



# Functional Analysis of Feedback Inhibition-Insensitive Variants of *N*-Acetyl Glutamate Kinase Found in Sake Yeast Mutants with Ornithine Overproduction

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**ABSTRACT** In the yeast *Saccharomyces cerevisiae*, *N*-acetyl glutamate kinase (NAGK), which catalyzes the phosphorylation of *N*-acetyl glutamate to form *N*-acetyl glutamyl-5-phosphate, is one of the rate-limiting enzymes in the ornithine and arginine biosynthetic pathways. NAGK activity is strictly regulated via feedback inhibition by the end product, arginine. We previously reported that the Thr340Ile variant of NAGK was insensitive to arginine feedback inhibition and that the interaction between Lys336 and Thr340 in NAGK may be important for arginine recognition. In the present study, we demonstrated that amino acid changes of Thr340 to Ala, Leu, Arg, Glu, Ile, and Asn removed arginine feedback inhibition, although the Thr340Ser variant was subject to the feedback inhibition. Therefore, these results indicate that the arginine-binding cavity formed via the interaction between the carbonyl group in the main chain of Lys336 and the hydroxyl group in the side chain of the residue at position 340 is critical for arginine recognition of NAGK. In addition, we newly identified two mutations in the *ARG5,6* gene encoding the Cys119Tyr or Val267Ala variant of NAGK of sake yeast mutants with intracellular ornithine accumulation. Although it is unlikely that Cys119 and Val267 are directly involved in arginine recognition, we found here that two variants of NAGK were insensitive to arginine feedback inhibition and contributed to high-level production of ornithine. Structural analysis of NAGK suggests that these two amino acid substitutions influence the sensitivity to Arg feedback inhibition through alterations in local conformation around each residue.

**IMPORTANCE** Ornithine has a number of physiological benefits in humans. Thus, an Orn-rich alcoholic beverage is expected to relieve feelings of fatigue after drinking. In the yeast *Saccharomyces cerevisiae*, *N*-acetyl glutamate kinase (NAGK) encoded by the *ARG5,6* gene catalyzes the second step in ornithine and arginine biosynthesis, and its activity is subjected to feedback inhibition by arginine. Here, we revealed a role of key residues in the formation of the arginine-binding cavity which is critical for arginine recognition of NAGK. In addition, we analyzed novel arginine feedback inhibition-insensitive variants of NAGK in sake yeast mutants with ornithine overproduction and proposed that the amino acid substitutions in the NAGK variants destabilize the arginine-binding cavity, leading to the lower sensitivity to arginine feedback inhibition of NAGK activity. These findings provide new insight into the allosteric regulation of NAGK activity and will help to construct superior industrial yeast strains for high-level production of ornithine.

**KEYWORDS** *Saccharomyces cerevisiae*, sake yeast, ornithine, *N*-acetyl glutamate kinase, allosteric regulation, feedback inhibition

Ornithine (Orn), a nonproteinogenic amino acid, functions in the urea cycle, which detoxifies ammonia generated by the degradation of amino acids in protein to urea. Various organisms and foods contain large amounts of Orn (1–3). In humans, Orn

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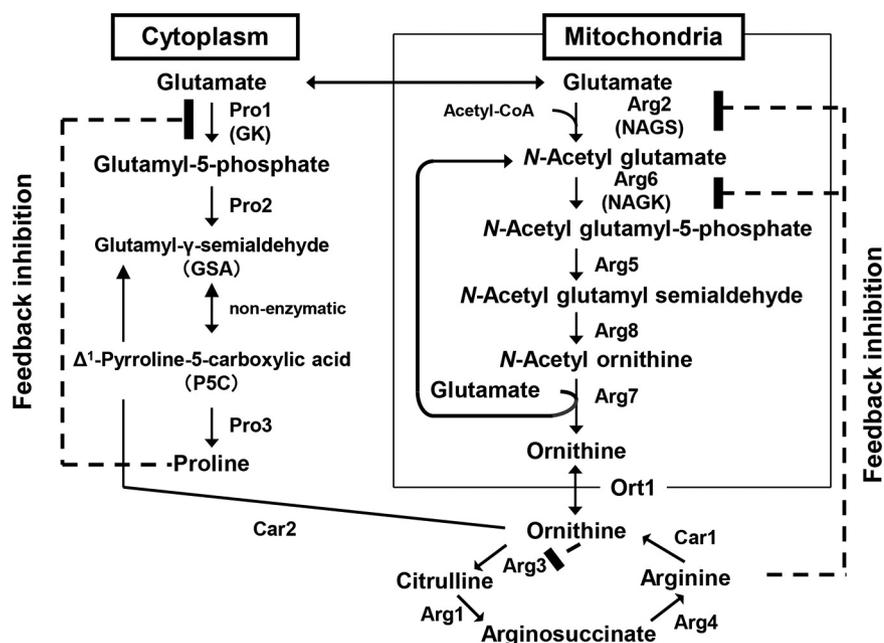
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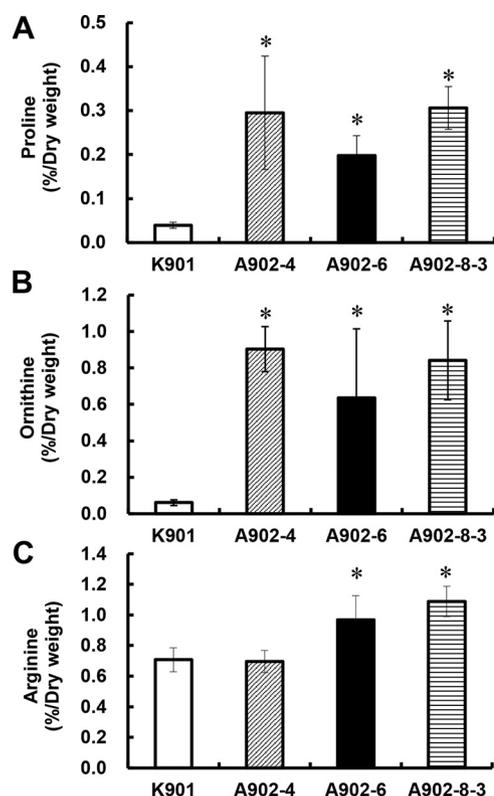


