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Original Article

Investigation of the Roles of Cysteine Desulfurases in the Molybdopterin Synthesis in *Escherichia coli*

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Summary

Molybdenum cofactors contain molybdenum and sulfur atoms as functionally essential components. These cofactors are required for the activities of the molybdoenzymes. It has been shown that CsdA, one of 3 cysteine desulfurases in *Escherichia coli*, can efficiently transfer sulfur from L-cysteine to the C-terminal thiocarboxylate of the smaller subunit of molybdopterin synthase in a defined *in vitro* system to generate the dithiolene group of molybdopterin from precursor Z. Precursor Z is the immediate precursor of molybdopterin in molybdenum cofactor biosynthesis. In this study, we found that a *csdA*-deletion strain of *E. coli* does not accumulate compound Z, a direct oxidation product of precursor Z, unlike a *ΔmoaD* strain. Although a two-dimensional PAGE analysis suggests that *csdA* is involved in L-cysteine metabolism, an assay of a molybdenum-dependent enzyme, namely, sulfite oxidase, revealed that the enzyme activity was not markedly different among the *ΔcsdA*, *ΔsufS*, and wild-type strains. In contrast, the activity of sulfite oxidase was greatly decreased in the *ΔiscS* strain. These results suggest that CsdA is not essential for the biosynthesis of molybdenum cofactor and IscS is a possible candidate for the physiological sulfur-donating enzyme in molybdopterin biosynthesis.

Introduction

Cysteine desulfurase is a pyridoxal 5'-phosphate-dependent enzyme that catalyzes the conversion of L-cysteine to L-alanine via the formation of a persulfide intermediate on a conserved cysteine residue[1]. The terminal sulfur (formally S^0) is transferred to the cysteine residues in acceptor proteins to form new persulfide groups. Subsequently these persulfide groups either directly participate in the biosynthesis of cofactors or thionucleosides, or are transferred to other protein acceptors for the eventual incorporation of sulfur into an end product[1−3]. *Escherichia coli* contains the following 3 cysteine desulfurases : CsdA (also termed as CSD), SufS (also termed as CsdB), and IscS[1,4]. These are classified into 2 groups−group I and group II−on the basis of differences in 4 sequence regions and the consensus sequence motif[5]. Unlike IscS, which has the consensus group I sequence SSGSACTS around the active site Cys-328, CsdA and SufS possess consensus group II sequences. CsdA is encoded by the *csdAE* operon, which is presumed to function in the maturation of an FeS protein[6]. The activity of CsdA is enhanced by CsdE via sulfur transfer from the persulfide group of the former to the cysteine residue of the latter[6]. The *sufABCDSE* operon contains the gene encoding SufS, which is specifically adapted to catalyze the synthesis of FeS clusters under conditions of iron starvation or oxidative stress[7,8]. The catalytic activity of SufS is strikingly lower than that of IscS and CsdA[9] but is enhanced by SufE through sulfur transfer from the persulfide group ; its catalytic activity is further enhanced by the SufBCD complex[10,11].

Molybdenum cofactor is a trace metal element-containing cofactor required for the activity of various molybdoenzymes, such as nitrate reductase, sulfite oxidase, and xanthine dehydrogenase[12,13]. A molybdenum cofactor consists of a mononuclear molybdenum covalently bound to the dithiolene moiety of a tricyclic pterin termed as molybdopterin (MPT). In *E. coli,* MPT is generated from precursor Z, which is the first intermediate formed from GTP in the biosynthesis of molybdopterin[14]. Under experimental conditions, precursor Z is rapidly oxidized to a 6-alkyl pterin, which is termed as compound Z, by air or iodine[15]. The dithiolene moiety is inserted into precursor Z by MPT synthase, a tetrameric protein comprising 2 small MoaD subunits and 2 large MoaE subunits[16]. Each of the MPT sulfurs is derived from one of the 2 MoaD subunits that carry a C-terminal thiocarboxylate[17]. MoaD with the C-terminal Gly-Gly motif is first adenylated by MoeB via for-

