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Analysis of Heme Biosynthetic Pathways in a Recombinant *Escherichia coli*

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Bacterial heme was produced from a genetic-engineered *Escherichia coli via* the porphyrin pathway and it was useful as an iron resource for animal feed. The amount of the *E. coli*-synthesized heme, however, was only few milligrams in a culture broth and it was not enough for industrial applications. To analyze heme biosynthetic pathways, an engineered *E. coli* artificially overexpressing ALA synthase (*hemA* from *Rhodobacter sphaeroides*) and pantothenate kinase (*coaA* gene from self geneome) was constructed as a bacterial heme-producing strain, and both the transcription levels of pathway genes and the intermediates concentrations were determined from batch and continuous cultures. Transcription levels of the pathway genes were not significantly changed among the tested conditions. Intracellular intermediate concentrations indicated that aminolevulinic acid (ALA) and coenzyme A (CoA) were enhanced by the *hemA-coaA* co-expression. Intracellular coproporphyrinogen I and protoporphyrin IX accumulation suggested that the bottleneck steps in the heme biosynthetic pathway could be the spontaneous conversion of HMB to coproporphyrinogen I and the limited conversion of protoporphyrin IX to heme, respectively. A strategy to increase the conversion of ALA to heme is discussed based on the results.

Keywords: Heme-iron, recombinant *E. coli*, porphyrin pathway, pantothenate pathway, bottleneck step

Introduction

Heme is an iron-associated prosthetic group in many organisms involving oxidative phosphorylation as an energy generating metabolism for its functions as a component of oxygen transfer in red blood cell, electron transfer in mitochondria, and redox reactions in enzymes [8]. Iron is reversibly stored within the human liver as ferritin and hemosiderin, whereas it is transported between different compartments in the body by protein ferritin. The main source of iron for human consumption is food containing heme-iron or non-heme-iron. Heme-iron sources are contained in red meat, poultry, and fish, whereas non heme-iron sources are vegetable and fruit. Heme-iron content can reach 40% of meat compared with non-heme-iron. Heme-iron is more readily absorbed by the body.

The structure of heme is a ring-form molecule containing four pyrrolic groups and a Fe^{2+} ion in the center. 5-Aminolevulinic acid (ALA) is a precursor for heme that is synthesized in mitochondria from succinyl-CoA and glycine [10] and catalyzed by ALA synthase. There are two pathways known for ALA synthesis; namely, the C4 pathway and the C5 pathway. The C5 pathway, which is found in photosynthetic algae and cyanobacteria, utilizes glutamate with ATP and NADPH as co-substrate. It requires a three-step reaction catalyzed by glutamyl-tRNA-synthetase, glutamyl-tRNA-reductase, and glutamate-1-semialdehyde aminotransferase [11]. The C4 pathway, which is found in photosynthetic bacteria, yeast, and mammalian cells, involves pyridoxal-5-phosphate as a cofactor in succinyl-CoA and glycine condensation. The well-known microorganism for the C4 pathway is *Rhodobacter sphaeroides* 2.4.1 that has the *hemA* gene to code for ALA synthase [12]. *R. sphaeroides* synthesizes both heme and bacteriochlorophyll

(Bchl), as well as vitamin B_{12}, a necessary cofactor in Bchl formation and also in methionine and cysteine biosynthesis.

Biologically synthesized heme-iron by recombinant bacteria, such as *E. coli*, is the promising method to make mass product of heme. Genetic engineering allows bacteria to produce heme-iron. An *E. coli* strain carrying the *hemA* gene from *R. spaeroides* synthesized heme-iron, and it could be used for an iron source for mouse feed. [2]. Lee *et al*. [6] additionally expressed the *coaA* gene that is involved in the pantothenate pathway and every gene from *hemB*, *hemC*, *hemD*, to *hemE* in the porphyrin pathway to produce a higher concentration of heme-iron, though the amount was still only a few milligrams per gram biomass that is too little for industrial-scale production [6], even in the optimized medium [5], and further pathway engineering is required.