**FIG 1** Metabolic pathways of proline (Pro), ornithine (Orn), and arginine (Arg) in *S. cerevisiae*. Protein names: Pro1,  $\gamma$ -glutamyl kinase (GK); Pro2,  $\gamma$ -glutamyl phosphate reductase; Pro3, P5C reductase; Arg2, *N*-acetyl glutamate synthase (NAGS); Arg6, *N*-acetyl glutamate kinase (NAGK); Arg5, *N*-acetyl glutamyl-5-phosphate reductase; Arg8, *N*-acetyl Orn aminotransferase; Arg7, *N*-acetyl Orn acetyltransferase; Ort1, Orn transporter; Arg3, Orn carbamoyltransferase; Arg1, arginosuccinate synthetase; Arg4, arginosuccinate lyase; Car1, arginase; Car2, Orn aminotransferase. Acetyl-CoA, acetyl coenzyme A.

has been reported to have a number of physiological benefits, such as reducing the plasma ammonia level after bicycle exercise (4), improving various negative feelings (4, 5), enhancing sleep quality (6, 7), and increasing growth hormone levels (4, 8).

The budding yeast *Saccharomyces cerevisiae* synthesizes Orn from glutamate (Glu) via intermediates such as *N*-acetyl L-glutamate (NAG) and *N*-acetyl glutamyl-5-phosphate, *N*-acetyl glutamyl- $\gamma$ -semialdehyde, and *N*-acetyl Orn in mitochondria (Fig. 1). The *ARG5,6* gene encodes a polyprotein precursor that is likely cleaved into two enzymes, one being *N*-acetyl glutamate kinase (NAGK, Arg6) and the other being *N*-acetyl glutamyl-5-phosphate reductase (Arg5), between residues 510 and 540 (9). NAGK is one of the rate-limiting enzymes of Orn and arginine (Arg) biosynthesis. This enzyme catalyzes the conversion of NAG into *N*-acetyl glutamyl-5-phosphate, and its activity is subject to feedback inhibition by Arg (10). *N*-Acetyl glutamate synthase (NAGS), which is encoded by the *ARG2* gene, is another rate-limiting step of Arg biosynthesis. NAGS transfers the acetyl group to Glu to produce NAG, and the protein or expression level of this enzyme is tightly regulated due to the Arg feedback inhibition (10) (Fig. 1). NAG can be regenerated by transferring the acetyl group from *N*-acetyl Orn to Glu with the production of Orn in *S. cerevisiae* (10) even if the synthesis of NAG by NAGS is limited by Arg (Fig. 1). Therefore, expressing NAGK variants that are desensitized to feedback inhibition is effective for overproduction of Orn, rather than expressing the feedback inhibition-insensitive NAGS variants. Alternatively, *S. cerevisiae* also synthesizes proline (Pro) from Glu.  $\gamma$ -Glutamyl kinase (GK) encoded by the *PRO1* gene is the rate-limiting enzyme of Pro biosynthesis from Glu, and its activity is strictly regulated by Pro feedback inhibition (11). Moreover, Pro can be biosynthesized from Orn via glutamyl- $\gamma$ -semialdehyde by Orn transaminase activity (12) (Fig. 1).

Using traditional chemical mutagenesis, we recently isolated two Orn- and Pro-accumulating mutants (strains A902-4 and A902-6) that were resistant to the Pro toxic analogue azetidine-2-carboxylate (AZC) derived from a diploid sake yeast strain of *S. cerevisiae* (13). In addition, strain A902-4 had a homoallelic mutation in the *ARG5,6* gene encoding the feedback inhibition-insensitive variant of NAGK (Thr340Ile). Furthermore, our structural analysis of the Thr340Ile variant of NAGK predicted that the Arg-binding cavity



**FIG 2** Intracellular Pro (A), Orn (B), and Arg (C) contents in sake yeast strains. Yeast cells were cultured in SD liquid medium at 30°C for 48 h. The values are means and standard deviations for results from three independent experiments. Asterisks indicate statistically significant differences between the parent strain K901 and the AZC-resistant mutant strains A902-4, A902-6, and A902-8-3 (Student's *t* test,  $P < 0.05$ ).

consists of Lys265, Ser285, Lys336, Glu337, Thr340, and Gly345 and that this cavity may be stabilized through the interaction between the hydroxyl group in the side chain of Thr340 and the carbonyl group in the main chain of Lys336 (13).

