Ophthalmic acid accumulation in an *Escherichia coli* mutant lacking the conserved pyridoxal 5′-phosphate-binding protein YggS

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[Running title]

yggS-deficient *E. coli* accumulates ophthalmic acid

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20 Keywords:

21 pyridoxal 5′-phosphate, ophthalmic acid, YggS, *Escherichia coli*, glutathione, amino acid

Abstract

Escherichia coli YggS is a highly conserved pyridoxal 5′-phosphate (PLP)-binding protein whose biochemical function is currently unknown. A previous study with a *yggS*-deficient *E. coli* strain (Δ*yggS*) demonstrated that YggS controls L-Ile- and L-Val-metabolism by modulating 2-ketobutyrate (2-KB), L-2-aminobutyrate (L-2-AB), and/or coenzyme A (CoA) availability in a PLP-dependent fashion. In this study, we found that Δ*yggS* accumulates an unknown metabolite as judged by amino acid analyses. LC/MS and MS/MS analyses of the compound with propyl chloroformate derivatization, and co-chromatography analysis identified this compound as γ-L-glutamyl-L-2-aminobutyryl-glycine (ophthalmic acid), a glutathione (GSH) analogue in which the L-Cys moiety is replaced by L-2-AB. We also determine the metabolic consequence of the *yggS* mutation. Absence of YggS initially increases L-2-AB availability, and then causes ophthalmic acid accumulation and CoA limitation in the cell. The expression of a γ-glutamylcysteine synthetase and a glutathione synthetase in a Δ*yggS* background causes high-level accumulation of ophthalmic acid in the cells (~1.2 nmol/mg cells) in a minimal synthetic medium. This opens the possibility of a first fermentative production of ophthalmic acid.

Introduction

Escherichia coli YggS is a pyridoxal 5'-phosphate (PLP)-binding protein that belongs to the fold-type III family of PLP-binding enzymes. YggS orthologs are highly conserved in a wide range of organisms, from bacteria to mammals, indicating that the YggS protein family plays a vital role in cellular function.

We have reported that in the log phase, a *yggS*-deficient *E. coli* strain (Δ*yggS*) accumulates 2-ketobutyrate (2-KB) and its aminated compound L-2-aminobutyrate (L-2-AB) and displays slightly decreased coenzyme A (CoA) levels compared to that in the parental strain. In the stationary phase, Δ*yggS* exhibits a significantly altered intracellular amino-acid pool and high levels of extracellular L-Val, probably due to altered L-2-AB, 2-KB, and/or CoA availability [1]. We also demonstrated the highly conserved PLP-dependent function of YggS by showing that the phenotypes of Δ*yggS* were completely reversed by plasmid-borne expression of YggS and orthologs from *Bacillus subtilis*, *Saccharomyces cerevisiae*, and human, but not by the expression of a mutant YggS lacking PLP-binding ability [1]. Nichols et al. reported that the double mutant of *yggS* and *glyA*, the latter encoding for serine hydroxymethyltransferase, is a synthetic lethal pair in *E. coli*, and that the Δ*yggS* is sensitive to sulfonamides, the latter targeting the enzyme dihydropteroate synthase (FolP) in the tetrahydrofolate biosynthesis pathway [2]. In *B. subtilis*, the *yggS* ortholog *ylmE* is reported to be involved in biofilm formation and its disassembly. The double mutant of *ylmE* and *racX* (the latter's product

58 exhibits sequence homology with Asp/Glu racemases) was blocked in D-Tyr production and
59 exhibited impaired D-Leu production [3]. However, racemase activity toward any of the
60 proteinogenic amino acids has not been detected in purified YggS or its orthologs, including
61 YlmE [1]. Very recently, Prunetti et al. suggested that *yggS* is involved in PLP homeostasis by
62 demonstrating that Δ*yggS* accumulates the PLP precursor pyridoxine 5'-phosphate (PNP) and
63 exhibits pyridoxine sensitivity [4]. Although several metabolic consequences of lacking this
64 protein in *E. coli* and in other organisms have been reported, the biochemical function of YggS
65 has not been identified.