The porphyrin pathway in the recombinant *E. coli* begins with the condensation of two molecules of ALA, synthesized from succinyl-CoA and glycine, to porphobilinogen (PBG) by zinc-dependent ALA dehydratase, also known as porphobilinogen synthase (coded by *hemB*) (Fig. 1). Four molecules of PBG subsequently form an unstable tetrapyrrolle-hydroxymethylbilane (HMB), catalyzed by porphobilinogen deaminase (coded by *hemC*). Porphobilinogen deaminase, which is also known as HMB synthase, contains dipyrromethane in its active site for the attachment of PBG. HMB can continue to two pathways. The first pathway is the conversion of HMB by uroporphyrinogen synthase (coded by *hemD*) to close the HMB macrocycle, leading to the conversion of tetrapyrolle to uroporphyrinogen III. Another pathway is the conversion of HMB by spontaneous cyclization to form uroporphyrinogen I.

Uroporphyrin decarboxylase (coded by *hemE*) then catalyzes the decarboxylation of all four acetate side chains of uroporphyrinogen III to methyl groups and produces coproporphyrinogen III. Coproporphyrinogen oxidase (coded by *hemF*) then catalyzes the conversion of coproporphyrinogen III to protoporphyrinogen IX with the release of H_2O_2 and CO_2. Protoporphyrinogen IX needs FAD-containing

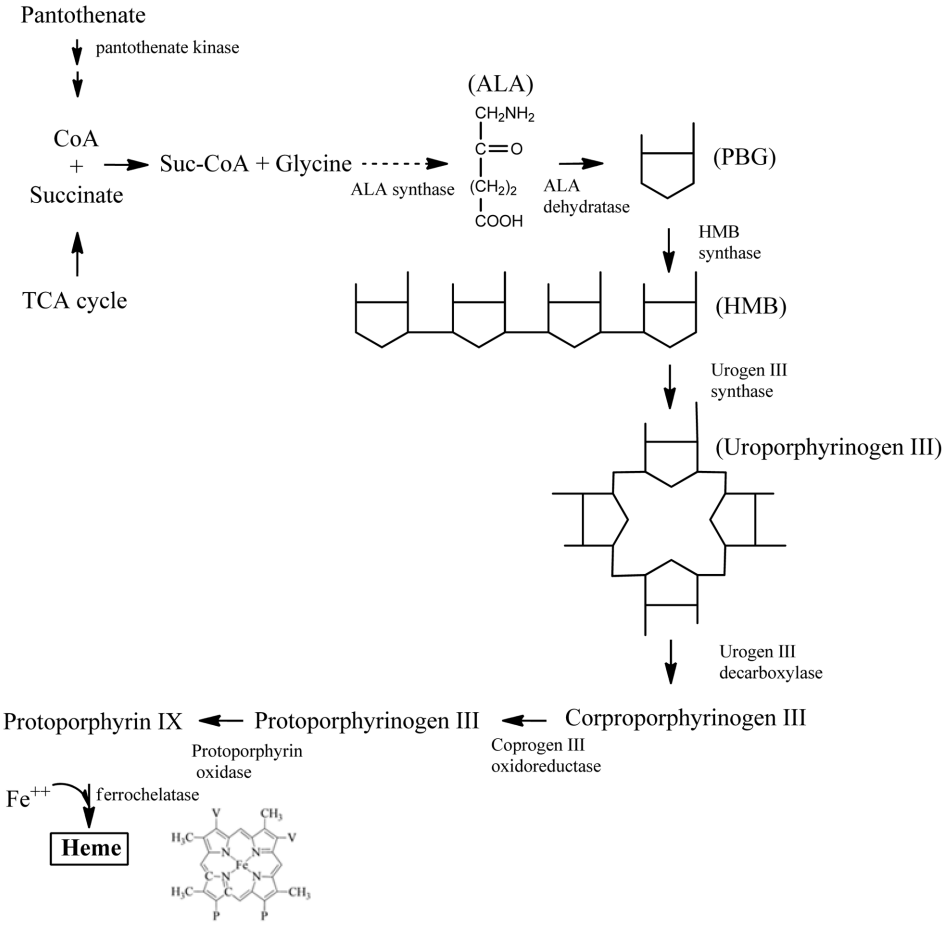

Fig. 1. Schematic of the heme biosynthetic pathway in a recombinant *Escherichia coli*.
Adapted from a previous publication [5].