In this study, to examine the importance of the amino acid residue at position 340, we analyzed the effects of amino acid substitution of Thr340 on NAGK activity and structure. In addition, we newly isolated another Orn-overproducing mutant (strain A902-8-3) that was resistant to AZC by conventional mutagenesis. Strains A902-6 and A902-8-3 each had a novel mutation in the *ARG5,6* gene encoding the Cys119Tyr and Val267Ala variants of NAGK, respectively, leading to an increase in intracellular Orn. Several genetically modified microorganisms, such as *Corynebacterium crenatum* (14) and *S. cerevisiae* (15), have been developed to overproduce Orn, using metabolic engineering or modular pathway rewiring. However, these strains have not been directly applied in food production because the food industry and customers still have not accepted genetically modified microorganisms. In contrast, the Orn-overproducing mutants (strains A902-4, A902-6, and A902-8-3) could be readily applied to sake brewing because they are not genetically modified.

## RESULTS AND DISCUSSION

**Isolation of sake yeast strains with intracellular Orn accumulation from AZC-resistant mutants.** In the present study, using traditional chemical mutagenesis, we isolated a new Pro- and Orn-accumulating strain, A902-8-3, from AZC-resistant mutants of diploid sake yeast strain K901 in addition to previously isolated strains A902-4 and A902-6 (13). As shown in Fig. 2A, the intracellular Pro contents in A902-4, A902-6, and A902-8-3 were 7.6-, 5.1-, and 7.8-fold higher, respectively, than that in the parent strain K901. Interestingly, these three strains accumulated Orn in the cells 14.8-, 10.4-, and

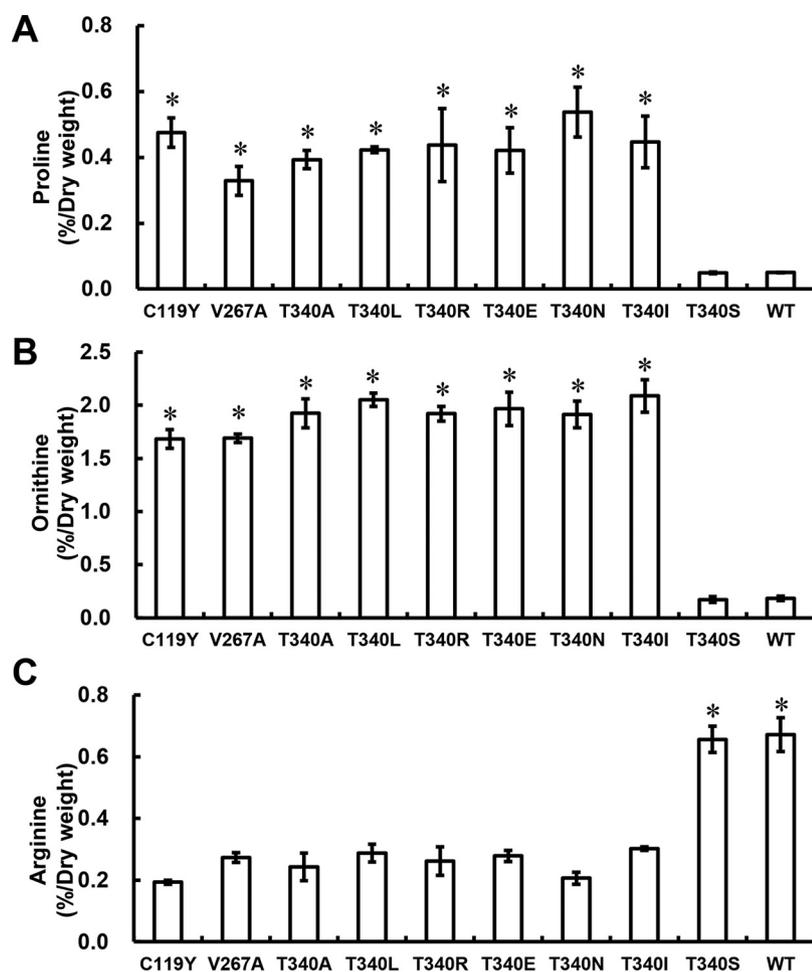
13.8-fold more than K901 (Fig. 2B). However, levels of intracellular Arg, which is biosynthesized from Orn, in these strains were not remarkably higher than in strain K901 (Fig. 2C).

**Identification of mutations in the *ARG5,6* gene of sake yeast strains with intracellular Orn accumulation.** Strain A902-4 was reported to carry a mutation in the *ARG5,6* gene encoding the Thr340Ile variant of NAGK. The NAGK activity of this variant was insensitive to feedback inhibition by Arg, leading to overproduction of Orn (13). Therefore, we assumed that two other strains, A902-6 and A902-8-3, also had one or more mutations in the *ARG5,6* gene. We therefore analyzed the nucleotide sequence of *ARG5,6* in each of these strains using direct PCR DNA sequencing (see Fig. S1 in the supplemental material). As expected, the *ARG5,6* gene sequence in A902-6 contained a homozygous mutation, which was a single base change at position 356, leading to one amino acid change of Cys to Tyr at position 119. On the other hand, A902-8-3 had a heterozygous mutation, i.e., a single base change at position 800, which led to one amino acid change of Val to Ala at position 267. In contrast, strains A902-6 and A902-8-3 harbored no other mutations in the genes encoding the rate-limiting steps for Orn (*ARG2*) and Pro (*PRO1*) biosynthesis (Fig. 1). Thus, we hypothesized that mutations identified in the *ARG5,6* gene of these two strains contribute to an increase in intracellular Pro and Orn levels.