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mation of an MoaD/MoeB complex[18]. The next step in the C-terminal thiocarboxylation of MoaD has not been completely established. In a previous study, Leimkühler and Rajagopalan suggested that L-cysteine can be used as a sulfur source and CsdA, a cysteine desulfurase in *E. coli,* serves as an efficient sulfur-donating enzyme for *in vitro* MPT synthesis[19]. However, the role of CsdA in the *in vivo* MPT synthesis remains unclear. In the present study, we investigated the role of CsdA in MPT biosynthesis using an *E. coli* mutant strain deficient in *csdA*.

Materials and Methods

1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *E. coli* cells were grown aerobically at 37℃ in Luria-Bertani (LB) medium, unless otherwise noted.

Table 1 *E. coil* struins used in this work

Strains	Genotype	Source
BW25113	*lacI*^{q}, *rrnBT14*, *ΔlacZWJ16*, *hsdR514*, *ΔaraBADAH33*, *ΔrhaBADLD78*	NBRP[1] collection
ΔcsdA	BW25113 *ΔcsdA* (JW2781)	NBRP[1] collection
ΔsufS	BW25113 *ΔsufS* (JW1670)	NBRP[1] collection
ΔiscS	BW25113 *ΔiscS* (JW2514)	NBRP[1] collection
ΔmoaD	BW25113 *ΔmoaD* (JW0767)	NBRP[1] collection

[1] National BioResource Project (NIG, Japan) : *E. coli*

2. Preparation of compound Z

(Method A) Compound Z was prepared according to the method of Johnson *et al.*[15] with a few modifications. Briefly, the pellet from 50 mL of aerobically grown *ΔmoaD* cells was resuspended in 1 mL H_2O. The cells were then oxidized by adding 34 μL of 4 N hydrochloric acid (HCl), 100 μL of 1 % iodine (I_2), and 2 % potassium iodide (KI) in H_2O. The preparation was incubated at room temperature in the dark for 30 min and then centrifuged ; the pH of the supernatant was adjusted to 7 with 1.0 M sodium hydroxide (NaOH) and lyophilized. The dried sample was suspended in 100 μL H_2O, centrifuged, and a portion of the supernatant (50 μL) was then analyzed by using high-performance liquid chromatography (HPLC).

(Method B) *E. coli* cells were cultured in LB medium for 18 h at 37℃ with vigorous shaking and harvested by centrifugation at 3,000 × *g* for 10 min. The cell pellets were washed twice with 30 mL water (H_2O), suspended in 3 mL of H_2O/g wet weight, and frozen at −30℃. Acid extracts of the cells were essentially prepared by following the procedure proposed by Wuebbens and Rajagopalan[20]. The extracts were concentrated up to 900 μL by rotary evaporation and centrifuged at 14,000 × *g* for 3 min to remove protein. A portion of the supernatant (12.5−25 μL) was analyzed by using HPLC after air-oxidation that enabled conversion of precursor Z to compound Z.

3. HPLC analysis

All HPLC analyses were performed at 25℃ using a Capcell Pak C_{18} SG120 column (4.6 × 250 mm ; Shiseido, Tokyo, Japan) and a Shimadzu HPLC system (Shimadzu, Kyoto, Japan). The column was equilibrated with 5 mM ammonium acetate (pH 5.0) with a flow rate of 1.0 mL/min. Eluents were monitored for absorption at 300 nm. Under these conditions, compound Z eluted as a major peak at 5.75 min.

4. Two-dimensional PAGE

Two-dimensional PAGE (2-DE) was performed according to the method described by Mihara *et al.,*[21] with the following changes. Crude extracts from wild-type and *ΔcsdA E. coli* cells were loaded onto 7-cm immobilized pH gradient strips (pH 4−7: Bio-Rad, Richmond, CA). SDS-PAGE on a 10 % separation gel was performed as the second-dimensional electrophoresis. After the second-dimensional electrophoresis, the gels were fixed and stained with Coomassie Brilliant Blue.