protoporphyrinogen oxidase (coded by *hemG*) to synthesize protoporphyrin IX. The reaction requires oxygen as a terminal electron acceptor and leads to removal of six hydrogen atoms from protoporphyrinogen IX. Ferrochelatase (coded by *hemH*) completes the pathway by insertion of Fe^{2+} to protoporphyrin IX to form heme [9]. Heme is formed in mitochondria and then exported to the cytosol for incorporation into hemoproteins. Heme degradation is catalyzed by the microsomal heme oxygenase 1 (HO-1) and its homologs HO-2 and HO-3 [10]. A side from the main heme synthesis pathway, the pantothenate pathway can be involved in molecular heme biosynthesis. Pantothenate kinase (which is coded by *coaA*) converts pantothenate into 4′-phosphopantothenate, and the end molecule produced through the next four consecutive steps is coenzyme A (CoA), which is used to form succinyl-CoA.

In this manuscript, we analyzed the mRNAs and intermediates of the heme biosynthetic pathway in batch and continuous cultures of *E. coli* artificially expressing the *hemA* and *coaA* genes. The potential bottleneck step and a strategy to enhance bacterial heme-iron production are also discussed.

Materials and Methods

Strains and Plasmids

Strains and plasmids in this study are listed in Table 1. *Escherichia coli* DH5α (Invitrogen, Life Technologies Co.), was used for DNA manipulation and W3110 (KCTC 2223) for heme-iron production, respectively. The vector expressing ALA synthase from *Rhodobacter spaeroides* was from a previous study [11]. Pantothenate kinase (*coaA*) was PCR-amplified with oligonucleotide of 5′-TTC TGC AGA GG**A GGA** ACA GAC ATG AGT ATA AAA GAG CAA ACG TTA ATG A-3′ (*Pst*I site undelined, ribosomal binding site in bold) and 5′-AAG CTT GTT ATT TGC GTA GTC TGA CCT CTT CTA-3′ (*Hin*dIII site underlined) based on *E. coli* genomic DNA template. Oligonucleotides were synthesized at Macrogen Co. (Seoul, Korea). The PCR product (980 bp) was subcloned into a T-blunt vector (Solgent Co., Daejeon, Korea) for sequencing (Macrogen Co.). The *Pst*I-*Hin*dIII double-digested DNA fragment was ligated to the same digested pTrc99a-*hemA*$^+$ after purification, and resulted in pTrc99a-*hemA*$^+$-*coaA*.

Culture Conditions

Luria-Bertani (LB) medium was used in the DNA manipulating process. The media for batch culture, continuous cultures, and enzyme activity determinations were composed of 5 g yeast extract, 10 g tryptone, 5 g KH_2PO_4, 2 g glycine, and 10 g succinate per liter medium supplemented with ampicillin (50 μg/ml) and IPTG (0.1 mM). The initial pH was set to 6.5.

A single colony was inoculated in a 15 ml tube containing 3 ml of LB medium and incubated for 16 h at 37°C, 230 rpm. For batch culture, culture broth (500 μl) was transferred to a 250 ml Erlenmeyer flask containing 50 ml of medium. For continuous culture, culture broth (1.5 ml) was transferred to a 250 ml jar containing 150 ml of medium. Medium feeding and outlet pumps were set to be 15, 45, and 75 ml/h (D = 0.1, 0.3, and 0.5 h^{-1}, respectively). Steady state cells were harvested for analysis after at least five turnover times.

Analysis

Biomass was measured by optical density at 600 nm (OD_{600}) with conversion coefficient (OD 1 = 0.31 g/l).

Cells were harvested by centrifugation (14,000 rpm for 5 min.) and the pellet was disrupted by sonic vibration on ice (70 W for 1 min. at 1 sec intervals) using a sonic vibrator (UP200S; Hielscher Ultrasonics GmbH, Teltow, Germany). Cell extract was prepared after removal of cell debris by centrifugation and used for the determination of enzyme activities [3]. The enzyme activity of ALA synthase was determined by measuring ALA formation as previously described [2]. The reaction mixture (1 ml; composed of 10 mM glycine, 10 mM succinyl-CoA, 1 mM pyrydoxal-5-phophate, cell extract 50 μl in a 50 mM Tris-HCl buffer (pH 7.5)) was incubated at 37°C for 30 min. ALA formation was measured by adding Ehrlich's reagent (200 μl; composed of 0.2 g ρ-dimethylaminobenzaldehyde,

Table 1. Strains and plasmids.