**Effects of the *ARG5,6* mutations on intracellular amino acid contents.** To analyze the *ARG5,6* gene mutation found in strains A902-6 and A902-8-3, we constructed expression plasmids for the wild-type (WT) and mutated *ARG5,6* genes (*ARG5,6*<sup>C119Y</sup> and *ARG5,6*<sup>V267A</sup>). In addition, the importance of Thr340 in the mechanism underlying the feedback inhibition of NAGK was still unknown. Therefore, we also designed a series of mutated *ARG5,6* genes (*ARG5,6*<sup>T340A</sup>, *ARG5,6*<sup>T340L</sup>, *ARG5,6*<sup>T340R</sup>, *ARG5,6*<sup>T340E</sup>, *ARG5,6*<sup>T340N</sup>, *ARG5,6*<sup>T340I</sup>, and *ARG5,6*<sup>T340S</sup>) that replaced Thr340 with various amino acids (Ala, Leu, Arg, Glu, Asn, Ile, and Ser) that have a side chain with a similar or different size, charge, and polarity.

We next determined the intracellular amino acid contents in strain BY4741 *arg5,6* $\Delta$  expressing the wild-type and mutated *ARG5,6* genes (Fig. 3). Yeast cells expressing the mutated *ARG5,6* genes except for *ARG5,6*<sup>T340S</sup> showed from 6.6- to 10.7-fold increases in Pro content compared to the wild-type *ARG5,6* gene (Fig. 3A). In addition, the expression of the mutated *ARG5,6* genes except for *ARG5,6*<sup>T340S</sup> increased the intracellular Orn content markedly, in the range of 9.2- to 11.5-fold compared with that of BY4741 *arg5,6* $\Delta$  cells harboring the wild-type *ARG5,6* gene (Fig. 3B). The Pro and Orn levels in yeast cells expressing the *ARG5,6*<sup>T340S</sup> gene were almost the same as those of cells expressing the wild-type *ARG5,6* gene (Fig. 3A and B). On the other hand, the Arg content in yeast cells expressing the mutated *ARG5,6* genes except for *ARG5,6*<sup>T340S</sup> was significantly lower than that in cells expressing the wild-type *ARG5,6* gene (Fig. 3C). *S. cerevisiae* cells synthesize Arg from Orn via intermediates such as citrulline and arginosuccinate via the enzymatic reactions catalyzed by Arg3, Arg1, and Arg4 (Fig. 1). Among them, Orn carbamoyltransferase, encoded by the *ARG3* gene, catalyzes the conversion of Orn into citrulline, and excess Orn inhibits its enzyme activity (16). Therefore, this inhibition by excess Orn may result in a decrease in Arg level in cells expressing the mutated *ARG5,6* genes except for *ARG5,6*<sup>T340S</sup>. However, intracellular Arg levels in Orn-accumulating mutants were similar to (A902-4) or higher than (A902-6 and A902-8-3) that in the parent strain, K901 (Fig. 2C), suggesting that these mutants might cancel the excess Orn-mediated inhibition of Orn carbamoyltransferase activity. Besides the inhibition of the enzymatic activity of Arg3 by Orn, transcription of the *ARG1* and *ARG3* genes is repressed by Arg (17). One possibility for differences in intracellular Arg levels among the Orn-accumulating mutants thus is that such Arg-mediated transcriptional repression of the *ARG1* and *ARG3* genes may be abolished in strains A902-6 and A902-8-3, enabling the expression of these genes even in the presence of Arg. Further studies are needed to clarify the underlying molecular mechanism.

These results in Fig. 3A and B indicate that amino acid changes from Cys to Tyr at position 119, from Val to Ala at position 267, and from Thr to Ala, Leu, Arg, Glu, Asn, and Ile at position 340 in NAGK confer Pro and Orn accumulation on yeast cells.



**FIG 3** Intracellular Pro (A), Orn (B), and Arg (C) contents in laboratory yeast strains. BY4741 *arg5,6Δ* cells expressing the wild-type (WT) and mutated *ARG5,6* (C119Y, V267A, T340A, T340L, T340R, T340E, T340N, T340I, and T340S) genes were cultured in SD+Leu+His+Met liquid medium at 30°C for 48 h. The values are means and standard deviations for results from three independent experiments. Asterisks indicate statistically significant differences between BY4741 *arg5,6Δ* cells expressing the WT and mutated *ARG5,6* genes (Student's *t* test,  $P < 0.05$ ).

