It has been shown that conversion of precursor Z to molybdopterin (MPT) by *Escherichia coli* MPT synthase entails the transfer of the sulfur atom of the C-terminal thioctic acid from the small subunit of the synthase to generate the dithiolene group of MPT and that the *moeB* mutant of *E. coli* contains inactive MPT synthase devoid of the thioctic acid. The data presented here demonstrate that L-cysteine can serve as the source of the sulfur for the biosynthesis of MPT *in vitro* but only in the presence of a persulfide-containing sulfurtransferase such as IscS, cysteine sulfinate desulfinase (CSD), or CsdB. A fully defined *in vitro* system has been developed in which an inactive form of MPT synthase can be activated by incubation with MoeB, Mg-ATP, L-cysteine, and one of the NifS-like sulfurtransferases, and the addition of precursor Z to the *in vitro* system gives rise to MPT formation. The use of radiolabeled L[^35]S-cysteine has demonstrated that both sulfurs of the dithiolene group of MPT originate from L-cysteine. It was found that MPT can be produced from precursor Z in an *E. coli* iscS mutant strain, indicating that IscS is not required for the *in vivo* sulfuration of MPT synthase. A comparison of the activity of the three sulfurtransferases to provide the sulfur for MPT formation showed the highest activity for CSD in the *in vitro* system.

In all molybdoenzymes with the exception of nitrogenase, molybdenum is coordinated by the sulfur atoms of the dithiolene group present in the molybdenum cofactor (MocO)\(^1\) (1). The biosynthetic pathway for MocO is evolutionary conserved, since many genes encoding highly homologous proteins involved in the pathway have been found in archaea, bacteria, higher plants, *Drosophila*, and higher animals including humans. The reactions of the Moc biosynthetic pathway comprise three stages, which are similar in all organisms utilizing molybdoenzymes. In the first step, a guanine nucleotide is converted into the dithiolene, and the transfer of sulfur from L-cysteine to MPT is catalyzed by MPT synthase, a tetrameric protein composed of two small MoaD subunits (8.8 kDa) and two large MoaE subunits (16.8 kDa). The recently solved high resolution crystal structure of *E. coli* MPT synthase has shown that the C terminus of each small subunit is inserted into one of the large subunits to form the active site (3). The small subunit of MPT synthase shows high structural similarity to the eukaryotic protein ubiquitin. In the activated form of MPT synthase, the C terminus of the small subunit is converted to a glycine thioctic acid that acts as the sulfur donor for the conversion of precursor Z to MPT (2, 3). Mass spectroscopy has identified that MPT synthase in its inactive form is lacking the thioctic acid at the C-terminal glycine of MoaD (2). Since the inactive form of MPT synthase could be purified from *moeB* mutant strains (2), it has been proposed that the MoeB protein is in fact MPT synthase sulfurate responsible for regenerating the active sulfur at the C-terminal glycine-thioctic acid of the small subunit of the synthase. This reaction has been shown to be ATP-dependent (4); however, details of the mechanism of action of the sulfurate, including the identity of the sulfur donor for the protein, remains as yet unknown.

This paper describes an *in vitro* system for the activation of inactive MPT synthase isolated from a *moeB* mutant strain. This activation was monitored by conversion of precursor Z to MPT *in vitro*. The data strongly suggest that L-cysteine is the likely physiological sulfur donor for the dithiolene group of MPT and demonstrate that an additional protein component is required for the transfer of sulfur from L-cysteine to MPT synthase.

It is known that NifS, a well characterized pyridoxal phosphate-dependent enzyme from *Azotobacter vinelandii*, is involved in iron-sulfur cluster formation for nitrogenase, converting L-cysteine to L-alanine and elemental sulfur (5). In *E. coli*, three NifS-like proteins resembling *A. vinelandii* NifS in amino acid sequence and catalytic properties have been identified (6–8). These proteins, designated IscS, CSD, and CsdB, are described as pyridoxal 5'-phosphate-dependent enzymes that catalyze the elimination of selenium and sulfur from L-selenocysteine and L-cysteine, respectively, to form L-alanine (9). IscS is a cysteine desulfurase and is proposed to play a general role in the formation of iron-sulfur clusters and additionally is required for the biosynthesis of 4-thiouridine, thiamin and NAD (6, 10, 11). In contrast, CSD, encoded by csdA, has been shown to act on L-selenocysteine,
Involvement of NifS-like Proteins in the Biosynthesis of MPT