Strains and plasmids	Description	Source
Strains		
W3110	Wild-type *E. coli λ⁻IN(rrnD-rrnE)1rph-1*	KCTC 2223
DH5α	F-ϕ80 d*lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *endAI recAI hsdR17* ($r_k^-m_k^+$) *deoR thi-I phoA supE44λ gyrA96 relAI*	Invitrogen Co.
Plasmids		
T-blunt vector	Cloning vector, Amp^r Kan^r	Solgent Co. Korea
pTrc99a	Expression vector, *trc* promoter, Amp^r	AP Biotech Co. USA
pTrc(P_{lac}*hemA*$^+$)	pTrc99a with additional *lac* promoter, *hemA* gene, and following 500 bp flanking region from *R. sphaeroides*	Shin *et al*. [11]
pTrc(P_{lac}*hemA*$^+$-*coaA*)	pTrc(P_{lac}*hemA*$^+$) with RBS and *coaA* from *E. coli* (*Pst*I-*Hin*dIII)	This study

8.4 ml of glacial acetic acid, and 1.6 ml of 70% (v/v) perchloric acid per liter) and the absorbance at 555 nm was detected [7]. The enzyme activity of pantothenate kinase was estimated by measuring ATP reduction. Cell extract (50 µl) was mixed with 5 mM Ca-D-pantothenate as substrate, 5 mM ATP, and 1 mM $MgCl_2$ in a Tris-HCl buffer (pH 7.5) and incubated at 37°C for 30 min. The remained ATP was estimated using a luminometer (20/20n Luminometer System; Turner Biosystem Inc., Sunnyvale, CA, USA) and ATP assay kit (Sigma-Aldrich Co.).

Metabolite concentrations from the sample were measured by colorimetric methods and LC/MS depending on the chemical nature of the intermediate. The intracellular ALA concentration in the cell extract was detected after visualization by Ehrlich's reagent as previously described [11]. Intracellular CoA concentrations were determined using the Coenzyme A Assay Kit (Biovision, San Francisco, CA, USA) and spectrophotometer at 570 nm according to the manufacturer's protocol. Heme was extracted from cells membrane after sonic vibration. The pellet of cell debris was resuspended in 6 N NaOH solution (200 µl) for re-disruption and mixed with DMSO:acetonitrile (1:4 (v/v)) solution (400 µl). The mixture was centrifuged at 13,000 ×*g* for 15 min, and the supernatant was subjected to heme quantification using the Quantichrom Heme Assay Kit (BioAssay System, Hayward, CA, USA) according to the manufacturer's instruction. Uroporphyrinogen III, coproporphyrinogen I, coproporphyrinogen III, and protoporphyrin IX concentrations were determined from the cell extract. Supernatant from sonic disrupted cells was mixed with ice-cold acid acetone (acetone: 6N HCl = 99.8:0.2, 500 µl) and vigorously vortexed. After centrifugation (13,000 ×*g*, 20 min), the supernatant was separated and dried in a speedvac overnight. The dried contents were dissolved in 10 N NaOH solution and further subjected to LC/MS analysis in a facility of MetaMass Co. (Seoul, Korea).

Transcription levels of all genes involved in the heme synthetic pathway (*coaA, hemA, hemB, hemC, hemD, hemE, hemF, hemG*, and *hemH*) were quantitated by RT-PCR [4]. RNAs were extracted from cells using a kit (Nucleic Acid and Protein Purification; Macherey-Nagel, Germany), and then converted to cDNA using the First Stand cDNA Synthesis Kit (Toyobo, Japan). Quantitative PCR was performed using MxQPCR (Agilent Technologies, Inc., CA, USA) with 16S rDNA as the reference gene and SYBR Green as dye. The template cDNA volume was set to 1 µl and specific oligonucleotides were designed for each gene with the same melting temperature (60°C). Relative quantities from the expression analysis were calculated with MxPro QPCR software ver. 4.10 (Agilent Technologies). The oligonucleotides used for RT-qPCR are listed in Table S1.