5. Enzyme assay

Sulfite oxidase activity was assayed as described previously[19,22]. *E. coli* cells were cultured in LB medium for 10 h at 37℃ with vigorous shaking. Cells were disrupted by sonication, and the crude extracts were used for enzyme assays.

Results

1. Compound Z accumulation in the *ΔmoaD* strain

A previous study has shown that precursor Z (or compound Z) is accumulated in a *moaD*-deficient strain due to the inability of this strain to convert precursor Z to MPT.[15] We first investigated the accumulation of compound Z in another *ΔmoaD* mutant, which has a genetic background different from that of the reported *moaD*-deficient strain.[15] The time courses of compound Z accumulation at 30℃ and 37℃ were examined with the *ΔmoaD* strain cultivated in LB medium. The amount of compound Z did not greatly change during the 10−18 h cultivation times (Fig. 1). The cultivation at 30℃ resulted in higher yields of compound Z than that at 37℃.

2. Investigation of a possible role of *CsdA* in MPT biosynthesis

Since the compound Z accumulation in our *ΔmoaD*

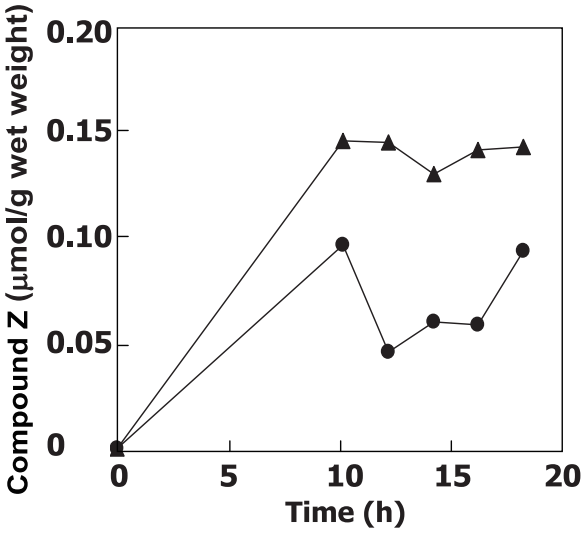

Fig. 1 Compound Z accumulation in the *ΔmoaD* strain. Compound Z was isolated from the *ΔmoaD* cells cultivated at 30℃ (▲) and 37℃ (●) at the indicated times by Method A described in Materials and Methods. The amount of compound Z was determined based on its molar extinction coefficient ($\varepsilon = 1.36 \times 10^4\ M^{-1}\cdot cm^{-1}$) at 370 nm at pH 12.

strain was confirmed, we next analyzed the acid extract of a *ΔcsdA* strain by HPLC to examine whether compound Z is also accumulated in this strain. HPLC analysis of the acid extracts of *E. coli* cells revealed that compound Z eluting at 5.75 min was accumulated in the *ΔmoaD* strain (Fig. 2); In contrast, such accumulation was not observed in the *ΔcsdA* and wild-type strains. This result suggests that CsdA is not essential as a sulfur-donating enzyme for MoaD in MPT biosynthesis.

To better understand the role of *csdA in vivo,* we examined whether the amount of any protein is affected by the *csdA* mutation and if CsdA is involved in the MPT biosynthesis pathway at a step other than sulfur-donation for MoaD. We performed 2-DE analysis of the crude extracts