66 In this study, we show that Δ*yggS* accumulates γ-L-glutamyl-L-2-aminobutyryl-glycine,
67 known as ophthalmic acid. The data indicate that absence of *yggS* initially causes an increase
68 in L-2-AB/2-KB availability in the cell, resulting in ophthalmic acid accumulation and decrease
69 in intracellular CoA content. The expression of *gshA* and/or *gshB* in the mutant cells readily
70 increases the intracellular levels of ophthalmic acid in M9-glucose medium. These results raise
71 the possibility of developing a first fermentative ophthalmic acid production method.
72

Materials and Methods

Materials

Amino acids were from Wako (Osaka, Japan). Ophthalmic acid was obtained from PH Japan Co., Ltd (Hiroshima, Japan).

Bacterial strains, plasmids and growth conditions

Construction of the Δ*yggS* of *E. coli* MG1655 and pU0 plasmid is described in [1, 5]. *E. coli* BW25113 and its *yggS*-deficient strain (*E. coli* JW2918, Δ*yggS* of *E. coli* BW25113) were obtained from the National Institute of Genetics (NIG, Shizuoka, Japan). Plasmids pCA24N-ilvA (ASKA clone: JW3746), pCA24N-avtA (JW5652), pCA24N-gshA (JW2663), pCA24N-gshB (JW2914), and the empty plasmid pCA24N were obtained from the ASKA library [6]. LB medium or M9-glucose medium [1] were used for cell growth. Ampicillin or chloramphenicol was added to the medium at a final concentration of 100 μg/ml or 30 μg/ml, respectively. If required, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 0.1 mM. For growth in the M9-glucose medium, log-phase *E. coli* cells cultivated in the LB medium (O.D.600 ~ 1.0) were used. Cells from the LB medium were collected, washed twice with PBS, and then inoculated in the M9-glucose medium at a final O.D.600 of 0.01 as described in [1]. All bacteria were grown at 30°C with shaking.

92 *Determination of amino acids*

93 *E. coli* cells were grown in the M9-glucose medium to a log-phase (O.D.600 ~0.5) and the
94 amino acids were extracted from the cells as described in [1]. Briefly, log-phase cells were
95 collected and disrupted in 5% trichloroacetic acid. Samples were extracted three times with
96 water-saturated diethylether, dried, and then resuspended in distilled water.

97 The amino acids were derivatized using the EZ:faast^{TM} amino acid analysis kit (Phenomenex,
98 USA) according to the manufacture's instructions and subsequently analyzed by LC-ESI-MS.
99 The system was Agilent 1100 Series HPLC system (Agilent Technologies, USA) with a
100 EZ:faast AAA-MS column (250 × 2.0 mm, 4 μm) which was provided with the EZ:faast kit.
101 Separation of amino acid derivatives were achieved using the following mobile phases, 10 mM
102 ammonium formate in water (solvent A) and 10 mM ammonium formate in methanol (solvent
103 B), flow rate 0.25 min/mL by a gradient elution (0 min 68% of solvent B, 15 min 71% B, 17
104 min 72% B, 20 min 83% B). UV detection was at 210 nm. ESI-MS was performed with an
105 Esquire 3000 ion trap system (Bruker Daltonics, USA). The instrument parameters used were
106 as follows: nebulizer, 30 psi N_2; dry gas, 7.0 L·min^{-1}; dry temperature, 320°C; compound
107 stability, 10%; Trap drive level, 100%; scanning range, 100–600 m/z.

108 Alternatively, amino acids were derivatized by Boc-L-cysteine (Boc-L-Cys) and *o*-
109 phtalaldehyde (OPA) or by *N*-acetyl-L-cysteine (NAC) and OPA, and separated as previously
110 described in [1] or [7], respectively. Briefly, for the amino acid analysis with Boc-L-Cys/OPA,