Results

Plasmid Construction and Enzyme Activities

To overexpress ALA synthase and pantothenate kinase, a plasmid carrying gene *hemA* plus the following 500 bp flanking region from *Rhodobacter sphaeroides* and gene *coaA* from *E. coli* was constructed and named pTrc-*hemA*$^+$-*coaA*. The constructed plasmid was transformed into the *E. coli* W3110 strain, and the enzyme activities of ALA synthase and pantothenate kinase were analyzed (Table 2). ALA synthase activity was found in the strains carrying the *hemA*$^+$ gene in the plasmids, whereas the strain carrying empty vector (pTrc99A) did not show any ALA synthase activity. Pantothenate kinase activities of the strains without the plasmid-originated *coaA* gene (pTrc99A and pTrc-*hemA*$^+$) were 2.71 and 2.74 U/$mg_{protein}$, respectively, whereas W3110 carrying pTrc-*hemA*$^+$-*coaA* showed a promoted activity of 3.77 U/$mg_{protein}$. Therefore, proper expression of ALA synthase and pantothenate kinase was confirmed by the pTrc-*hemA*$^+$-*coaA* plasmid in the W3110 strain.

Transcription Levels of the Genes in Heme Biosynthetic Pathway

The heme biosynthetic pathway in the recombinant *E. coli* (W3110 carrying pTrc-*hemA*+-*coaA*) is consisted of plasmid-originated ALA synthase (*hemA*) and plasmid/genome-originated pantothenate kinase (*coaA*) as well as genome-originated genes of ALA dehydratase (*hemB*), HMB synthase (*hemC*), uroporphyrinogen III synthase (*hemD*), uroporphyrinogen III decarboxylase (*hemE*), coproporphyrinogen III oxidoreductase (*hemF*), protoporphyrin oxidase (*hemG*), and ferrochelatase (*hemH*). Transcription levels in the heme biosynthetic pathway were analyzed in the cells from batch culture (12 h) and steady-state cells from continuous cultures (Table 3). Plasmid-derived *hemA* transcription and both plasmid- and genome-derived *coaA* transcription

Table 2. Enzyme activities in the cell extract of *E. coli* harboring pTrc-*hemA*+-*coaA*.

	ALA synthase (*hemA*) (U/$mg_{protein}$)[a]	Pantothenate kinase (*coaA*) (U/$mg_{protein}$)
Strain carrying pTrc99A	0	2.71 ± 0.19
Strain carrying pTrc-*hemA*$^+$	54.0 ± 0.50	2.74 ± 0.21
Strain carrying pTrc-*hemA*$^+$-*coaA*	55.0 ± 0.50	3.77 ± 0.13

[a]Unit/$mg_{protein}$ was defined as the amount of ALA formed (nmol) per minute reaction per mg-protein.
Data are represented as the mean ± SD from at least three independent repeats.

Table 3. Transcription levels of genes in the heme biosynthetic pathway.

	Control 16S rRNA	*coaA* (P/G)	*hemA* (P)[a]	*hemB* (G)[b]	*hemC* (G)	*hemD* (G)	*hemE* (G)	*hemF* (G)	*hemG* (G)	*hemH* (G)
Batch 12 h[c]	1.00	275 ± 29.8	592 ± 46.0	1.46 ± 0.15	1.42 ± 0.20	1.33 ± 0.13	1.48 ± 0.10	1.26 ± 0.08	1.19 ± 0.03	1.32 ± 0.16
D = 0.1 h^{-1}[d]	1.00	3.75 ± 0.19	22.4 ± 0.11	0.46 ± 0.06	0.87 ± 0.09	0.61 ± 0.04	0.63 ± 0.02	0.47 ± 0.13	0.59 ± 0.05	0.55 ± 0.07
D = 0.3 h^{-1}[e]	1.00	47.1 ± 4.48	20.9 ± 0.17	0.55 ± 0.02	0.54 ± 0.02	0.60 ± 0.07	0.53 ± 0.04	0.59 ± 0.08	0.51 ± 0.01	0.59 ± 0.06
D = 0.3 h^{-1}[f]	1.00	60.3 ± 8.52	19.9 ± 0.03	0.45 ± 0.03	0.26 ± 0.01	0.49 ± 0.06	0.43 ± 0.06	0.46 ± 0.04	0.36 ± 0.06	0.47 ± 0.05

RNAs were harvested from *E. coli* W3110 carrying pTrc-*hemA*$^+$-*coaA* from various cultures. Data represent the average ± SD from three analyses.

