

IscS Functions as a Primary Sulfur-donating Enzyme by Interacting Specifically with MoeB and MoadD in the Biosynthesis of Molybdopterin in *Escherichia coli**[§]

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The persulfide sulfur formed on an active site cysteine residue of pyridoxal 5'-phosphate-dependent cysteine desulfurases is subsequently incorporated into the biosynthetic pathways of a variety of sulfur-containing cofactors and thionucleosides. In molybdenum cofactor biosynthesis, MoeB activates the C terminus of the MoadD subunit of molybdopterin (MPT) synthase to form MoadD-adenylate, which is subsequently converted to a thiocarboxylate for the generation of the dithiolene group of MPT. It has been shown that three cysteine desulfurases (CsdA, SufS, and IscS) of *Escherichia coli* can transfer sulfur from L-cysteine to the thiocarboxylate of MoadD *in vitro*. Here, we demonstrate by surface plasmon resonance analyses that IscS, but not CsdA or SufS, interacts with MoeB and MoadD. MoeB and MoadD can stimulate the IscS activity up to 1.6-fold. Analysis of the sulfuration level of MoadD isolated from strains defective in cysteine desulfurases shows a largely decreased sulfuration level of the protein in an *iscS* deletion strain but not in a *csdA/sufS* deletion strain. We also show that another *iscS* deletion strain of *E. coli* accumulates compound Z, a direct oxidation product of the immediate precursor of MPT, to the same extent as an MPT synthase-deficient strain. In contrast, analysis of the content of compound Z in Δ *csdA* and Δ *sufS* strains revealed no such accumulation. These findings indicate that IscS is the primary physiological sulfur-donating enzyme for the generation of the thiocarboxylate of MPT synthase in MPT biosynthesis.

The presence of sulfur in cofactors and RNA thionucleoside confers significant functionality to these biomolecules because of its unique chemical properties. The sulfur atoms of thiamin,

lipoic acid, biotin, iron-sulfur (FeS)² clusters, and molybdenum cofactors participate in stabilizing reaction intermediates (1, 2), alternating between the reduced dithiol and oxidized disulfide, facilitating electron transfer, or modulating the redox potential of the cofactor (3). The thionucleosides in tRNAs fine-tune the efficiency or accuracy of translation (4, 5), serve as recognition sites for aminoacyl-tRNA synthetase (6), or act as photosensors (7). Despite the diverse chemical environments in which the sulfur atoms reside in cofactors and thionucleosides, it has been found that the incorporation of sulfur into them involves a few enzymes with the persulfide (R-S-SH) groups on the cysteine residues of these enzymes; thus, these enzymes act as the key agents of sulfur transfer (8, 9).

Protein persulfide groups are generated by a family of cysteine desulfurases; these use the cofactor pyridoxal 5'-phosphate to form a persulfide group on an active site cysteine residue by using the free amino acid L-cysteine and generate L-alanine as the second product (10). The terminal sulfur (formally S⁰) is transferred to the cysteine residues in acceptor proteins to form new persulfide groups that are subsequently used directly in the biosynthesis of cofactors or thionucleosides or transferred to other protein acceptors for the eventual incorporation of sulfur into an end product (8–10).

Escherichia coli contains the following three cysteine desulfurases: CsdA (also termed CSD), SufS (also termed CsdB), and IscS (10, 11). These are classified into 2 groups, I and II, based on differences in four sequence regions and the consensus sequence motif (12). 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 (13). 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 (13). The *sufABCDSE* operon containing the gene encoding SufS is specifically adapted to synthesize FeS clusters under conditions of iron starvation or oxidative stress (14, 15). The catalytic activity of SufS is significantly lower than that of CsdA and IscS (16), but it is enhanced by SufE through persulfide sulfur transfer; its catalytic activity is further enhanced by the SufBCD complex

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² The abbreviations used are: FeS, iron-sulfur; MPT, molybdopterin; Moco, molybdenum cofactors; PLP, pyridoxal 5'-phosphate; HPLC, high performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry.