**Experimental Procedures**

**Bacterial Strains, Media, and Growth Conditions**—E. coli strains and plasmids used in this study are listed in Table I. E. coli cell strains were grown aerobically at 30 °C in LB medium. Cell strains containing expression plasmids were grown in the presence of 150 μg/ml ampicillin. For expression of pET15b-based plasmids, the ΔDE3 lysogenization kit from Novagen was used to integrate the gene for T7 RNA polymerase into the chromosome of the E. coli strains. To determine nitrate reductase activity, cells were grown aerobically in LB medium supplemented with 15 mM NaNO₃.

**Purification of the Reaction Components**—Precursor Z was isolated from E. coli moaD cells using high performance liquid chromatography (HPLC) with reverse phase and anion exchange columns (12). Cloned MPT synthase was expressed in a pET15b vector (Novagen) in E. coli moeB (DE3) cells, and the protein was purified by ammonium sulfate precipitation and gel filtration after the procedure described in Ref. 3. Cloning of MoeB was expressed in a pET15b vector (Novagen) in E. coli moaD (DE3) cells, and the protein was purified by ammonium sulfate precipitation, ion exchange chromatography, hydrophobic interaction chromatography, and gel filtration.2 Human sulfite oxidase was cloned into a pTRE- His vector (Amersham Pharmacia Biotech), generating an N-terminal fusion to a His₆ tag, expressed in E. coli moaA, MC1061, and CL100(ΔmoeB). Human isocitrate lyase was purified by Ni²⁺-nitrilotriacetic acid chromatography (13).

**Cloning of the icss, csdA, and csdB Genes from the E. coli Genome**—The DNA fragments containing icss, csdA, and csdB were cloned from chromosomal E. coli DNA by polymerase chain reaction. Oligonucleotide primers used were as follows: 5′-CGATGGACTTATCGAAGGTTATCCGAGTTCGTC-3′ and 5′-GGACCCATTACTACTACTACT-3′ for cloning icss into the Ncol and BamHI sites of pET15b. During cloning, the second amino acid of IscS was changed from a lysine to a glutamate. The resulting plasmid was designated pSL209. 2) Primers 5′-CGGTGGATCCAGGAGGCAGGATGATATGAAAC-3′ and 5′-GGACCCATTACTACTACTACT-3′ were used for cloning csdA into the Ncol and BamHI sites of pET15b. The resulting plasmid was designated pSL215. 2) Primers 5′-CATGATGACTTCTCTGGGAAATGCGG-3′ and 5′-GGATGACTTCTCTGGGAAATGCGG-3′ were used for cloning csdB into the Ncol and BamHI sites of pET15b. The resulting plasmid was designated pSL213. The corresponding restriction sites used for cloning are underlined.

**Expression and Purification of icss, csdA, and csdB**—E. coli BL21 (DE3) cells containing the corresponding expression plasmids were grown in 2 liters of LB medium to an A₆₀₀ of 0.6. At this point, protein expression was induced by the addition of 100 μM isopropyl-β-D-thiogalactoside. After 4 h, cells were harvested by centrifugation at 5000 × g for 5.0 × g x h. The His₆ tag-containing CadB and Csd were purified by Ni²⁺-nitrilotriacetic acid chromatography. The cell pellets were resuspended in 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, and lysed by several passages through a French pressure cell. After centrifugation at 17,000 × g for 25 min, imidazole was added to the supernatant to a final concentration of 10 mM. The supernatant was then combined with 1.5 ml of Ni²⁺-nitrilotriacetic acid resin (Qiagen) per liter of cell growth, and the slurry was equilibrated with gentle stirring at 4 °C for 30 min. The slurry was poured into a column and washed with 2 column volumes of 10 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, followed by a wash with 10 column volumes of the same buffer with 20 mM imidazole. The His-tagged proteins were eluted with 100 mM imidazole in 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. Fractions containing CadB or Csd were combined and dialyzed against 50 mM Tris, 1 mM EDTA, pH 7.5.