111 we used the following gradient elution for the separation of the diastereomers; Buffer A: 0.1 M
112 sodium acetate buffer (pH 6.0) and 9 % acetonitrile; Buffer B: 0.1 M sodium acetate buffer (pH
113 6.0) and 50% (v/v) acetonitrile; linear gradient 0% B to 17.5% in 10 min, 17.5% B to 33% in
114 40 min. Cosmosil 5C18-AR column (4.6 x 150 mm, Nacalai Tesque) was used. The elution
115 condition for the amino acid analysis with NAC/OPA was follows; Buffer A: 10 mM sodium
116 phosphate buffer (pH 6.5); Buffer B: 10 mM sodium phosphate buffer (pH 6.5) and 60% (v/v)
117 methanol; linear gradient 15% B to 35% in 25 min, 35% B to 65% in 15 min. A C18 column
118 (Mightysil RP-18 GP II 4.6 x 250 mm ID, 5 μm, Kanto chemical) was used. Column
119 temperature was 25°C and the flow rate was 0.8 ml/min. A Shimadzu HPLC system equipped
120 with fluorescence detector was used. Excitation and emission wavelength were 350 nm and
121 450 nm, respectively.
122
123

Results and Discussion

*Accumulation of an unknown metabolite in Δ*yggS*

Our previous study showed that Δ*yggS* of *E. coli* MG1655 accumulates L-2-AB and L-Val in the cells in the log phase and excretes high levels of L-Val into the culture medium in the stationary phase [1]. Further study showed that, in the log phase, the Δ*yggS* accumulates an additional metabolite (eluted at retention time (RT) 21 min in Fig. 1A), expected to be an amino acid, amine, or peptide, as judged by amino acid analysis. In the mutant strain, the intracellular concentration of the metabolite was ~20 times higher than that in control, an abundance comparable to some other free proteinogenic amino acids such as L-Asp and L-Ser (Fig. 1A). We found that another *yggS*-deficient *E. coli* strain with a different genetic background (Δ*yggS* of *E. coli* BW25113) neither displays significant difference in cellular L-Val and L-2-AB concentrations in the log phase (1.1 nmol/mg cells and 1.4 nmol/mg cells for L-Val in WT and the Δ*yggS*, respectively, and 0.29 nmol/mg cells and 0.33 nmol/mg cells for L-2-AB in WT and the Δ*yggS*, respectively) nor excretes L-Val into the medium in the stationary phase, however, it also accumulates the unknown metabolite (RT 21 min, Fig. 1B). In the two Δ*yggS* strains, intracellular levels of this metabolite were decreased to the same extent as those in parental strains upon plasmid-borne expression of *yggS* (data not shown), confirming that YggS participates in the metabolism of the unknown compound.

143 *Ophthalmic acid accumulation in Δ*yggS*

144 We reasoned that identification of the unknown metabolite may give insights as to the function
145 of YggS. We initially tried to identify the metabolite by co-chromatography with more than 70
146 kinds of D- and L-amino acids (using commercially available amino acid mixture and D-amino
147 acids) using the Boc-L-Cys/OPA and the NAC/OPA derivatization methods. However, no
148 amino acid was co-eluted with the metabolite (data not shown).

149 To obtain structural information of the metabolite, we then analyzed the intracellular amino
150 acids by the EZ:faast™ procedure. In this method, amino acids are derivatized by propyl
151 chloroformate and are then analyzed by LC/MS. As shown in Fig. 2A, Δ*yggS* of *E. coli*
152 MG1655 accumulated two molecules in the cells, whose derivatives have *m/z* values of 460
153 (Fig. 2B) and 246 at RT 7.0 min and RT 11 min, respectively. The Δ*yggS* of *E. coli* BW25113
154 accumulated a molecule whose derivative had a *m/z* value of 460 (Fig. 2C and 2D). The elution
155 profile, MS, and MS/MS data confirmed that the ion having a *m/z* value of 246 is the derivative
156 of L-Val. The precursor ion with a *m/z* value of 460 probably belonged to the unknown
157 metabolite.

158 MS/MS analysis of the fragment ion at *m/z* 460 gave rise to 6 prominent fragment ions of *m/z*
159 values of 400.4, 343.4, 315.4, 258.3, 203.3, and 172.2 (Fig. 2E). The manufacturer's manual
160 suggests that fragment ions whose *m/z* are 258 and 172 belong to the major MS/MS fragment
161 ions of Glu derivatives. In addition, the fragment ion of *m/z* 343 indicated that the molecule

may contain a Gly moiety. Combined, these results suggested that the metabolite was a tripeptide of Glu, 2-AB, and Gly, or of Glu, γ-aminobutyrate (GABA), and Gly.