**Effect of amino acid substitutions on NAGK activity.** We previously showed that the mutated *ARG5,6*<sup>T340I</sup> gene encoding the Thr340Ile variant of NAGK elevated the intracellular Orn level due to the desensitization of feedback inhibition of NAGK activity (13). We therefore hypothesized that the amino acid substitutions at positions 119, 267, and 340 led to the desensitization of feedback inhibition of NAGK by Arg. To prove this hypothesis, we expressed the wild-type and mutated *ARG5,6* genes in *Escherichia coli* BL21(DE3) cells, purified the recombinant wild type (WT) and variants (C119Y, V267A, T340A, T340L, T340R, T340E, T340N, T340I, and T340S) of NAGK tagged with 6×His at the amino terminus from *E. coli* cell extracts (Fig. S2), and measured enzyme activities of NAGKs under conditions without or with Arg. As shown in Table 1, the specific activities of all nine NAGK variants, which ranged from 0.96 to 1.39 U/mg, were slightly lower than that of WT (1.57 U/mg) in the absence of Arg.

Judging from the results of Arg inhibition kinetics (Table 1), however, the half-maximal inhibitory concentration values of Arg ( $IC_{50}^{Arg}$ ) of WT and T340S were less than 0.2 mM. In contrast, the newly identified variants (C119Y and V267A) exhibited higher  $IC_{50}^{Arg}$  (0.78 mM and 2.83 mM, respectively) than that of WT, suggesting that Cys119 and Val267 are important for the regulation of NAGK activity. These results indicate that the novel NAGK variants (C119Y and V267A) were less sensitive to feedback inhibition by Arg than WT. Moreover, in the NAGK variants at position 340 (T340A, T340L, T340R, T340E, and T340N), each  $IC_{50}^{Arg}$  (ranging from 0.78 mM to 23.98 mM) was higher than those of the WT and T340S enzymes.

**TABLE 1** Specific activities and arginine inhibition kinetics

NAGK	Sp act <sup>a</sup> (U/mg)	IC <sub>50</sub> <sup>Argb</sup> (mM)
C119Y	0.96 ± 0.01	0.78 ± 0.05
V267A	1.39 ± 0.01	2.83 ± 0.16
T340A	1.27 ± 0.09	2.25 ± 0.20
T340L	1.35 ± 0.06	10.46 ± 0.80
T340R	1.16 ± 0.09	2.48 ± 0.67
T340E	0.98 ± 0.04	6.87 ± 1.00
T340N	1.20 ± 0.04	23.98 ± 5.36
T340I	1.27 ± 0.07	>100
T340S	1.25 ± 0.02	0.17 ± 0.12
WT	1.57 ± 0.03	0.09 ± 0.38

<sup>a</sup>Specific activity in the absence of arginine.

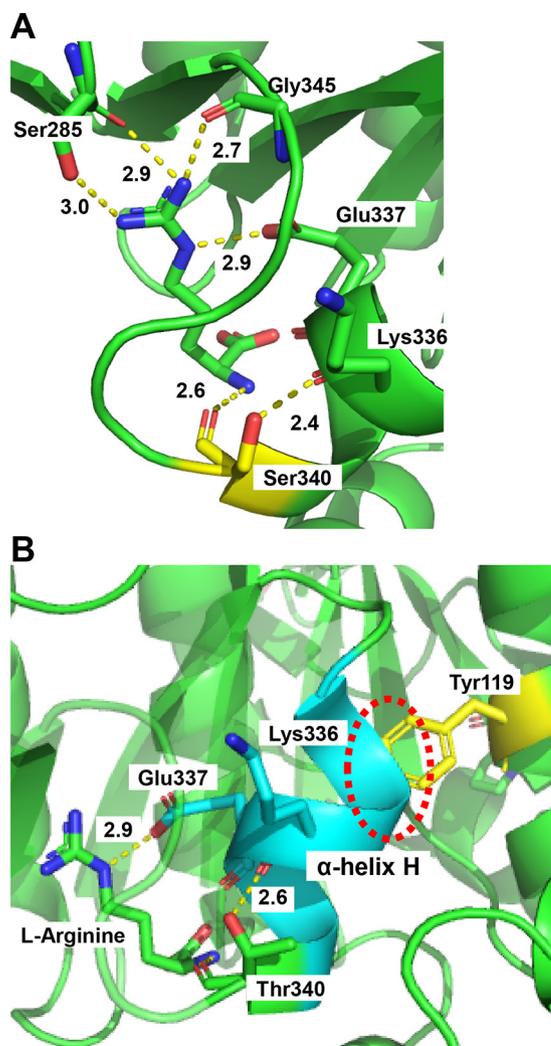
<sup>b</sup>The IC<sub>50</sub><sup>Arg</sup> value is an arginine concentration which gives 50% inhibition.

It should be noted that the IC<sub>50</sub><sup>Arg</sup> of the T340I variant (13) was the highest among all enzymes (>100 mM). We demonstrated that amino acid changes at position 340 (T340A, T340L, T340R, T340E, and T340N) resulted in less sensitivity to the Arg feedback inhibition of NAGK activity, leading to the accumulation of Orn in yeast cells.