For the purification of IscS, the cell pellet was resuspended in 50 mM Tris, 1 mM EDTA, pH 7.5, and lysed by several passages through a French pressure cell. After centrifugation at 17,000 × g for 25 min, imidazole was added to the supernatant to a final concentration of 10 mM. The supernatant was then combined with 1.5 ml of Ni²⁺-nitrilotriacetic acid resin (Qiagen) per liter of cell growth, and the slurry was equilibrated with gentle stirring at 4 °C for 30 min. The slurry was poured into a column and washed with 2 column volumes of 10 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, followed by a wash with 10 column volumes of the same buffer with 20 mM imidazole. The His-tagged proteins were eluted with 100 mM imidazole in 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. Fractions containing CadB or Csd were combined and dialyzed against 50 mM Tris, 1 mM EDTA, pH 7.5. For the purification of IscS, the cell pellet was resuspended in 50 mM Tris, 1 mM EDTA, pH 7.5, and lysed by several passages through a French pressure cell. After centrifugation at 17,000 × g for 25 min, imidazole was added to the supernatant to a final concentration of 10 mM. The supernatant was then combined with 1.5 ml of Ni²⁺-nitrilotriacetic acid resin (Qiagen) per liter of cell growth, and the slurry was equilibrated with gentle stirring at 4 °C for 30 min. The slurry was poured into a column and washed with 2 column volumes of 10 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, followed by a wash with 10 column volumes of the same buffer with 20 mM imidazole. The His-tagged proteins were eluted with 100 mM imidazole in 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. Fractions containing CadB or Csd were combined and dialyzed against 50 mM Tris, 1 mM EDTA, pH 7.5.

**In Vitro Activation of Inactive MPT Synthase by MoeB**—For the in vitro production of MPT, 150–200 μM precursor Z (in 10 mM sodium citrate buffer adjusted to pH 7.2) was applied to the MPT synthase complex containing 3.5–31 μM MoeB; 2.5 mM MgCl₂; 2.5 mM ATP; 4 mM to 5 μM IscS, CsdA, or CsdB; and 2.5 mM L-cysteine were incubated in a total volume of 400 μl of 100 mM Tris, pH 7.2. For a standard incubation assay, all reactants were allowed to react at room temperature for 30 min under aerobic conditions. The reaction was stopped by the addition of acidic trichloroacetic acid and analyzed for the formation of form A afterward (16). Generation of an IscS, CsdA, or CsdB-bound Persulfide—For the generation of an IscS, CsdA, or CsdB-bound persulfide, 3 mM of IscS, CsdA, or CsdB were incubated with 2 mM L-cysteine for 5 min at 4 °C, gel-filtered using a PD10 column equilibrated with 100 mM Tris, pH 7.2, and immediately added to the in vitro activation mixtures.

**MPT Analysis and Quantification of MPT by Generation of Form A (Diphospho)−In vitro production of MPT was quantified by its conversion to the stable, fluorescent degradation product form A. For this conversion, the incubation mixtures were adjusted to pH 2.5, and excess iodide was added as described in Refs. 16 and 17. The amount of form A was then quantitated by HPLC analysis by comparison with a standard sample obtained from purified human sulfite oxidase.

**In Vitro Insertion of Radiolabeled 35S into MPT**—For the in vitro insertion of 35S from labeled L-cysteine into precursor Z, incubation...
In Vitro Activation of Inactive MPT Synthase Purified from a moeB Mutant Strain—It has been shown earlier that MPT can be synthesized in vitro by incubation of purified precursor Z with the active form of MPT synthase (17, 19). After the transfer of sulfur from MPT synthase to precursor Z, MPT synthase is present in an inactive, desulfurated form lacking the C-terminal thioester group at the MoaD subunit of the protein. In order to define the sulfur transfer pathway involved in resulfuration of MPT synthase, the in vitro system was modified to include inactive MPT synthase and purified MoeB protein. MoeB has been proposed to regenerate the active sulfur at the glycine-carboxylate group of MPT synthase in an ATP-dependent reaction and has been designated as MPT synthase sulfurase (4). Precursor Z was purified from a moaD mutant strain unable to convert the precursor to MPT (12). Inactive, recombinant MPT synthase was purified from a moeB mutant strain as described earlier (17). MoeB was expressed in cells that contain a mutation in moeB and purified afterward (experimental procedures). The activation of MPT synthase was assayed by the ability of the synthase to convert precursor Z to MPT in vitro.