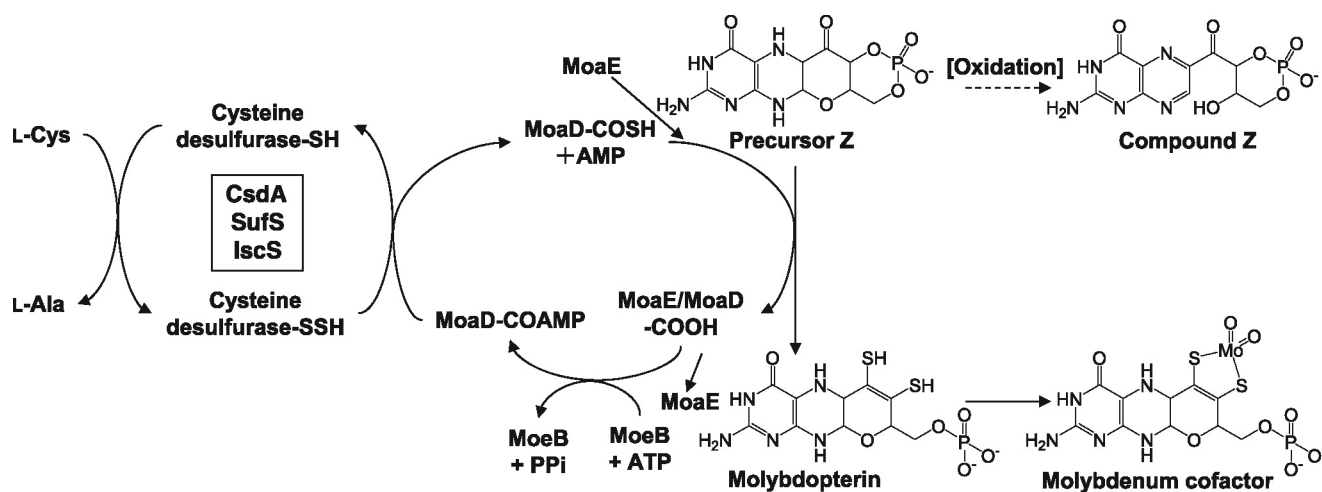


FIGURE 1. **Proposed pathway for the biosynthesis of molybdenum cofactor in *E. coli*.** The structures of precursor Z, MPT, molybdenum cofactor, and the oxidation product of precursor Z, i.e. compound Z, are shown. The incorporation of sulfur atoms into precursor Z is catalyzed by MPT synthase (MoaE/MoaD). Three cysteine desulfurases (CsdA, SufS, and IscS) transfer the sulfur atom from L-cysteine to the C-terminal thiocarboxylate of MoaD *in vitro* (36, 41).

(17, 18). IscS, which is the central sulfur-providing enzyme, is involved in the formation of the FeS cluster (19–22), thiamin (23–25), and the thionucleosides 4-thiouridine and 5-methylaminomethyl-2-thiouridine in tRNAs (23, 25–29) via a specific interaction with different proteins. The interaction of IscS with IscU enables the assembly of FeS clusters (21, 22), whereas IscS interacts with ThiI to form ThiS-COSH, which subsequently transfers the sulfur to thiamin (24, 25). The formation of persulfide in ThiI by IscS is a critical event in the biosynthesis of 4-thiouridine in tRNA (25). Unlike the synthesis of 4-thiouridine, the generation of 2-thiouridine requires 5 polypeptides as sulfur carriers between IscS and tRNA-binding MnmA (29). 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.

Molybdenum cofactors (Moco) also contain a sulfur atom as a functionally essential component. These cofactors are required for the activity of a variety of molybdoenzymes, including nitrate reductase, sulfite oxidase, and xanthine dehydrogenase (30, 31). A molybdenum cofactor consists of a mononuclear molybdenum covalently bound to the dithiolene moiety of a tricyclic pterin referred to as molybdopterin (MPT) (Fig. 1). In *E. coli*, MPT is generated from precursor Z, which is the first intermediate formed from GTP (32). Under experimental conditions, precursor Z is rapidly oxidized to 6-alkyl pterin, which is termed compound Z, by air or iodine (33). The dithiolene moiety is inserted into precursor Z by MPT synthase, a tetrameric protein comprising 2 small MoaD subunits and 2 large MoaE subunits (34). Each of the MPT sulfurs is derived from one of the 2 MoaD subunits that carry a C-terminal thiocarboxylate (35). MoaD with the C-terminal Gly-Gly motif is adenylated by MoeB via formation of the MoaD/MoeB complex (36). The next step in the C-terminal thiocarboxylation of MoaD has not been completely established. In a previous study, it is suggested that L-cysteine can be used as a sulfur source, and cysteine desulfurases in *E. coli* serve as a sulfur-donating enzyme for the synthesis of MPT in the *in vitro* system (36). However, the contributions of three cysteine desulfurases to MPT biosynthesis *in vivo* are unknown. Recent findings that

IscS plays a role in sulfur transfer via protein-protein interaction with a certain partner (8, 9) led us to reconsider cysteine desulfurases involved in *in vivo* MPT synthesis.

In the present study, we have investigated the functional and physical interactions of three cysteine desulfurases with MoeB and MoaD. Each of three cysteine desulfurases could catalyze the transfer of ^{35}S from [^{35}S]cysteine to MoaD in the presence of MoeB and Mg-ATP. However, only IscS was activated by MoeB. MoaD further enhanced the IscS activity in the presence of MoeB. Surface plasmon resonance studies further revealed that IscS specifically binds to MoeB and MoaD. In contrast, neither CsdA nor SufS interacted with MoeB or MoaD. We also show that the sulfuration level of MoaD isolated from strains defective in IscS exhibits a large decrease in contrast to that of MoaD expressed in a *csdA/sufS* deletion strain. In accord with this result, a genetic study exhibits that an ΔiscS strain, but not the ΔcsdA and ΔsufS strains, accumulates compound Z. Based on the results presented here, we propose that IscS is the primary physiological sulfur-donating enzyme in the conversion of precursor Z to MPT.

