ residue was changed to aspartic acid (E32D), which has a side chain shorter by one methylene group, the $k_{cat}$ value recovered to 65% of that of the wild-type enzyme. Comparison of the $k_{cat}/K_m$ for each enzyme indicates that enzyme possessing a negative charge at position 32 could catalyze the transamination reaction 10 times more efficiently than that with a neutral side chain. From these results, it was thought that the negative charge of the $Glu^{32}$ side chain would play an important role in the catalysis of D-AAT.

### 3.3. Spectral characteristics of $Glu^{32}$ mutant enzymes

D-AAT forms a Schiff base through the ε-amino group of the $Lys^{145}$ residue with the aldehyde group of pyridoxal 5′-phosphate [3,9]. It has been reported that the imine nitrogen of Schiff base is protonated at neutral pH and shows an absorption band at 415 nm [10,11]. Fig. 2 presents the absorption spectra of the wild-type and $Glu^{32}$ mutant enzymes at different pH values. In the case of the wild-type enzyme, the absorption band at 415 nm decreased along with increase in pH from 7.6 to 10.9 (Fig. 2a). On the other hand, there was a small concomitant increase in the 335 nm band. This observation suggests that deprotonation of the Schiff base does not lead to the increase in absorption at 375 nm. However, for the $Glu^{32}$ mutant enzymes, the region around 375 nm increased along with increasing pH. This 375 nm band corresponds to the non-protonated internal aldimine. For aspartate aminotransferase (AspAT) or tryptophanase, the non-protonated internal aldimine structure is known to appear at around 360–365 nm [12–14]. The 335 nm band might correspond to either an enolimine or substituted aldamine (Fig. 3) [15]. To assign the 335 nm band species, the fluorescence spectra were observed. It is known that the enolimine structure emits maximum intensity at around 510 nm when excited at 335 nm, whereas the substituted aldamine results in a maximum emission wavelength at 385 nm [15,16]. In the fluorescence spectra,

there was no emission band at 510 nm when excited at 335 nm for both the wild-type and mutant enzymes (Fig. 4), strongly implying that the 335 nm band represents a substituted aldamine structure. In addition, the fluorescence intensity was affected by the environmental conditions around the internal aldimine structure. The short and non-polar side chain of E32A mutant may reduce the degree of fluorescence quenching, resulting in a higher intensity than that of the wild-type or other mutant enzymes. The possibility that the E32A mutant has a higher content of the substituted aldamine structure should not be excluded.

All the mutant enzymes showed a marked increase in absorbance at 375 nm with increasing pH compared to wild-type enzyme. Therefore, it seems that the imine nitrogen of the mutant enzymes is deprotonated at lower pH because the 375 nm band represents the non-protonated internal aldimine structure. In particular, the E32A and E32Q enzymes exhibited an increase in the 375 nm band at around pH 8.5.

From the results described above, the $Glu^{32}$ residue of D-AAT seems to be closely linked with the protonation and deprotonation of the imine nitrogen, playing a similar role to the $Tyr^{70}$ residue of AspAT [17,18]. In the case of AspAT, the $pK_a$ value of the imine nitrogen is around 6.7 [18], but D-AAT seems to retain its proton up to considerably high pH. This higher $pK_a$ value of the imine nitrogen of D-AAT than AspAT might be attributed to either the absence of the amino acid residue enhancing the electron-withdrawing capacity of PLP like the $Asp^{222}$ residue of AspAT [19] or the presence of a group that stabilizes the positive charge of the protonated aldimine. The latter is more likely for the role of $Glu^{32}$ of D-AAT because the negative charge of the δ-carboxylic group of $Glu^{32}$ enables the imine nitrogen to hold the proton even at fairly high pH. The observation that the mutant enzymes were deprotonated at lower pH than the wild-type enzyme supports the above presumption. In the catalysis of aminotransferase, one of the rate-determining steps is known to be the deprotonation of the α-carbon of D-amino acid by the ε-amino group of $Lys^{145}$ [20]. In this case, the ε-amino group becomes positively charged by receiving a proton. Thus, variation in the $k_{cat}$ values of the mutant enzymes strongly implies that the positive charge of the ε-amino group of $Lys^{145}$ might be stabilized by the negatively charged δ-carboxylic group of $Glu^{32}$, which clearly demonstrates significant reduction in the $k_{cat}$ values of E32Q and E32A compared with that of the wild-type or E32D mutant enzyme.

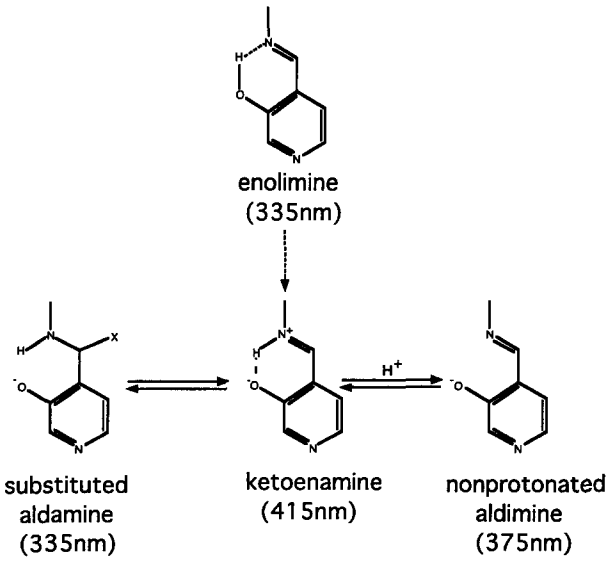


Fig. 3. Schiff base structures formed between D-AAT and PLP and their maximum absorption wavelengths.

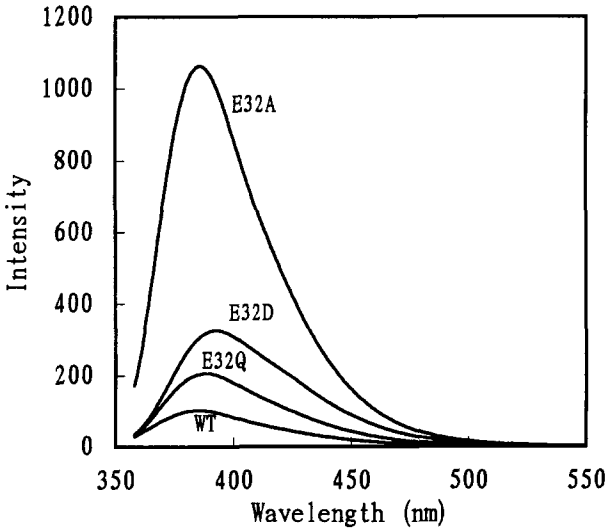


Fig. 4. Fluorescence emission spectra of the wild-type (WT) and $Glu^{32}$ mutant enzymes. The excitation wavelength was 335 nm, and the spectra were measured at 25°C in 30 mM Tris-HCl (pH 7.3).

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