[a]P: plasmid-derived origin and IPTG-induced.

[b]G: genome-derived and self promoter-induced.

[c]Sample from batch culture at 12 h.

[d]Sample from steady state at D = 0.1 h^{-1}.

[e]Sample from steady state at D = 0.3 h^{-1}.

[f]Sample from steady state at D = 0.5 h^{-1}.

were highly expressed compared with control (16S rRNA) and genome-derived genes. Transcription of gene *coaA* was increased when *E. coli* was cultured in a higher dilution rate during chemostat culture. For genome-derived genes, *hemB* to *hemH*, all of them were down-regulated in chemostat culture but up-regulated in batch culture, though there was no significant variation. Therefore, all genes in the heme biosynthetic pathway were transcribed regardless of the culture type and growth rate.

Intermediate Concentrations in Heme Biosynthetic Pathway

Intracellular ALA and CoA concentrations of the strain carrying pTrc-*hemA*$^+$-*coaA* were compared with that of the strain carrying pTrc-*hemA*$^+$. ALA concentration from the strain expressing both ALA synthase and pantothenate kinase was greater than that from the strain expressing only ALA synthase throughout the batch culture period (Fig. 2A). Because ALA synthase mediates combined glycine and succinyl-CoA, more CoA availability by pantothenate kinase expression would have led to promotion of the ALA concentration. The intracellular CoA concentration profile agreed with the assumption (Fig. 2B). During the growing period (0–12 h), the CoA concentration from the artificially pantothenate kinase expressing strain was greater than that from the control, though the trend was turned upside down during the non-growing period (24–48 h). Therefore, pantothenate kinase expression resulted in the increase of the CoA pool during the growth phase and further increase of the ALA pool.

Three intermediates were detected in the LC-MS analysis; namely, coproporphyrinogen I, coproporphyrinogen III, and protoporphyrin IX (Fig. 3). Intermediate concentrations

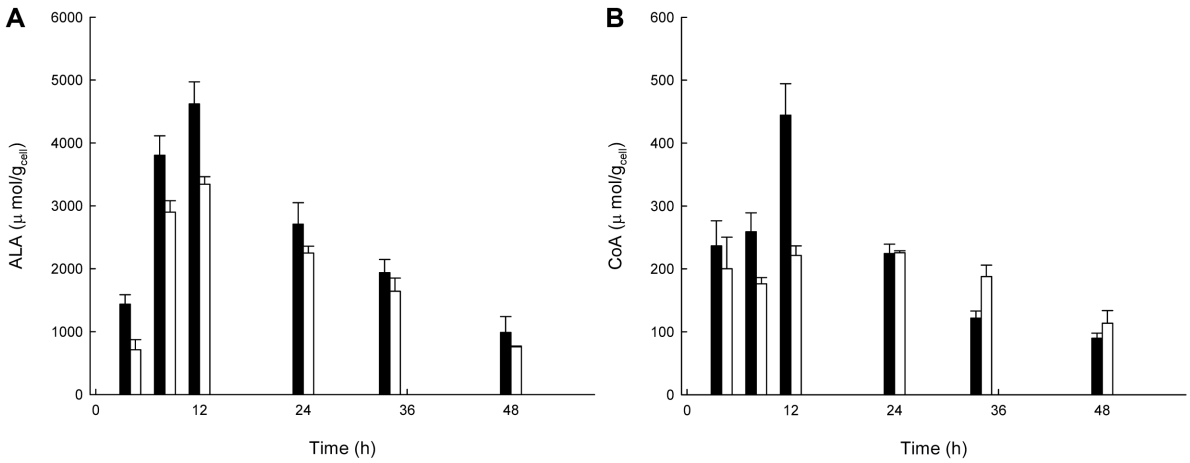

Fig. 2. Variation of intracellular ALA and CoA concentrations in *E. coli* harboring pTrc-*hemA*$^+$-*coaA*.