EXPERIMENTAL PROCEDURES

Bacteria, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* cells were grown aerobically at 37 °C in a Luria-Bertani (LB) medium unless otherwise noted.

Construction of Plasmids—A DNA fragment containing the *iscS* gene and a Shine-Dalgarno sequence was cleaved from the XbaI and HindIII sites of pEF1 (11) and inserted into the XbaI and HindIII sites of pUC118 to yield pIscS. The DNA fragment containing *moaD* was PCR-amplified from the chromosomal DNA of *E. coli* K-12 MG1655 with specific primers (5'-GGGA-ATTCCATATGATTAAGTTCTTTTTTCGC-3' and 5'-CGCGGATCCTTAACCTCCGGTTACCGGCG-5') and cloned into the NdeI and BamHI sites of pET14b to give a plasmid, pMOAD. For the cloning of *moeB*, a PCR product obtained with primers 5'-GGGAATTCCATATGGCGGAACCTCAGCG-ATCAGG-3' and 5'-CGCGGATCCTTACTGCCACACACCTC-3' was ligated into pET14b to yield a plasmid, pMOEB.

IscS Is Essential for Molybdopterin Biosynthesis

TABLE 1

E. coli strains and plasmids used in this study

Strains	Genotype	Source
BW25113	<i>lac^q, rrmBT14, ΔlacZWJ16, hsdR514, ΔaraBADAH33, ΔrhaBADLD78</i>	NBRP ^a collection
<i>ΔcsdA</i>	BW25113 <i>ΔcsdA</i>	NBRP ^a collection
<i>ΔsufS</i>	BW25113 <i>ΔsufS</i>	NBRP ^a collection
<i>ΔiscS</i>	BW25113 <i>ΔiscS</i>	NBRP ^a collection
<i>ΔmoaD</i>	BW25113 <i>ΔmoaD</i>	NBRP ^a collection
MC1061	<i>F'araD139 Δ(ara leu)7696 Δ(lacY74) galU galK hsdR hsdM+ strA</i>	(23)
CL100	MC1061 <i>ΔiscS</i>	(23)
CL102	MC1061 <i>ΔcsdA/ΔsufS</i>	(26)
Plasmids	Property	Source
pUC118	<i>lac</i> promoter-based expression vector	Takara Shuzo
pET14b	T7 RNA polymerase-based expression vector	Novagen
pCSD1	<i>csdA</i> gene cloned into EcoRI and PstI sites of pUC118	(12)
pCSDB	<i>sufS</i> gene cloned into EcoRI and HindIII sites of pUC118	(16)
pEF1	<i>iscS</i> gene cloned into NdeI and HindIII sites of pET21a	(11)
pIscS	An XbaI-HindIII <i>iscS</i> fragment of pEF1 was ligated into pUC118	This work
pMoaD	<i>moaD</i> gene cloned into NdeI and BamHI sites of pET14b	This work
pMoeB	<i>moeB</i> gene cloned into NdeI and BamHI sites of pET14b	This work
pMW15aD	<i>moaD</i> gene cloned into NcoI and BamHI sites of pET15b	(35)

^a National BioResource Project (NIG, Japan):*E. coli*.

Expression and Purification of Proteins—pCSD1 (12) and pCSDB (16) were introduced into *E. coli* JM109 to give CsdA- and SufS-overexpressing strains, respectively. *E. coli* BL21(DE3)/pLysS carrying pEF1 and *E. coli* BL21(DE3) carrying pMOAD and pMOEB were used for the expression of IscS, MoaD, and MoeB, respectively. Cells were cultured in LB with 100 mg/liter of ampicillin at 26 °C for 5 h. After induction by 1 mM isopropyl-β-D-thiogalactopyranoside for 6 h, the cells were harvested by centrifugation, disrupted by sonication, and purified by column chromatography as detailed in [supplemental Experimental Procedures](#). For the quantification of thiocarboxylated MoaD in different *E. coli* strains, MoaD was expressed from pMW15aD (35) in the DE3 lysogens of *E. coli* strains MC1061 (23), CL100 (*iscS* deletion mutant) (23), and CL102 (*csdA/sufS* deletion double mutant) (26). Strains were grown in an LB medium supplemented with 100 μM isopropyl-1-thio-β-D-galactopyranoside and 150 mg/liter ampicillin at 30 °C to an $A_{600} = 0.8$ and harvested by centrifugation at 5,000 × g. MoaD was purified as described previously (34).