For the production of MPT in vitro, precursor Z, inactive MPT synthase, MoeB, and Mg-ATP were incubated at room temperature as described under “Experimental Procedures.” Acidic iodine treatment converts MPT to its oxidized fluorescent degradation product form A (Fig. 1) (16). HPLC analysis revealed that no form A was formed under these conditions (Fig. 2A). This finding indicated that MoeB by itself was not able to sulfurate inactive MPT synthase in vitro. In contrast, when a crude cell extract prepared from a moeB mutant strain was included in the in vitro incubation mixture, form A was obtained (Fig. 2B). To identify the component in the crude extract necessary for activation of MPT synthase, the extract was separated into a protein fraction and a low molecular weight fraction by gel filtration and ultrafiltration, respectively. As shown in Fig. 2, C and D, neither the protein fraction nor the low molecular weight fraction alone was able to provide the missing component in the in vitro system. This result led to the conclusion that an as yet unidentified protein component as well as a low molecular weight substance are necessary for the activation of inactive MPT synthase by MoeB, with the low molecular weight substance presumably providing the sulfur source for the sulfuration of the synthase.

In order to identify possible physiological sulfur donors, dif-
With the identification of L-cysteine as a sulfur donor for the sulfuration of MPT synthase—requiring MoeB as well. In order to determine whether both sulfur atoms of the dithiolene group of MPT originate from L-cysteine, radiolabeled L-[35S]cysteine was added to the in vitro activation mixture consisting of IscS, MoeB, MPT synthase, precursor Z, Mg-ATP, and L-[35S]cysteine. The mixture was incubated (see “Experimental Procedures”). In order to stabilize the MPT produced, aposulfite oxidase was added after the 30-min incubation. We have previously reported that in vitro synthesized MPT can reconstitute a cofactor-free form of recombinant human sulfite oxidase, and in the presence of molybdate, active sulfite oxidase is obtained (17). Since the cloned sulfite oxidase contains a His6 tag, it can be easily purified from the in vitro incubation mixture (19). To test the relative abilities of the three proteins to utilize sulfur from L-cysteine for the sulfuration of MPT synthase in vitro, IscS, CSD, and CsdB were cloned from the E. coli genome, designated IscS, CSD, and CsdB (6–8). While all three enzymes can desulfurate L-cysteine, they displayed different substrate specificities. IscS has the highest activity with L-cysteine sulfinate as substrate (7). CsdB has a much higher activity with L-cysteine desulfurases. As shown in Table II, the catalytic activities of IscS, CSD, and CsdB varied markedly. The mixture containing CSD produced the highest amount of MPT (34.04 nmol of MPT/nM CSD). IscS produced much less MPT (29.5%) compared with CSD (10.05 nmol of MPT/nM IscS), whereas CsdB showed the lowest activity, with about 1.2% MPT formed compared with CSD (0.40 nmol of MPT/nM CsdB). The differences in the activities of the three enzymes in the transfer of sulfur from L-cysteine to MPT synthase are in conformity with their specific activities estimated by the production of elemental sulfur from L-cysteine as reported by Mihara et al. (8). In sum, these experiments delineate a sulfur transfer pathway from L-cysteine to MPT synthase, which in turn converts precursor Z to MPT in the presence of Mg-ATP. All components described above are essential for the in vitro assembly of MPT, since in the absence of either MoeB, MPT synthase, Mg-ATP, L-cysteine, or a sulfurtransferase no MPT was formed (data not shown).