Desensitization to the Arg feedback inhibition of NAGK led to overproduction of *N*-acetyl Orn and thereby enabled regeneration of NAG from Glu and *N*-acetyl Orn even though the synthesis of NAG is limited by the Arg feedback inhibition of NAGS, leading to Orn overproduction in strains A902-6 and A902-8-3 (Fig. 2B). In addition, strains A902-6 and A902-8-3 accumulated not only Orn but also Pro (Fig. 2A). Strains A902-4, A902-6, and A902-8-3 carry no mutations associated with Pro accumulation in the *PRO1* gene; thus, GK in these mutants is subject to feedback inhibition by Pro. These strains accumulated Pro probably due to biosynthesis of Pro from excess Orn (Fig. 1).

**Effects of amino acid substitutions on the NAGK structure.** Comparison of amino acid sequences among Arg-sensitive and -insensitive NAGKs demonstrates that Thr or Ser at position 340 is highly conserved among Arg-sensitive NAGKs including *S. cerevisiae* NAGK (18–20), whereas the corresponding residues contain no hydroxyl group in the side chain, such as Asn or Val, among Arg-insensitive NAGKs (21, 22) (Fig. S3). In addition, our results showed that NAGKs with the hydroxyl group in the side chain at position 340 (e.g., Thr and Ser) were sensitive to Arg feedback inhibition, suggesting the importance of the interaction between the hydroxyl group in the side chain of the residue at position 340 and the carbonyl group in the main chain of Lys336 for Arg recognition as described in our previous study (13).

To confirm this hypothesis, we next predicted the structures of the Arg-binding sites of NAGK variants in which Thr340 was replaced with Ala, Leu, Arg, Glu, Asn, Ser, or Ile using the crystal structure of *S. cerevisiae* NAGK (PDB identifier [ID], 3ZZH) as a template. The results showed that replacement of Thr340 with Ser can still form the hydrogen bond with Lys336 as well as the WT enzyme because the hydroxyl group in the side chain of Ser340 is located near the carbonyl group in the main chain of Lys336 (2.4 Å) (Fig. 4A), resulting in sensitivity to feedback inhibition by Arg (Table 1). In contrast, the amino acid changes of Thr340 to Ala, Leu, Arg, Glu, Asn, and Ile cause disruption of the interaction of Lys336 with each of these amino acid residues as follows. The substitutions by Ala, Leu, and Ile cannot form the hydrogen bond with Lys336 due to the lack of a hydroxyl group in the side chain of these amino acids, whereas those by Arg, Glu, and Asn are also unlikely to form based on the predicted distances from the carbonyl group in the main chain of Lys336 to the imino group (4.4 Å), the carboxyl group (5.9 Å), and the amino group (5.0 Å) in the side chain of Arg340, Glu340, and Asn340, respectively. These results indicate that the interaction between the hydroxyl group in the side chain of the residue at position 340 and the carbonyl group in the main chain of Lys336 is important for the stabilization of the structure of the Arg-binding site in the *S. cerevisiae* NAGK. The IC<sub>50</sub><sup>Arg</sup> of uncharged and bulky amino acids (Ile, Asn, and Leu) in the side chain was higher than that of the acidic (Glu), the basic (Arg), or the small (Ala)



**FIG 4** Predicted structure of Arg-binding site in *S. cerevisiae* NAGK variants. The Arg-binding sites of two NAGK variants, Thr340Ser (A) and Cys119Tyr (B), predicted from the structure of the *S. cerevisiae* NAGK (PDB ID, 3ZZH), are shown by the cartoon model. Arg, an inhibitor bound to NAGK, and the residues which interact with the inhibitor Arg are shown by the stick model. The replaced residues Ser340 (A) and Tyr119 (B) are shown in yellow. The expected hydrogen bonds are shown in yellow dots with their distance in angstroms. The  $\alpha$ H-helix (Asp333-Phe339) including Lys336 is shown in cyan (B). A steric constraint is likely to arise from the  $\alpha$ H-helix and the phenyl group of Tyr119 as shown by the dotted circle.

amino acid in the side chain (Table 1). These findings can result from the differences in the effects of neutral and bulky amino acids and charged or small amino acids on the Arg-binding cavity. Particularly, Ile340 might completely disrupt the Arg-binding cavity, judging from the high  $IC_{50}^{Arg}$ .

Moreover, we predicted the structure of the Arg-binding site in the C119Y and V267A variant. It is unlikely that Cys119 and Val267 are directly involved in Arg recognition. However, the replacement of Cys119 with tyrosine may cause the steric constraint between the phenolic group of Tyr119 and the  $\alpha$ H-helix (Asp333-Phe339) including Lys336 and Glu337 involved in the interaction with Arg (Fig. 4B). The steric constraint caused by Cys-to-Tyr alteration thus could affect the structure of the Arg-binding site in NAGK, leading to the lower sensitivity to Arg feedback inhibition of NAGK in the C119Y variant. On the other hand, the previous structural analysis of *S. cerevisiae* NAGK reported that the binding of inhibitor Arg caused a conformational change with an axis that crosses obliquely the central  $\beta$  sheet ( $\beta$ 11-sheet) including Val267-Tyr268, resulting in transition from the closed-active form to the open-inactive form (Fig. S4A)

(23). One possibility is that substitution for Val267 by Ala may influence a local structure around the  $\beta$ 11-sheet because of the smaller side chain of Ala than that of Val (Fig. S4B and C), and the presumed conformational change around the  $\beta$ 11-sheet may affect the transition from the close-active to the open-inactive form by Arg, resulting in the desensitization to the Arg feedback inhibition. However, further structural studies, such as molecular dynamics analysis, are needed to clarify the mechanism underlying replacements of Cys119 with Tyr and of Val267 with Ala.