³⁵S Transfer Assay—All the experiments were performed as described by Loiseau *et al.* (13) with modifications. Under anaerobic conditions, purified proteins were pretreated with 10 mM dithiothreitol and then repurified by gel filtration before use. 20 μM cysteine desulfurase was incubated with 10 μM PLP and 200 μM 2 mCi/mmol L-[³⁵S]cysteine (1,075 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, diluted by nonradioactive L-cysteine) at 37 °C for 30 min. The excess of cysteine was removed by desalting the protein over a Micro Bio-spin 6 column to give a persulfide form of cysteine desulfurase. A standard reaction mixture contains 10 μM persulfide-form cysteine desulfurase, 10 μM MoeB, 10 μM MoaD, 0.575 mM ATP, and 5.75 mM MgCl₂ in 20 mM Tris-HCl, pH 7.5 in a final volume of 12 μl. After incubation at 37 °C for 30 min, the solution was mixed with an SDS-PAGE loading buffer without a reductant. Samples were immediately analyzed by gel electrophoresis using a 15% polyacrylamide gel containing 0.1% SDS but in the absence of an added reducing agent. ³⁵S was visualized by exposure on a phosphor screen and analyzed using Typhoon 9210 (GE Healthcare).

Enzyme Assays—The cysteine desulfurase activities of CsdA, SufS, and IscS were quantitated by assaying the amount of sul-

fide formed from cysteine as described previously (22). A reaction mixture containing 1 mM L-cysteine, 10 μM PLP, 10 mM MgCl₂, 1 mM ATP, 5 mM dithiothreitol, and the enzyme in a 50 mM Tris-HCl buffer, pH 8.0, was incubated at 30 °C.

Surface Plasmon Resonance Analysis—Surface plasmon resonance analysis was carried out with BIACORE X (Biacore AB, Uppsala, Sweden). MoaD or MoeB in a 10 mM acetate buffer (pH 6.0) was immobilized on a sensor chip NTA (Biacore AB). The chip was blocked with a purified N-terminal His₆-tagged protein 2,4-diaminopentanoate dehydrogenase (37) from *Thermoanaerobacter ethanolicus*. The flow rate was 20 μl/min, and the sensor chip was regenerated with 350 mM EDTA and 500 mM NiCl₂.

Analysis of the Sulfuration Level of MoaD from Different *E. coli* Strains—75 μM purified MoaD from different *E. coli* strains, MC1061, CL100, and CL102, 75 μM MoaE (34), and precursor Z (38) in excess amounts were incubated for 30 min in 400 μl of 100 mM Tris, pH 7.2. The MPT produced was oxidized to Form A and quantified following published procedures (36, 39, 40).

Preparation of Acid Extracts of *E. coli* Cells—*E. coli* cells were cultured in an LB medium for 18 h at 37 °C with vigorous shaking and harvested by centrifugation. The cell pellets were washed twice with 30 ml of water (H₂O), suspended in 3 ml of H₂O/g wet weight, and frozen at -30 °C. Acid extracts of the cells were prepared by essentially following the procedure proposed by Wuebbens and Rajagopalan (38). The extracts were concentrated to 900 μl by rotary evaporation and centrifuged to remove the precipitated protein. A portion of the supernatant (12.5–25 μl) was analyzed using high-performance liquid chromatography (HPLC) following air oxidation, which enabled the conversion of precursor Z to compound Z.

HPLC Analysis—All HPLC analyses were performed at 25 °C using a Capcell Pak C₁₈ SG120 column (4.6 × 250 mm, Shiseido, Japan) and a Shimadzu HPLC system (Shimadzu, 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.

RESULTS AND DISCUSSION

³⁵S Transfer from L-Cysteine to the MoaD Thiocarboxylate Catalyzed by Cysteine Desulfurases—We examined the relative abilities of CsdA, SufS, and IscS to transfer sulfur from L-cys-