Three NifS-like Sulfurtransferases Can Catalyze the Activation of MPT Synthase—With the identification of L-cysteine as the likely physiological sulfur donor for the sulfuration of MPT synthase, it was of further interest to identify the sulfurtransferase required for the mobilization of this sulfur. Since this protein has to act as an L-cysteine desulfurase, it appeared that a NifS-like protein might be involved in this reaction. Three NifS-like proteins have been identified in the E. coli genome sequence, designated IscS, CSD, and CsdB (6–8). While all three enzymes can desulfurate L-cysteine, they displayed different substrate specificities. IscS has the highest activity with L-cysteine, whereas CSD and CsdB, described as a sulfinate desulfinase, prefers L-cysteine sulfinate as substrate (7). CsdB has a much higher activity with L-selenocysteine than L-cysteine and is regarded as the E. coli counterpart of mammalian selenocysteine lyase (8). To test the relative abilities of the three proteins to utilize sulfur from L-cysteine for the sulfuration of MPT synthase in vitro, IscS, CSD, and CsdB were cloned from the E. coli genome and purified after expression in BL21(DE3) cells as described under “Experimental Procedures.” The effectiveness of the three enzymes for in vitro MPT production was examined using reaction mixtures containing L-cysteine as a sulfur source, one of the three NifS-like sulfurtransferases, MoeB, inactives MPT synthase, precursor Z, and Mg-ATP. The sulfurtransferase activity was assessed by the amount of MPT produced in vitro by equivalent amounts of the sulfurtransferases. As shown in Table II, the catalytic activities of IscS, CSD, and CsdB varied markedly. The mixture containing CSD produced the highest amount of MPT (34.04 nmol of MPT/nM CSD). IscS produced much less MPT (29.5%) compared with CSD (10.05 nmol of MPT/nM IscS), whereas CsdB showed the lowest activity, with about 1.2% MPT formed compared with CSD (0.40 nmol of MPT/nM CsdB). The differences in the activities of the three enzymes in the transfer of sulfur from L-cysteine to MPT synthase are in conformity with their specific activities estimated by the production of elemental sulfur from L-cysteine as reported by Mihara et al. (8). In sum, these experiments delineate a sulfur transfer pathway from L-cysteine to MPT synthase, which in turn converts precursor Z to MPT in the presence of Mg-ATP. All components described above are essential for the in vitro assembly of MPT, since in the absence of either MoeB, MPT synthase, Mg-ATP, L-cysteine, or a sulfurtransferase no MPT was formed (data not shown).

Direct Evidence for the Transfer of Sulfur from L-Cysteine to the Dithiolene Group of MPT—In order to determine whether both sulfur atoms of the dithiolene group of MPT originate from L-cysteine, radiolabeled L-[35S]cysteine was added to the in vitro activation mixture consisting of IscS, MoeB, MPT synthase, precursor Z, Mg-ATP, and L-[35S]cysteine. The mixture was incubated under aerobic conditions for 30 min at room temperature (see “Experimental Procedures”). In order to stabilize the MPT produced, aposulfite oxidase was added after the 30-min incubation. We have previously reported that in vitro synthesized MPT can reconstitute a cofactor-free form of recombinant human sulfite oxidase, and in the presence of molybdate, active sulfite oxidase is obtained (17). Since the cloned sulfite oxidase contains a His6 tag, it can be easily purified from the in vitro incubation mixture (19). To test the relative abilities of the three proteins to utilize sulfur from L-cysteine for the sulfuration of MPT synthase in vitro, IscS, CSD, and CsdB were cloned from the E. coli genome and purified after expression in BL21(DE3) cells as described under “Experimental Procedures.” The effectiveness of the three enzymes for in vitro MPT production was examined using reaction mixtures containing L-cysteine as a sulfur source, one of the three NifS-like sulfurtransferases, MoeB, inactives MPT synthase, precursor Z, and Mg-ATP. The sulfurtransferase activity was assessed by the amount of MPT produced in vitro by equivalent amounts of the sulfurtransferases. As shown in Table II, the catalytic activities of IscS, CSD, and CsdB varied markedly. The mixture containing CSD produced the highest amount of MPT (34.04 nmol of MPT/nM CSD). IscS produced much less MPT (29.5%) compared with CSD (10.05 nmol of MPT/nM IscS), whereas CsdB showed the lowest activity, with about 1.2% MPT formed compared with CSD (0.40 nmol of MPT/nM CsdB). The differences in the activities of the three enzymes in the transfer of sulfur from L-cysteine to MPT synthase are in conformity with their specific activities estimated by the production of elemental sulfur from L-cysteine as reported by Mihara et al. (8). In sum, these experiments delineate a sulfur transfer pathway from L-cysteine to MPT synthase, which in turn converts precursor Z to MPT in the presence of Mg-ATP. All components described above are essential for the in vitro assembly of MPT, since in the absence of either MoeB, MPT synthase, Mg-ATP, L-cysteine, or a sulfurtransferase no MPT was formed (data not shown).
In vivo activation of inactive MPT synthase with MoeB and the high molecular weight fraction of moeb x extract. HPLC elution profiles of a reversed phase C18 column after the in vitro incubation of 3.4 μM inactive MPT synthase with 2.5 mM Mg-ATP, 200 μM precursor Z, 15 μM MoeB, 20 μl (2 mg/ml) of the high molecular weight fraction of moeb x extract after the addition of 250 μM sodium sulfide (A), 250 μM sodium thiocyanate (B), 250 μM L-cysteine (C), 250 μM L-cysteine (E), or 250 μM sodium sulfide (F) without the addition of the high molecular weight fraction of moeb x extract.