(**A**) ALA concentration; (**B**) CoA concentration. Black bar: metabolite from *E. coli* harboring pTrc-*hemA*$^+$-*coaA*; White bar: metabolite from *E. coli* harboring pTrc-*hemA*$^+$. Data represent the average ± SD from three biological repeats.

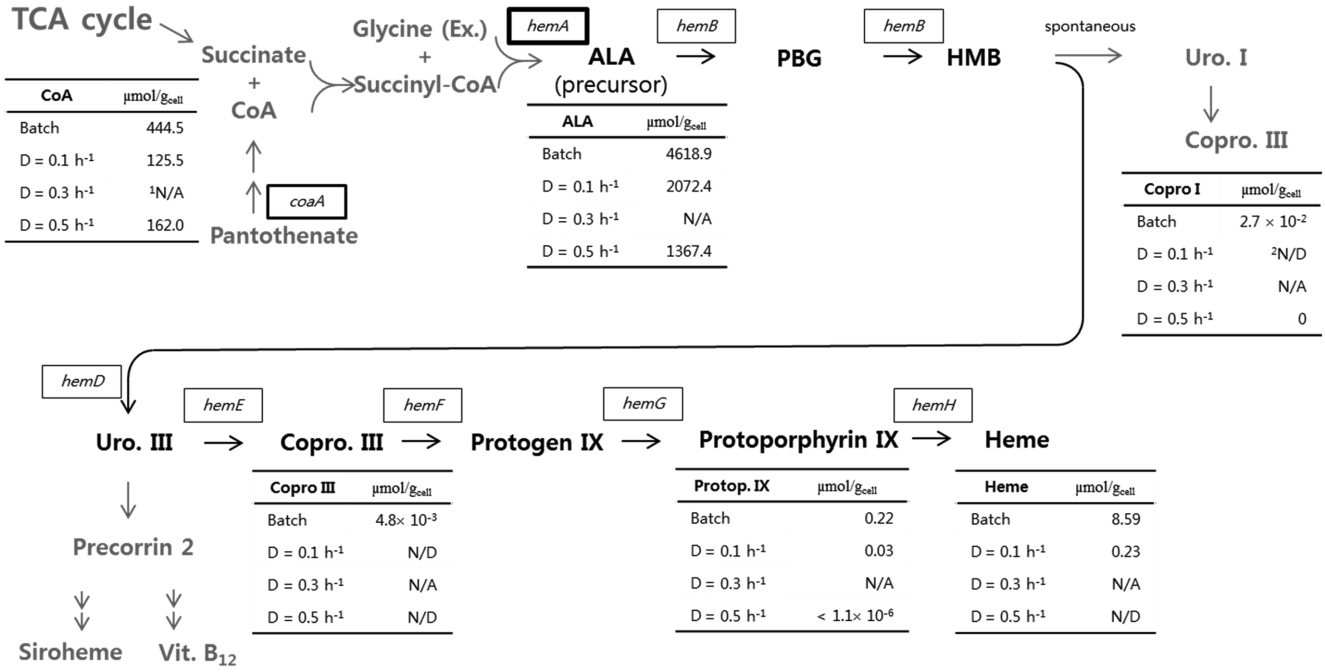

Fig. 3. Intermediates concentrations of the heme biosynthetic pathway.

Tables represent the intracellular concentrations of molecules at each culture condition in μmol/g_{cell} unit. Batch data are from culture time at 12 h, and chemostat data at each dilution rate are from steady-state cells. Intermediates in the heme pathway are in black and the branch pathways in grey. The thickness of the gene names represents mRNA level. Ex (external). [1]Metabolite concentrations at D = 0.3 h^{-1} were not accounted for technical reason. [2]N/D: not detected.

from steady-state cells were drastically lower than those from batch culture sample. Protoporphyrin IX, the last intermediate in the heme biosynthetic pathway, was 0.222 μmol/g_{cell} for batch culture and <0.028 μmol/g_{cell} for continuous cultures. Although the protoporphyrin IX concentration was the highest between other metabolites, it was still far lower than its precursor (ALA) and CoA concentrations, indicating very limited ALA was used for heme biosynthesis.