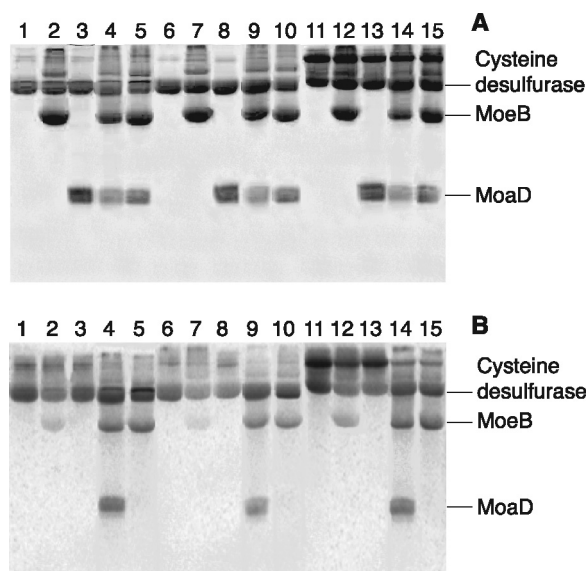


FIGURE 2. Analysis of the sulfur transfer from cysteine desulfurases to MoaD by ³⁵S-labeled L-cysteine. Lane 1, CsdA; lane 2, CsdA and MoeB; lane 3, CsdA and MoaD; lane 4, CsdA, MoeB, and MoaD; lane 5, CsdA, MoeB, and MoaD (without ATP); lane 6, SufS; lane 7, SufS and MoeB; lane 8, SufS and MoaD; lane 9, SufS, MoeB, and MoaD; lane 10, SufS, MoeB, and MoaD (without ATP); lane 11, IscS; lane 12, IscS and MoeB; lane 13, IscS and MoaD; lane 14, IscS, MoeB, and MoaD; lane 15, IscS, MoeB, and MoaD (without ATP). The gel was stained with Coomassie Brilliant Blue (A), and the ³⁵S radioactivity was visualized on a phosphorimaging plate (B).

teine to the C-terminal group of MoaD. To visualize sulfur flow in the reaction, persulfide forms of the cysteine desulfurases were incubated with MoeB and MoaD under anaerobic conditions and subjected to SDS-PAGE analysis without a reducing agent (Fig. 2). The tracer assay of ³⁵S radioactivity visualized by the phosphorimager analysis shows that sulfur was transferred from each of three cysteine desulfurases to MoaD in the presence of MoeB and Mg-ATP (Fig. 2, lanes 4, 9, and 14). No such transfer to MoaD was seen in the absence of either MoeB or Mg-ATP (Fig. 2, lanes 3, 5, 8, 10, 13, and 15). These results showed that all three of the cysteine desulfurases, CsdA, SufS, and IscS, could similarly transfer the persulfide sulfur to the C-terminal group of MoaD in the defined *in vitro* reaction and are consistent with the previous observation (36). MoeB was slightly labeled by ³⁵S in the presence of either of the cysteine desulfurases (Fig. 2, lanes 2, 7, and 12). Multiple sequence alignment analysis of MoeBs from various organisms showed that there were two highly conserved cysteine residues (Cys-142 and Cys-187) (supplemental Fig. S2). We performed the sulfur transfer experiment to MoaD using a C142A/C187A double mutant of MoeB. The result showed that the mutation had no effect on the sulfur transfer to MoaD, and even the C142A/C187A mutant MoeB was also labeled by ³⁵S (supplemental Fig. S3). Thus, the ³⁵S-labeling of MoeB is most probably caused by nonspecific transfer of persulfide of cysteine desulfurases to one or more of the cysteine residues exposed on the surface of MoeB. This is in accordance with previous study suggesting that MoeB itself is not a carrier of sulfur for the sulfur transfer reaction to active MPT synthase (41).

Activation of IscS by MoeB and MoaD—Previous studies have shown that the activity of cysteine desulfurases is enhanced by their physiological interacting partners, such as CsdE for CsdA

(13), SufE-SufBCD for SufS (17, 18), and IscU (42), ThiI (43), and TusA (29) for IscS. The mechanism of the activation is not completely understood, but it is proposed that the binding by partner proteins probably induces a conformational change of the flexible loop carrying the persulfide-forming cysteine residue in cysteine desulfurase to facilitate persulfide formation and/or its subsequent cleavage (44, 45). Therefore, it seemed possible that MoeB and/or MoaD could alter the enzyme activity of a physiologically relevant cysteine desulfurase. To determine whether MoeB and MoaD affect the activity of three cysteine desulfurases, we measured the activity of CsdA, SufS, and IscS in the presence of MoeB and MoaD. The specific activities of purified CsdA, SufS, and IscS were 0.2 units/mg, 0.01 units/mg, and 0.24 units/mg, respectively. The addition of MoeB increased the desulfurase activity of IscS (1.6-fold in the presence of 10-fold molar excess of MoeB) but had no significant effect on the activity of CsdA and SufS (Fig. 3). The addition of MoaD to CsdA, SufS, and IscS had little effect on the desulfurase activity. However, the addition of MoaD to a mixture of IscS and MoeB detectably enhanced the desulfurase activity to levels much higher than those observed for the addition of MoeB alone to IscS (Fig. 3C). The enhancement of desulfurase activity by MoaD in the presence of MoeB is specific for IscS, as the addition of MoaD together with MoeB to CsdA and SufS had only a slight effect on their activities (Fig. 3, A and B). The activation of IscS by MoeB and further enhancement of the activity by MoaD resembles the activation of SufS by SufE and the additional enhancement of the SufS-SufE complex by SufBCD (18). In the activation of SufS by SufE-SufBCD, the persulfide sulfur of SufS is transferred to SufE and subsequently to SufBCD via protein-protein interactions (18, 46). However, the mechanism of activation of IscS by MoeB and MoaD may be different from that of SufS activation by SufE-SufBCD because specific sulfuration of MoeB was not observed (Fig. 3) (41).