**FIG. 3.** Identification of the physiological sulfur donor required for the in vitro activation of inactive MPT synthase with MoeB and the high molecular weight fraction of moeb x extract. HPLC elution profiles of a reversed phase C18 column after the in vitro incubation of 3.4 μM inactive MPT synthase with 2.5 mM Mg-ATP, 200 μM precursor Z, 15 μM MoeB, 20 μl (2 mg/ml) of the high molecular weight fraction of moeb x extract after the addition of 250 μM sodium sulfide (A), 250 μM sodium thiocyanate (B), 250 μM L-cysteine (C), 250 μM L-cysteine (E), or 250 μM sodium sulfide (F) without the addition of the high molecular weight fraction of moeb x extract.

TABLE II
Abilities of E. coli NifS-like proteins to provide the sulfur for the formation of MPT

| Enzyme | Enzyme activity \(\text{formation of nmol MPT/nM enzyme} \times 10^6 \text{ enzyme} \) | Relative activity |
|--------|-------------------------------------------------|------------------|
| CSD    | 34.04                                           | 100              |
| IscS   | 10.05                                           | 29.5             |
| CsdB   | 0.40                                            | 1.2              |

*Enzyme activity was determined by the amount of form A produced in in vitro activation assays containing 175 nM MPT synthase, 30.2 μM MoeB, 150 μM precursor Z, 2.5 mM Mg-ATP, and 2.5 mM cysteine. The amounts of sulfurtransferase present in each assay were varied for CSD from 1.4 to 42 nM, for IscS from 13 to 133 nM, and for CsdB from 0.65 to 1.3 μM. Mean values of MPT produced per enzyme were estimated as an average from up to six independent measurements with varying amounts of sulfurtransferase. All reactants were allowed to react for 30 min at room temperature before the addition of acidic iodine (see "Experimental Procedures").

30 min at 95 °C for the production of form B, and the other fraction was denatured with SDS in the presence of iodoacetamide for the formation of camMPT. As shown in Table III, analysis of the radioactivity present in purified form B and camMPT revealed a ratio of 1.173, correlating with the number of sulfur atoms present in the two MPT derivatives. This ratio remained the same in the presence or absence of sodium molybdate in the in vitro incubation mixture (data not shown). These results showed conclusively that both sulfur atoms present in the dithiolene group of MPT are derived from MPT, in contrast, analysis of activity of human sulfite oxidase expressed in these strains revealed that active sulfite oxidase is produced in strain CL100(iscS<sup>-</sup>), but only to the extent of 10% in comparison with the parental strain MC1061 (Table IV). Measurement of the cofactor content of purified sulfite oxidase revealed that the lower activity of sulfite oxidase in CL100(iscS<sup>-</sup>) corresponded with its cofactor content (Table V). In addition, the amount of total MPT determined in whole cells of these two strains showed a significantly lower amount of MPT in CL100(iscS<sup>-</sup>) in comparison with MC1061 (Table V). It therefore appeared that the reduced activity of sulfite oxidase in strain CL100(iscS<sup>-</sup>) is based on an impaired ability of this strain to produce MPT.