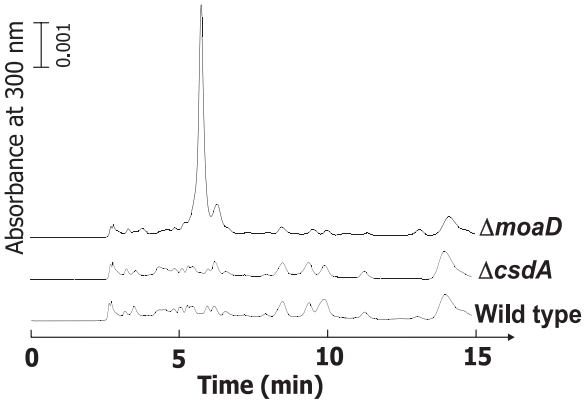

Fig. 2 Analysis of compound Z in the acid extracts of *E. coli* cells. The acid extracts of the wild-type, *ΔcsdA*, and *ΔmoaD* strains prepared by Method B (Materials and Methods) were analyzed by HPLC with a Capcell Pak C_{18} SG120 column after air oxidation. Volumes of the analyzed samples were 12.5 μL for the *ΔmoaD* strain and 25 μL for other strains.

of a *csdA*-deficient strain and its parent strain. We found that 1 spot (Fig. 3B, arrowhead) was markedly induced in the *csdA* mutant, with good reproducibility. No other clearly visible spot that depended on *csdA* was obtained. The N-terminal amino acid sequence of the protein derived from the spot was MENFKHLPEP. BLAST[23] search revealed that the sequence was identical to that of tryptophanase, which is encoded by the *tnaA* gene[24]. In *E. coli,* tryptophanase is involved in L-cysteine metabolism. It catalyzes the hydrolysis of L-cysteine to generate pyruvate, ammonia, and hydrogen sulfide[25]. A recent study also indicated that the tryptophanase expression was induced by L-cysteine[26]. The deficiency of *csdA* probably increased the concentration of L-cysteine in the cells. Thus, the increased L-cysteine levels in the *ΔcsdA* strain probably resulted in the induction of tryptophanase expression. The results suggest that *csdA* is involved in L-cysteine metabolism, although a specific role of the enzyme in MPT biosynthesis could not be demonstrated.

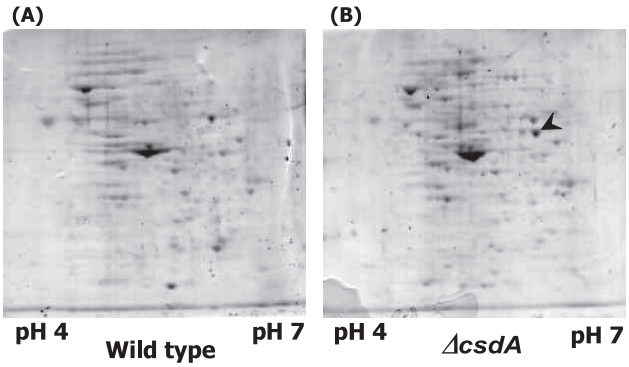

Fig. 3 2-DE of crude extracts of the wild-type (A) and *ΔcsdA* (B) strain. The gels were stained with Coomassie Brilliant Blue. The protein induced in the *csdA*$^-$ strain (indicated by arrowhead) was subjected to N-terminal sequence analysis and identified to be tryptophanase.

3. Activity of the molybdenum enzyme sulfite oxidase in various cysteine desulfurase-deficient mutants

The results described above show no evidence of the involvement of CsdA in MPT biosynthesis. Therefore, we investigated whether the other 2 cysteine desulfurases (IscS and SufS) are involved in MPT biosynthesis. The activity of sulfite oxidase, a molybdenum cofactor-containing enzyme, was assayed using crude extracts of the cysteine desulfurase-deficient mutants (*ΔcsdA*, *ΔsufS*, and *ΔiscS*) and the *ΔmoaD* strain. No apparent difference in the activity of sulfite oxidase was observed for the wild-type, *ΔcsdA*, and *ΔsufS* strains. In contrast, the sulfite oxidase activity in the *ΔiscS* strain was markedly decreased (Fig. 4). The level of the sulfite oxidase activity in the *ΔiscS* strain was almost the same as that in the *ΔmoaD* strain.