Evidence for the Specific Interaction of IscS with MoeB and MoaD—An intrinsic function of a protein *in vivo* is highly dependent on its specific interactions with other proteins. In fact, the activation of a cysteine desulfurase by a partner protein is coincident with the specific interaction between them (13, 18, 42, 46, 47). To investigate whether the observed activation of IscS by MoeB and MoaD involves specific physical protein-protein interactions, we conducted surface plasmon resonance measurements. MoeB or MoaD was immobilized on a sensor chip, while CsdA, SufS, or IscS was added to a solution as the analyte. For both MoeB and MoaD, the response unit was increased by increasing the concentration of IscS (Fig. 4). Binding of IscS to MoaD and MoeB showed K_D values of 21 and 75 nM, respectively. In contrast, no measurable interaction was observed when SufS or CsdA was used as an analyte (Fig. 4). These data imply that the MoeB-MoaD complex presumably formed *in vivo* (41, 48) preferentially binds to IscS during the thiocarboxylation of MoaD.

Analysis of the Sulfuration Level of MoaD Isolated from Strains Defective in Cysteine Desulfurases—Because the MoaD-thiocarboxylate group is the direct sulfur donor for the conversion of precursor Z to MPT, it was of interest to analyze the sulfuration level of MoaD isolated from cysteine desulfurase mutants. The purified protein MoaD from the MC1061, CL100,

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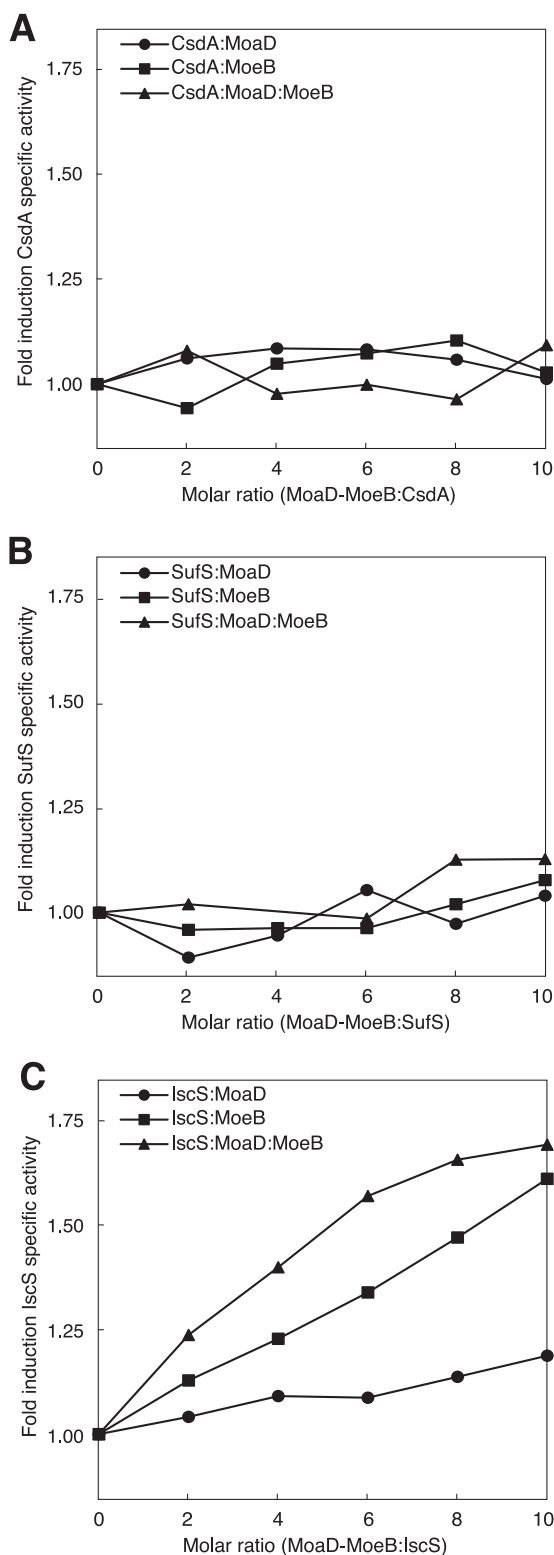


FIGURE 3. **Activation of cysteine desulfurase activity by MoaD or MoeB.** Formations of sulfide from L-cysteine were measured after 30 min for CsdA (A) and IscS (C) and 90 min for SufS (B).

and CL102 strains was incubated with purified MoaE and precursor Z. Produced MPT was quantified by a conversion to Form A. As shown in Fig. 5, a largely reduced level of MPT formation was detected (quantified as Form A) when MoaD was purified from the *iscS*-deficient strain CL100, whereas