**Conclusion.** In the present study, functional analysis of the Thr340 variants of NAGK revealed that the Arg-binding cavity formed through the interaction between Lys336 and Thr340 is critical for Arg recognition of NAGK. In addition, we newly analyzed diploid sake yeast mutants A906-2 and A902-8-3, which overproduced not only Pro but also Orn. These two strains possessed novel mutations in the *ARG5,6* genes encoding the Cys119Tyr and Val267Ala variants of NAGK. These amino acid substitutions weakened the Arg feedback inhibition of NAGK, leading to high-level production of both Pro and Orn in yeast cells. Structural analysis suggests that the two amino acid replacements Cys119Tyr and Val267Ala desensitize Arg feedback inhibition through alterations in local conformation around each residue. These results will contribute to the development of superior industrial yeast strains for high-level production of Orn, which has a number of physiological benefits.

## MATERIALS AND METHODS

**Strains and media.** We used *S. cerevisiae* strain BY4741 (*MATa his3 $\Delta$ 1 leu2 $\Delta$ 0 met15 $\Delta$ 0 ura3 $\Delta$ 0*) derived from strain S288C and diploid strain Kyokai no. 901 (K901) in this study. Strain BY4741 *arg5,6 $\Delta$*  (BY4741 *arg5,6::kanMX6*), which was provided by EUROSCARF (Oberursel, Germany), was used for the expression of the WT and mutated *ARG5,6* genes. The media used for growth of *S. cerevisiae* were a nutrient-rich YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose), a synthetic dextrose (SD) minimal medium (1.7 g/L nitrogen base without amino acid and ammonium sulfate [Difco Laboratories, Detroit, MI, USA], 5 g/L ammonium sulfate, and 20 g/L glucose), and SD medium containing 0.04% leucine, 0.008% histidine, and 0.008% methionine (SD+Leu+His+Met). AZC was added as a supplement at a concentration of 1 mg/mL for the screening of Orn-accumulating cells.

*Escherichia coli* strains DH5 $\alpha$  [*F<sup>-</sup>  $\lambda$   $\phi$ 80lacZ $\Delta$ M15  $\Delta$ (lacZYA argF) U169 deoR recA1 endA1 hsdR17 (r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>) supE44 thi-1 gyrA96*] and BL21(DE3) [*F<sup>-</sup> ompT hsdS(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm  $\lambda$ (DE3) (lacI lacUV5-T7 gene1 ind1 sam7 nin5)*] were used for construction of expression plasmids and for expression of the recombinant NAGKs, respectively. *E. coli* strains were cultured in Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) containing 100  $\mu$ g/mL ampicillin. If necessary, 2% agar was added to solidify the medium.

**Isolation of Orn-accumulating mutants.** Orn-accumulating mutants were isolated by conventional mutagenesis of the diploid sake strain K901 as described previously (13).

**Measurement of intracellular amino acid content.** We quantified intracellular amino acid content of *S. cerevisiae* cells with a UF-Amino Station (Shimadzu, Kyoto, Japan) as described previously (13).

**Construction of expression plasmids for the *ARG5,6* genes.** To construct plasmids for expressing the *ARG5,6* genes, the DNA fragment including 1,000 bp both upstream and then downstream of the open reading frame of the *ARG5,6* gene was amplified from the genomic DNA of *S. cerevisiae* BY4741 by high-fidelity PCR with primers ARG5,6FW (HindIII) and ARG5,6Rv (BamHI) (see Table S1 in the supplemental material) and was introduced into the HindIII-BamHI site of yeast centromere plasmid pRS416 by the In-Fusion HD cloning kit (TaKaRa Bio, Shiga, Japan) to construct pRS416-*ARG5,6*. In-Fusion products were then digested with *DpnI* before introduction into *E. coli* DH5 $\alpha$  cells. The point mutations were introduced into the *ARG5,6* gene on pRS416-*ARG5,6* with mutagenic primers listed in Table S1, leading to the T340A, T340L, T340R, T340E, T340N, T340S, T340I, V267A, and C119Y substitutions on NAGK. PCR products were then digested with *DpnI* before introduction into *E. coli* DH5 $\alpha$  cells. Plasmids of pRS416 harboring the wild-type or mutated *ARG5,6* gene were further confirmed by DNA sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Waltham, MA, USA). Strain BY4741 *arg5,6 $\Delta$*  was transformed with pRS416 harboring the wild-type or mutated *ARG5,6* gene by the lithium acetate method with slight modification (24). Yeast transformants were selected on SD+Leu+His+Met agar plates. To express and purify the recombinant NAGKs from *E. coli*, we constructed expression plasmids as described below. The *ARG5,6* gene encodes a polyprotein precursor with the mitochondrial targeting signal sequence (residues 1 to 57), the amino-terminal NAGK (residues 58 to 513), and the carboxyl-terminal *N*-acetyl glutamyl phosphate reductase (23). The polyprotein precursor is cleaved into the two enzymes in the mitochondria (9), and then deletion of the amino-terminal mitochondrial targeting signal sequence results in yeast mature NAGK by proteolytic processing. To produce NAGK, which lacks the mitochondrial targeting sequence, the DNA sequence encoding enzyme residues 58 to 513 was amplified from pRS416-*ARG5,6* (T340A, T340L, T340R, T340E, T340N, T340S, T340I, V267A, and C119Y) by high-fidelity PCR using KOD Plus DNA polymerase with primers pQE-2 NdHi Fw (NdeI) and pQE-2 NdHi Rv (HindIII) (Table S1) and was cloned into the NdeI-HindIII site of pQE-2 (Qiagen, Hilden, Germany) by the In-Fusion HD cloning kit to construct pQE-2-NAGK-WT or pQE-2-NAGK-variants. In-Fusion products were then