Our previous study showed that the Δ*yggS* of *E. coli* MG1655 accumulates L-2-AB [1]. Thus, the metabolite most probably contains L-2-AB rather than GABA. We next cultured wild-type *E. coli* BW25113 cells in the presence of L-2-AB or GABA, whereupon the intracellular amino acid compositions were evaluated. In the presence of L-2-AB, the *E. coli* cells accumulated high levels of a compound whose retention time agreed with that of the unknown metabolite (Fig. 3, line d). No increase in the intracellular levels of this compound was observed with GABA (Fig. 3, line c). These results suggest that the compound is a tripeptide of L-Glu, L-2-AB, and Gly, rather than Glu, GABA, and Gly.

L-2-AB is known to serve as a substrate of γ-glutamylcysteine synthase (GshA) [8]. γ-L-glutamyl-L-2-aminobutyryl-glycine, known as ophthalmic acid, is found in various living organisms as an endogenous glutathione (GSH) analogue [9, 10]. We compared the chemical properties of the metabolite accumulated in the Δ*yggS* to that of synthetic ophthalmic acid. The retention time of ophthalmic acid in an amino acid analysis with Boc-L-Cys/OPA derivatization was identical to that of the unknown metabolite (Fig. 3B). In the EZ:faast™ procedure, the elution profile of the derivatives of ophthalmic acid standard (i.e. the RT and the *m/z* value (460) of the precursor ion) was also identical to that of the unknown metabolite. In addition, the MS/MS spectra of the precursor ion of ophthalmic acid derivative (*m/z* 460) were identical

to that of the unknown molecule (Fig. 2F). Identical MS/MS fragmentation pattern was also observed with derivatives of GSH (data not shown). These results demonstrate that the metabolite accumulated in the Δ*yggS* is ophthalmic acid.

YggS is involved in the regulation of L-2-AB rather than of CoA

In mouse serum and liver, ophthalmic acid levels are increased significantly in conjunction with GSH consumption [11]. Therefore, the ophthalmic acid accumulation in the Δ*yggS* can be expected to stem from altered GSH levels and/or its biosynthetic enzymes (GshA and GshB). In Δ*yggS*, GSH levels were not significantly affected (Fig. 2A and 2C). This indicates that ophthalmic acid accumulation is not caused by the limitation of GSH and/or activation of its biosynthetic enzymes (GshA and/or glutathione synthase (GshB)). Although less likely, ophthalmic acid may be an enzymatic substrate of YggS. To examine this, recombinant C-terminal His-tagged YggS protein was incubated with amino acids extract of Δ*yggS* in a buffer (100 mM sodium phosphate buffer, 20 μM PLP, pH 7.0) for 13 h at 30°C, whereupon the change in ophthalmic acid concentration was analyzed by HPLC. No reactivity of YggS with ophthalmic acid was observed under the conditions examined (data not shown). We previously found that the levels of the enzymes involved in 2-KB and L-2-AB metabolism (i.e. threonine dehydratase (IlvA), transaminase B (IlvE), and transaminase C (avtA)) are all elevated in Δ*yggS* [1]. Extrapolating these results, we may estimate that the ophthalmic acid accumulation

200 is due to increase in L-2-AB availability.

201 We previously showed that the *yggS* mutation of *E. coli* MG1655 leads to a ~10% decrease
202 in intracellular coenzyme A (CoA) concentration. Addition of the CoA precursor pantothenate
203 to the culture medium effectively abolished the high-level excretion of L-Val by the Δ*yggS* [1].
204 From these results we have speculated that YggS is involved in the regulation of CoA levels;
205 the decrease in CoA leads to accumulation of 2-KB and/or L-2-AB and then induces L-Val
206 excretion [1]. However, this hypothesis remains to be confirmed.

207 To examine the causal relationship between L-2-AB/2-KB accumulation and CoA limitation,
208 productivity of ophthalmic acid was analyzed in the presence or absence of exogenous
209 pantothenate. Exogenous pantothenate is probably effective to restore the decreased CoA levels
210 in the cells [12]. As shown in Fig. 4, supplementation with pantothenate showed no significant
211 decrease of ophthalmic acid in the *yggS*-mutant. This suggests that CoA levels do not influence
212 L-2-AB availability.