Analysis of the Ability of Extracts from Strains CL100(iscS<sup>-</sup>) and MC1061 to Convert Added Precursor Z to MPT—It remained possible that the reduced ability of CL100(iscS<sup>-</sup>) to produce MPT is due to a reduced ability of strain CL100(iscS<sup>-</sup>) to transport the sulfate formed in the strain to the position of the synthase. Analyses of the Activities of Sulfite Oxidase in E. coli Strain CL100(iscS<sup>-</sup>)—The data presented above have shown the requirement for an NifS-like protein to mobilize the sulfur atom of cysteine for the biosynthesis of MPT. Lauhon and Kambampati (11) reported the successful construction of an E. coli strain with an in-frame deletion of the iscS gene (Table I). To determine whether IscS is required for the synthesis of MPT in vivo, we analyzed the activities of different molybdoenzymes in the E. coli strain CL100(iscS<sup>-</sup>) and the corresponding parental strain MC1061. As shown in Table IV, nitrate reductase activity was detected in the strain MC1061 but not in strain CL100(iscS<sup>-</sup>). In contrast, analysis of activity of human sulfite oxidase expressed in these strains revealed that active sulfite oxidase is produced in strain CL100(iscS<sup>-</sup>), but only to the extent of 10% in comparison with the parental strain MC1061 (Table IV). Measurement of the cofactor content of purified sulfite oxidase revealed that the lower activity of sulfite oxidase in CL100(iscS<sup>-</sup>) corresponded with its cofactor content (Table V). In addition, the amount of total MPT determined in whole cells of these two strains showed a significantly lower amount of MPT in CL100(iscS<sup>-</sup>) in comparison with MC1061 (Table V). It therefore appeared that the reduced activity of sulfite oxidase in strain CL100(iscS<sup>-</sup>) is based on an impaired ability of this strain to produce MPT.

Analysis of the Ability of Extracts from Strains CL100(iscS<sup>-</sup>) and MC1061 to Convert Added Precursor Z to MPT—It remained possible that the reduced ability of CL100(iscS<sup>-</sup>) to produce MPT is based on the limited ability of the strain to produce the sulfated form of MPT synthase. It has been shown that mutant strains in moaD or moeB, which lack MPT synthase or produce an unsulfurated form of the synthase, respectively, accumulate precursor Z (12). Analysis of the precursor Z content of CL100(iscS<sup>-</sup>) revealed no such accumulation (data not shown), indicating that all precursor Z produced in CL100(iscS<sup>-</sup>) is completely converted to MPT by a sulfated form of MPT synthase. Thus, it could be concluded that the inability of strain CL100(iscS<sup>-</sup>) to produce larger amounts of MPT is due to a reduced ability of this strain to synthesize precursor Z.
IscS forms protein-bound persulfides by transfer of sulfur from *A. vinelandii* iscS strain CL100 (MC1061). This finding showed that either CSD or CsdB in addition of precursor Z to extracts of CL100 (Fig. 4), the same amounts of form A were formed after the vert externally added precursor Z to MPT. Conclusively, the reduced ability of strain CL100(iscS) to provide the Fe-S clusters for MoaA, a protein required for the synthesis of precursor Z from a guanosine nucleotide.

It was of further interest to determine whether the two remaining sulfurtransferases, CSD and CsdB, are sufficient to provide the sulfur for the conversion of larger amounts of precursor Z to MPT. For this purpose, the extracts of CL100(iscS) and MC1061 were tested for their ability to convert externally added precursor Z to MPT *in vitro*. As shown in Fig. 4, the same amounts of form A were formed after the addition of precursor Z to extracts of CL100(iscS) and MC1061. This finding showed that either CSD or CsdB in strain CL100(iscS) is sufficient for producing a sulfurated form of MPT synthase, which in turn can convert all precursor Z present to MPT. Conclusively, the reduced ability of strain CL100(iscS) to produce precursor Z may be due to the inability of this strain to provide the Fe-S clusters for MoaA, a protein required for the synthesis of precursor Z from a guanosine nucleotide.

**The Sulfur Is Transferred as a Protein-bound Persulfide**—In *A. vinelandii*, it has been shown that the two proteins NifS and IscS form protein-bound persulfides by transfer of sulfur from free l-cysteine to an cysteine thiol group of the protein (6, 21). This protein-bound persulfide acts as the sulfur donor for the sulfuration of the corresponding substrates of these proteins. To determine whether a persulfide bound to *E. coli* IscS, CSD, or CsdB can act as the sulfur donor for the sulfuration of inactive MPT synthase *in vitro*, IscS, CSD, and CsdB were incubated with l-cysteine as described under “Experimental Procedures,” and excess l-cysteine was removed by gel filtration. Reaction mixtures containing the putative persulfide-containing proteins IscS, CSD or CsdB, MPT synthase, Moeb, precursor Z, and Mg-ATP were tested for their abilities to produce MPT without the addition of l-cysteine as a sulfur source. The amount of MPT produced in the *in vitro* incubation mixtures was again determined by conversion of MPT to form A. As shown in Fig. 5, form A production was observed in all three incubation mixtures, indicating that the sulfur for the sulfuration of MPT synthase is indeed being transferred from a sulfurtransferase in the form of a protein-bound persulfide. However, Fig. 5 shows that the abilities of the three sulfurtransferases to transfer the sulfur are significantly different. The *in vitro* assay containing the CSD-bound persulfide produced the highest amount of form A (Fig. 5A), while IscS-persulfided produced only 37% of the amount of form A in comparison with CSD (Fig. 5C), and CsdB-bound persulfide produced only 2% form A compared with CSD (Fig. 5B). These results are in agreement with the results shown in Table II, where CSD showed the highest catalytic activity in the desulfuration of l-cysteine for the production of MPT, followed by IscS and CsdB. These findings indicate that CSD has a high ability to