digested with *DpnI* before introduction into DH5 $\alpha$  cells. Plasmids pQE-2-NAGK-WT and pQE-2-NAGK-variants were further confirmed by DNA sequencing. *E. coli* strain BL21(DE3) was transformed with pQE-2-NAGK-WT or pQE-2-NAGK-variants.

**Expression and purification of recombinant NAGKs.** *E. coli* BL21(DE3) cells harboring pQE-2-NAGK-WT or pQE-2-NAGK-variants were cultivated in 5 mL of LB medium containing 100  $\mu$ g/mL ampicillin and grown at 37°C to an optical density at 600 nm ( $OD_{600}$ ) of 0.6 to 0.8. Protein expression was induced by adding isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, and cells were cultured at 18°C for 18 h. Cells were harvested by being placed on ice for 5 min and centrifuged at 4°C for 10 min at 3,000 rpm. The cell pellet was washed with ice-cold sonication buffer (10 mM MgCl<sub>2</sub>, 0.25 M NaCl, 20 mM Tris-HCl [pH 7.5]), and the intact cells were stored at -80°C if they were not used immediately. For purification of recombinant NAGKs, cell pellets were suspended in ice-cold sonication buffer. The cell mixture was subjected to sonication (output = 7, 10 s/on ice 30 s for 1 cycle) by a US-150T ultrasonic generator (Nihonseiki, Tokyo, Japan), was subsequently centrifuged at 15,000 rpm for 20 min at 4°C, and then filtered with an 0.45- $\mu$ m-diameter filter. Crude protein was loaded by gravity on Ni-Sepharose 6 Fast Flow resin (GE Healthcare, Chicago, IL, USA), equilibrated with sonication buffer. The resin was washed in sonication buffer with 20 mM imidazole and then with 70 mM imidazole-containing sonication buffer. Purified proteins were eluted by 500 mM imidazole-containing sonication buffer. Proteins were quantified using Bio-Rad protein assay (Hercules, CA, USA) and subjected to SDS-polyacrylamide gel electrophoresis (Fig. S2).

**Assay of NAGK activity.** NAGK activity was measured by an enzyme-coupled system with lactate dehydrogenase (LDH) and pyruvate kinase (PK) as previously described (17). Briefly, the production of ADP by NAGK was detected with the LDH-dependent oxidation of reduced NADH in the presence of phosphoenol pyruvate (PEP) and PK using a Synergy HTX microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The NAGK reaction mixture contained 25 mM NAG, 5 mM ATP, 10 mM MgCl<sub>2</sub>, 1 mM PEP, 20 U PK, 30 U LDH enzymes from rabbit muscle (Sigma-Aldrich, St. Louis, MO, USA), 0.25 mM NADH, 100 mM HEPES (pH 7.5), and 5  $\mu$ g of NAGK in a final volume of 300  $\mu$ L. The NAGK reaction mixture was prewarmed at 30°C for 1 min without NAG, and then NAG was added to start the NAGK reaction. The rate of the decrease in absorption at 340 nm was measured consecutively. NAGK activity was calculated from the initial linear rate with the extinction coefficient ( $\epsilon_{340\text{ nm}} = 6.22\text{ mM}^{-1}\text{ cm}^{-1}$ ) of NADH. One unit of activity was defined as the amount of enzyme required to produce 1  $\mu$ mol of NAD<sup>+</sup> per min. To determine the 50% inhibitory concentration ( $IC_{50}$ ) values of NAGK activity, NAGK activity was measured by adding Arg, and kinetic parameters of each enzyme were calculated with GraphPad Prism version 9 (GraphPad Software) using nonlinear regression analysis.

**Structural analysis.** To analyze the effect of amino acid substitution on NAGK, we predicted the structure of the Arg-binding site in the variant NAGKs using the crystal structure of NAGK bound to Arg from *S. cerevisiae* (PDB ID, 3ZZH) as the template structure as follows: Thr340 was replaced with Ala, Leu, Arg, Glu, Asn, Ser, and Ile, respectively, or Cys119 and Val267 were changed to Tyr119 and Ala267, respectively, using PyMOL (<http://www.pymol.org>). Figures of NAGK variant structures were drawn using PyMOL.

**Data availability.** The data underlying this article are available in the article.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.9 MB.

## ACKNOWLEDGMENT

We declare no conflicts of interest.

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