213 Previously, Powers et al. and Primerano et al. reported that CoA synthesis is compromised by
214 2-KB accumulation by showing that 2-KB is a competitive substrate for ketopantoate
215 hydroxymethyltransferase, the first enzyme in pantothenate biosynthesis [13, 14]. We, too,
216 confirmed that 2-KB lowers intracellular CoA levels in *E. coli* [1]. These observations suggest
217 that YggS participates in the regulation of L-2-AB and/or 2-KB rather than that of CoA, and
218 the CoA limitation observed in the Δ*yggS* stems from high levels of 2-KB.

219

220 *ΔyggS opens the possibility for fermentative production of ophthalmic acid*

221 The Δ*yggS* is probably the first microorganism that produces high levels of ophthalmic acid.
222 Ophthalmic acid is a potent inhibitor for glyoxalase I [15] and a high-affinity ligand for a
223 calcium-sensing receptor in humans involved in the perception of *kokumi*, a type of taste [16].
224 Furthermore, it is suggested to be a potent indicator of oxidative stress that senses depletion of
225 hepatic GSH [11]. Indeed, ophthalmic acid has several potential uses in research and industry,
226 but it is currently highly expensive (~ $1,500/g). Low-cost supply of ophthalmic acid will
227 enable further study and industrial application of the compound.

228 Ophthalmic acid is probably synthesized via sequential reactions catalyzed by GshA and
229 GshB in *E. coli* (Fig. 5). Overexpression of GshA and/or GshB may further increase the
230 productivity of ophthalmic acid. As shown in Fig. 6, we found that the plasmid-borne
231 expression of *gshA* or *gshB* in the Δ*yggS* of *E. coli* BW25113 further increased the intracellular
232 ophthalmic acid levels even in the M9 medium lacking L-2-AB (Fig. 6). The concentration of
233 ophthalmic acid in the Δ*yggS* expressing *gshA* reached 1.2 nmol/mg cells, which is 20 times
234 higher than that in WT, and indeed much higher than most free proteinogenic amino acids. This
235 suggests that the reactions catalyzed by GshA and GshB are rate-limiting steps in ophthalmic
236 acid synthesis. Threonine dehydratase (IlvA) catalyzes the dehydration of L-Thr to generate 2-
237 KB and ammonia. Transaminase C (avtA) converts 2-KB to L-2-AB and vice versa. These two

enzymes are potentially capable of elevating intracellular L-2-AB levels (Fig. 6). The plasmid-borne expression of *ilvA* and *avtA*, however, did not increase ophthalmic acid production under the conditions examined (Fig. 6). The reason of the decreased productivity of ophthalmic acid remains to be elucidated. We observed that the enzyme levels of IlvA and avtA are both significantly elevated in Δ*yggS* (2.7- and 2.4-fold, respectively) compared with WT [1]. Simultaneous expression of the two enzymes may be required to further increase the productivity of ophthalmic acid.

Suzuki et al. reported direct fermentation of GSH by using an *E. coli* strain overexpressing *gshA* and *gshB*, and lacking γ-glutamyltransferase (*ggt*) and GSH importers (*yliAB*) [17]. Zhang et al. described the fermentative production of L-2-AB by overexpressing a mutant glutamate dehydrogenase and threonine dehydratase from *B. subtilis* in a modified threonine-hyperproducing *E. coli* strain [18]. Combining these strategies will help to develop a first fermentative production method for ophthalmic acid.

Acknowledgments

This work was supported in part by JSPS KAKENHI Grant Numbers 24780098 and Noda Institute for Scientific Research Grant (to T. I.).

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310 **Figure Legends**

311 **Fig. 1 Elution profile of intracellular amino acid**

312 Wild-type *E. coli* cells (MG1655 or BW25113) and the Δ*yggS* mutant cells those harboring
313 pU0 plasmid were grown in M9-glucose medium at 30°C. Cells were collected at log-phase.
314 Intracellular amino acids were extracted, derivatized by the Boc-L-Cys and OPA, and separated
315 by a C18 column as described in Materials and Methods. The HPLC chromatograms of wild-
316 type (solid line) and the Δ*yggS* mutant (broken line) of *E. coli* MG1655 are shown in (A) and
317 those of wild-type (solid line) and the Δ*yggS* mutant (broken line) of *E. coli* BW25113 are (B).
318 The arrows points to the unknown metabolite (later identified as ophthalmic acid) accumulated
319 in the Δ*yggS*.