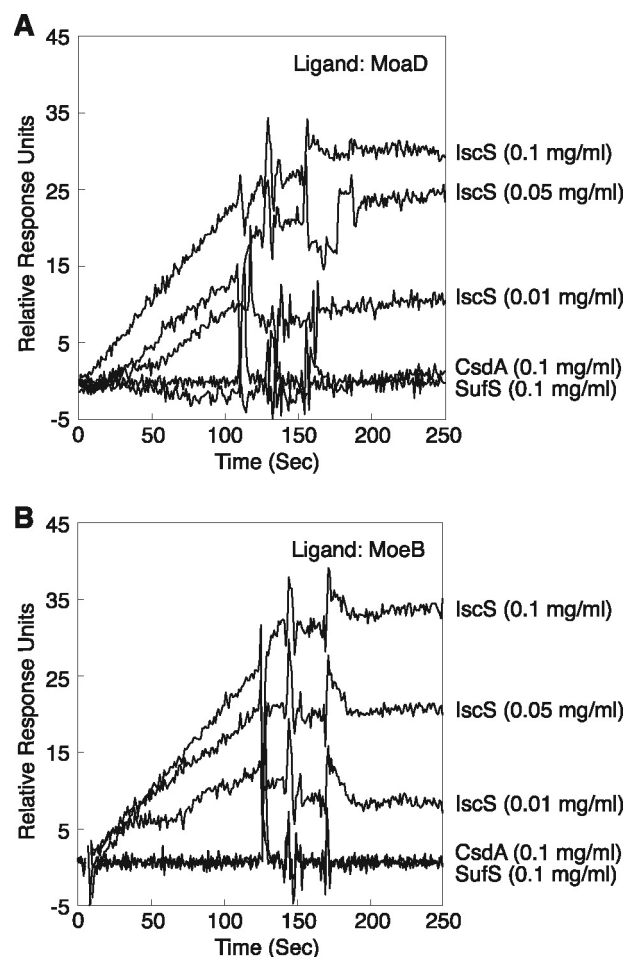


FIGURE 4. **Surface plasmon resonance analysis to examine the interaction between MoaD (A) or MoeB (B) and three cysteine desulfurases of *E. coli*.**

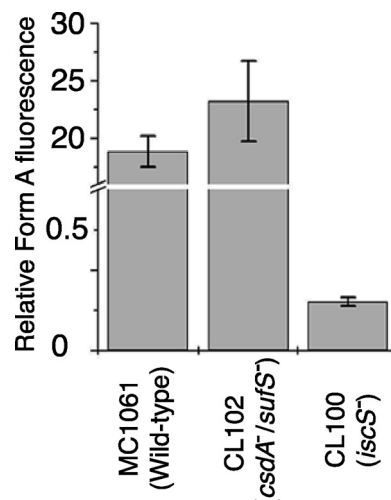


FIGURE 5. **Detection of the sulfuration level of MoaD after expression in different *E. coli* mutant strains.** The sulfuration level of MoaD was determined by the amount of MPT formed after incubation of purified MoaD, MoaE, and precursor Z in excess. The amount of MPT formed during the reaction was quantified as Form A using the peak height.

MoaD isolated from the *csdA/sufS* deletion mutant strain CL102 showed the same activity as the corresponding wild-type strain. The amount of about 1% of activity of MoaD purified

from the CL100 strain is consistent with previous results, which show that the CL100 strain contained only a level of 10% Moco of wild-type strain (36). This result strongly suggests that IscS is involved in the sulfuration of Moco. Because no effect on the sulfuration level of Moco was obtained using the CL102 strain, CsdA and SufS are not involved in Moco biosynthesis *in vivo*.

Accumulation of Compound Z in the $\Delta iscS$ Strain—Previously, Leimkühler and Rajagopalan (36) showed that precursor Z is not accumulated in the $iscS^-$ strain; however, the crude extract of the $iscS^-$ strain was able to convert externally added precursor Z to MPT to the same level as the parental strain. Accordingly, they suggested that the low level of MPT in an $iscS^-$ strain is due to the impaired activity of the Fe-S protein MoaA involved in the synthesis of precursor Z, and, therefore, it could not be clearly determined whether IscS is required for the *in vivo* sulfuration of MPT synthase. Because the amount of Moco in the $iscS$ mutant was determined to be 10% of that in the wild-type strain, it was concluded that CsdA, SufS, or other sulfur transferases in the cells are able to compensate to some extent for the function of IscS in the Fe-S cluster formation of MoaA and the conversion of precursor Z to MPT. Therefore, the amount of precursor Z in an $iscS^-$ strain seemed to differ depending on the balance of CsdA, SufS, or other sulfur transferases available for Fe-S cluster synthesis and for the sulfuration of MoaD.