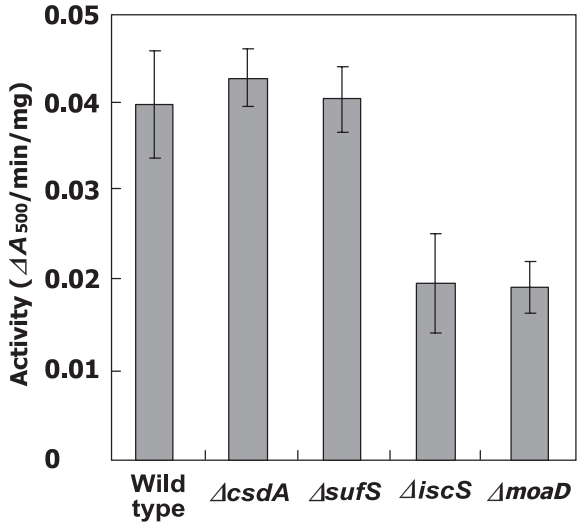

Fig. 4 Sulfite oxidase activity in the wild-type, *ΔcsdA*, *ΔsufS*, *ΔiscS*, and *ΔmoaD* strains. Sulfite oxidase activity was measured using crude extracts of the strains, and 1 unit of sulfite oxidase activity is expressed as an absorbance change (ΔA_{550}) of 1 per minute per milligram of protein. An MPT-independent background activity of 0.019 was observed in the *ΔmoaD* strain.

Discussion

In a previous study, Leimkühler and Rajagopalan suggested that L-cysteine can serve as a sulfur source and CsdA, a cysteine desulfurase in *E. coli,* serves as an efficient sulfur-donating enzyme in *in vitro* MPT synthesis[19]. However, our data indicate that CsdA is not directly involved in *in vivo* MPT synthesis as a sulfur-donating enzyme for MoaD thiocarboxylation. Our results indicate that IscS is likely to be involved in MPT biosynthesis.

IscS is the central sulfur-donating enzyme for the formation of FeS clusters[27,28], thiamin [29], and the thionucleosides 4-thiouridine and 5-methylaminomethyl-2-thiouridine in tRNAs[29–32] via a specific interaction with different proteins. The interaction of IscS with IscU enables the assembly of FeS clusters[33,34], while IscS interacts with ThiI to form ThiS-COSH, which subsequently transfers the sulfur to thiamin[35,36]. The formation of persulfide in ThiI by IscS is a critical event in the biosynthesis of 4-thiouridine in tRNA[36]. Unlike the synthesis of 4-thiouridine, the generation of 2-thiouridine requires 5 polypeptides as sulfur carriers between IscS and tRNA bound to MnmA[37]. Thus, the elucidation of donor-acceptor pairs and specific structural elements that specify the protein-protein interactions remains a fascinating aspect of sulfur transfer systems.

In the *in vitro* system, CsdA shows the highest activity as a sulfurtransferase in MPT formation, while SufS exhibits low efficiency[19]. However, our data suggests that these enzyme activities do not compensate for the loss of IscS (Fig. 4). Therefore, there must be a mechanism to facilitate the IscS-specific sulfur transfer to MoaD. Similarly, IscS and not CsdA or SufS is essential for the biosynthesis of 5-methylaminomethyl-2-thiouridine[31]. Recently, Ikeuchi *et al.* showed that 5 sulfur carrier proteins are involved in the transfer of sulfur from IscS to MnmA in the biosynthesis of 5-methylaminomethyl-2-thiouridine[37], although IscS and MnmA were sufficient for the *in vitro* generation of 2-thiouridine in the anticodon of tRNALys [38]. The inclusion of additional sulfur-trafficking proteins probably enables the cell to control the flow of sulfur along various pathways by regulating the level of sulfur transfer proteins[2].

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