320

321 **Fig. 2 LC/MS and MS/MS analyses of the unknown metabolite**

322 All *E. coli* strains harboring pU0 plasmid were cultivated in M9-glucose medium at 30°C. Log-
323 phase cells were collected and the intracellular amino acids were analyzed with EZ:faast kit.
324 (A and C) Total-ion chromatogram (TIC) of the positive-ion mass spectra of intracellular amino
325 acids of wild-type (solid line) and the Δ*yggS* mutant (broken line) of *E. coli* MG1655 (A) and
326 BW25113 (C). (B and D) Extracted-ion chromatograms (EIC) of m/z 460. (E and F) MS/MS
327 spectra of the compound whose precursor ion of m/z 460 (of Δ*yggS* mutant of BW25113) (E)
328 and that for the synthetic ophthalmic acid (F). (G) Estimated derivative of ophthalmic acid with

propyl chloroformate. Putative sites of cleavage by MS/MS analysis is shown as (a) to (d) in Figs F and G. The arrows in panels A and C points to the unknown metabolite (later identified as ophthalmic acid). Retention times of L-Val and L-2-AB are also indicated.

Fig. 3 Identification of the ophthalmic acid accumulation in Δ*yggS*

(A) Elution profiles of intracellular amino acids of *E. coli* BW25113 wild-type (line a) and Δ*yggS* (b) in the M9-glucose medium, and that of the *E. coli* BW25113 wild-type cultivated in the M9-glucose medium supplemented with 1 mM GABA (c) and 1 mM L-2-AB (d). Amino acids were derivatized by Boc-L-Cys and OPA, and separated by C18 column.

(B) Comparison of the retention time between the metabolite accumulated in the Δ*yggS* mutant cells and ophthalmic acid standard. Elution profile of intracellular amino acid of *E. coli* BW25113 wild-type (line a) and of the Δ*yggS* (b). Amino acid standard (Wako Co. Ltd, type H) (d) and that contains synthetic ophthalmic acid (c) were used as controls. Amino acids were derivatized by NAC and OPA, and separated with a ODS column. The arrows points to the unknown metabolite (or ophthalmic acid).

Fig. 4 Effect of pantothenate on the ophthalmic acid productivity

Wild-type and Δ*yggS* cells of *E. coli* BW25113 were cultivated in M9-glucose medium in the presence or absence of pantothenate at 30°C. Pantothenate was added to the culture medium at

348 a final concentration of 0.1 mM. Cells were collected at log-phase and intracellular ophthalmic
349 acid concentrations were analyzed by HPLC using Boc-L-Cys and OPA derivatization method.
350 The arrows points to the unknown metabolite (or ophthalmic acid).
351
352 **Fig. 5 Biosynthesis of ophthalmic acid**
353 Ophthalmic acid is synthesized from L-Glu, L-2-AB, and Gly via two consecutive enzymatic
354 reactions catalyzed by γ-glutamylcysteine synthetase (GshA) and glutathione synthetase
355 (GshB). L-2-AB is produced from 2-KB by transaminase reaction catalyzed by transaminase B
356 (IlvE) and/or transaminase C (avtA). Threonine dehydratase (IlvA) deaminates L-Thr to yield
357 2-KB.
358
359 **Fig. 6 Enhancement of ophthalmic acid production by the overexpression of *gshA*, *gshB*,**
360 ***ilvA*, or *avtA*.**
361 BW25113 wild-type or Δ*yggS* strains harboring pCN24N or its derivatives (pCA24N-avtA,
362 pCA24N-ilvA, pCN24N-gshA, pCA24N-gshB) were cultivated in M9-glucose medium at
363 30°C and the cells were harvested at log-phase (O.D.600 = ~0.5). IPTG was added to the
364 medium at a final concentration of 0.1 mM. The intracellular concentration of ophthalmic acid
365 was determined by amino acid analysis using NAC and OPA derivatization method. Each value
366 is an average of at least two independent experiments.