Discussion

Cells tightly regulate biosyntheses by multilayered controls such as transcriptional, post-transcriptional, translational, and post-translational levels. Even though artificial *hemA* and *coaA* gene expression enabled *E. coli* to accumulate precursor ALA, the production amount of heme, the end-product of the pathway, was limited (Fig. 3). If all ALA at D = 0.1 h^{-1} (2,072.4 $\mu mol_{ALA}/g_{cell}$) had been converted to heme, it could have been corresponding to 259 $\mu mol_{heme}/g_{cell}$; however, only 1/1,000 of heme (0.23 $\mu mol_{heme}/g_{cell}$) was actually found. Therefore, much carbon flow of ALA did not go through the porphyrin pathway, even though all necessary genes were transcribed (Table 3). These results led to a hypothesis that there might be feedback controls on the enzyme by intermediates that restrict excessive heme production. Another hypothesis is that the carbon of ALA might have flown to other pathways from intermediate metabolites, such as siroheme or vitamin B_{12} branch pathways. Another limiting step observed from continuous cultures was the conversion of protoporphyrin IX to the heme step, because there was protoporphyrin IX accumulation. The CoA and ALA concentrations in chemostat culture were lower than those in batch culture (Fig. 3). The transcription level of *coaA* showed that the highest expression level was in the fastest dilution rate in the chemostat (Table 3), though the CoA concentration showed the opposite result (Fig. 3). This result represented that CoA was circulated more rapidly in higher growth rate. Introducing the heterologous *coaA* gene could be a strategy to avoid feedback controls on the self-*coaA* or to acquire higher pantothenate kinase activity, considering the overexpression of self-*coaA* increased only 38% activity whereas the overexpression of foreign-originated *hemA* led to an increase of activity in dozens-fold (Table 2). In addition, the highest heme content was obtained after 48 h

batch culture. Therefore, the heme accumulation rate is low in actively growing cells. Other pathways branched from the heme synthesis pathway are spontaneous conversion of HMB to coproporphyrinogen I and conversion of uroporphyrinogen III to siroheme (Fig. 3). Coproporphyrinogen I was detected in the LC/MS analysis, and this fact represented there might have been some amount of HMB spontaneously converted to uroporphyrinogen I that went to coproporphyrinogen I. Siroheme is a heme-like prosthetic group for sulfite reductases that are required for methionine and cysteine synthesis. It is synthesized from uroporphyrinogen III in four enzymatic steps; two *S*-adenosyl-L-methionine-dependent transmethylations, a dehydrogenation, and a ferrochelation. In *E. coli*, CysG protein performs all these enzymatic reactions [1].

Ferrochelatase (*hemH*) mediates ferrous ion incorporation into protoporphyrin IX, and the ferrous ion shortage condition would have limited the heme production and accumulation of protoporphyrin IX (Fig. 3). To prove this hypothesis, we performed a preliminary test with batch culture of W3110 harboring pTrc-*hemA*$^{+}$-*coaA* in a medium supplemented with ferrous ion. Cells were cultured in the same batch culture medium with and without addition of 2 μM $FeCl_3 \cdot 6H_2O$ and the heme content was analyzed. The preliminary result showed the ferrous ion addition enabled the strain to produce 5.8% more heme (9.1 $\mu mol/g_{cell}$) than the case of control. Therefore, ferrous ions have been shortened, though it does not explain all the reasons why many portions of ALA have not flown to heme.

In conclusion, the overexpression of ALA synthase and pantothenate kinase enabled *E. coli* to produce ALA, the heme precursor, though most of ALA was not used for heme biosynthesis. Since all heme biosynthetic genes were transcribed, we hypothesized that there might be strong feedback control to the enzymes, many portions of ALA might be used for branch pathways, ferrous ion might be limited, or there is a combination of the above reasons. Measuring all enzyme activities and all ALA flux analysis would give detailed information about the bottleneck step in the heme biosynthesis. Further study is under way to increase heme production.

Acknowledgments

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