To test this possibility, we examined whether compound Z is accumulated in *E. coli* strains deficient in *csdA*, *sufS*, or *iscS*. An earlier study demonstrated that the accumulated compound Z could be released by direct acidification of whole-cell suspensions of appropriate *E. coli* MPT-deficient mutants, such as the $\Delta moaD$ and $\Delta moeB$ strains (33). HPLC analysis of the acid extracts of *E. coli* cells revealed that the peak absorbing at 300 nm, which eluted at 5.75 min, was observed in the $\Delta iscS$ strain (Fig. 6A); the $\Delta moaD$ strain also accumulated compounds absorbing at 300 nm with the same retention time as the $\Delta iscS$ strain (Fig. 6B). In contrast, such accumulation was not observed in the $\Delta csdA$ and $\Delta sufS$ strains as well as in the wild-type strain (Fig. 6A). HPLC/ESI-MS, ESI-MS/MS, and UV-Vis absorption analyses clearly identified the accumulated 300 nm absorbing material to be compound Z (supplemental Fig. S2, S3, and S4). A genetic complementation analysis of the $\Delta iscS$ strain by the expression of IscS further confirmed that the accumulation of compound Z is specifically caused by the deletion of the *iscS* gene (supplemental Fig. S5). These results indicate that MoaA is active in the strain. CsdA, SufS, or other sulfur transferases presumably support the assembly of the FeS cluster in MoaA present in the $\Delta iscS$ strain. However, the contributions of the three cysteine desulfurases for it are still unclear and will be interesting to study in the future.

The studies presented here identify the protein involved in the sulfur transfer for the formation of the dithiolene moiety of Moco in *E. coli*. Earlier work demonstrated that any of the three NifS-like sulfur transferases, namely IscS, CsdA, or SufS, is capable of mobilizing and transferring sulfur from L-cysteine to MoaD *in vitro* (36). The minimal requirements for the activation of inactive MPT synthase *in vitro* were shown to be MoeB, Mg-ATP, L-cysteine, and a sulfur transferase. Thus, the specific protein involved in sulfur transfer to MoaD remained unknown in *E. coli*. Using different approaches, we show the IscS is the specific cysteine desulfurase transferring the sulfur to the Moco protein.

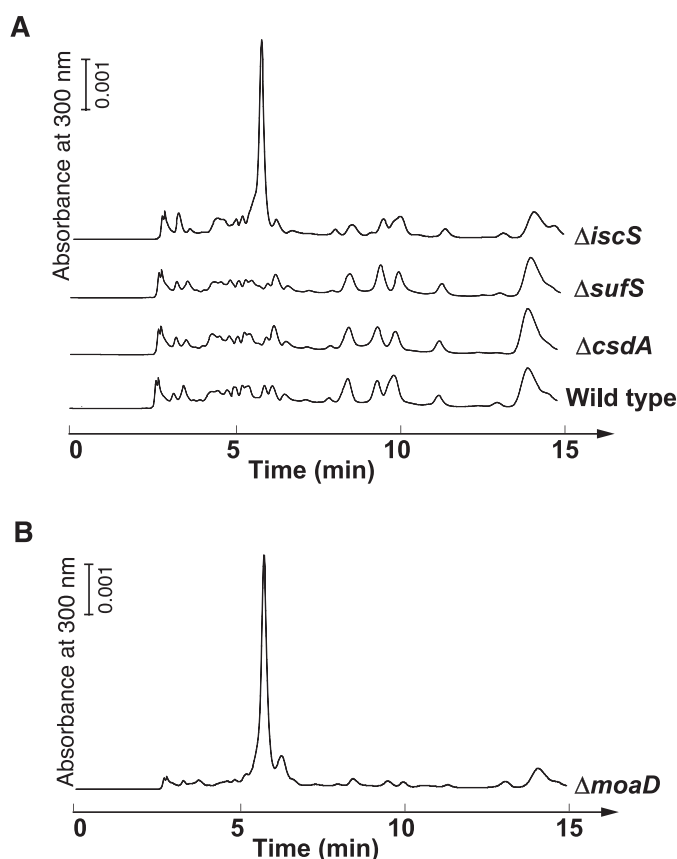


FIGURE 6. Analysis of the acid extracts of *E. coli* cells. The acid extracts of the wild-type, $\Delta csdA$, $\Delta sufS$, $\Delta iscS$, and $\Delta moaD$ strains were analyzed using HPLC with a Capcell Pak C₁₈ SG120 column after air oxidation. The volumes of the analyzed samples were 12.5 μ l for the $\Delta moaD$ strain and 25 μ l for the other strains.

It was recently shown that, in humans, the cysteine desulfurase Nfs1 acts as direct sulfur donor for MOCS3 in the cytosol (49). Thus, in humans and *E. coli*, the same protein components participate in the sulfur transfer pathway to MPT synthase during Moco biosynthesis: L-cysteine is the direct sulfur donor for a cysteine desulfurase, which transfers the sulfur to the adenylated small subunit of MPT synthase. In humans, this sulfur transfer occurs with the aid of the C-terminal rhodanese-like domain of MOCS3. For this additional sulfur transfer step, there must have been an evolutionary advantage for eukaryotes, which, likely, made this reaction more specific.

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