![FIG. 4. Analysis of the ability of CL100(iscS) and MC1061 extracts to convert externally added precursor Z to MPT. Shown are HPLC elution profiles of form A obtained after the in vitro incubation of L-[35S]cysteine, and 50 mM precurser Z, and Mg-ATP with 50 mM MC1061 extract (2 mg/ml) (A) and 50 mM of CL100(iscS) extract (2 mg/ml) (B).](image)

**TABLE III**

| MPT derivative | nCi per nM MPT derivative | Ratio | Sulfur | atoms |
|---------------|---------------------------|-------|--------|-------|
| Form B        | 0.03                      | 0.054 | 1      | 1     |
| camMPT        | 0.026                     | 0.052 | 1.73   | 2     |

* The conversion of MPT into form B and camMPT, respectively, was carried out as described under “Experimental Procedures.”

* Radioactivity was determined with a Beckman LS 1801 liquid scintillation counter.

**TABLE IV**

| Strain         | Enzyme activity | Nitrate reductase | Sulfite oxidase | Sulfite oxidase activity |
|----------------|-----------------|------------------|----------------|--------------------------|
| MC1061         | 1.34            | —                | 771.7          |
| CL100(iscS)    | —               | 70               |

* Nitrate reductase activity was estimated in crude extracts, and one unit of nitrate reductase activity is expressed as μmol of nitrate reduced per min per mg of protein.

* Sulfite oxidase activity was estimated with the purified protein, and 1 unit of sulfite oxidase activity is expressed as an absorbance change (ΔA) of 1 per min per mg of enzyme.

* Below the limit of detection.

**TABLE V**

| Strain         | MPT in sulfite oxidase | Total MPT in cell extract |
|----------------|------------------------|---------------------------|
| MC1061         | 100%                   | 100%                      |
| CL100(iscS)    | 9.8%                   | 33%                       |

* The MPT content of sulfite oxidase was determined as described under “Experimental Procedures.”

* The total MPT content of cell extracts from MC1061 and CL100(iscS) was determined after the method described in Ref. 16.

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**Involvement of NifS-like Proteins in the Biosynthesis of MPT**

The *in vitro* activation mixture for the incorporation of 35S into MPT contained 73.8 μM MPT synthase, 31.3 μM MoeB, 5.2 μM IscS, 400 μM precursor Z, 20 mM Na2MoO4, 2 mM Mg-ATP, 1 mM L-cysteine, 1 μCi of L-[35S]cysteine, and 51 μM sulfite oxidase.

**FIG. 4. Analysis of the ability of CL100(iscS) and MC1061 extracts to convert externally added precursor Z to MPT. Shown are HPLC elution profiles of form A obtained after the in vitro incubation of 1.7 μM inactive MPT synthase, 3 μM MoeB, 200 μM precursor Z, and 2.5 mM Mg-ATP with 50 μM of MC1061 extract (2 mg/ml) (A) and 50 μM of CL100(iscS) extract (2 mg/ml) (B).**
Involvement of NifS-like Proteins in the Biosynthesis of MPT

The studies presented here delineate an in vitro system using purified components for studying the mechanism of assembly of the dithiolene group of MPT synthase. While all NifS-like proteins tested in this system, IscS, CSD, and CsdB, is capable of mobilizing and transferring sulfur from L-cysteine to precursor Z. The minimal requirement for the activation of inactive MPT synthase was shown to be MoeB, Mg-ATP, L-cysteine, and a sulfurtransferase. After the addition of excess precursor Z, the reaction was shown to be catalytic rather than stoichiometric, since with the amounts of precursor used up to 30 times more MPT was produced than MPT synthase present in the system (data not shown), showing clearly that the components of the in vitro system are getting turned over during the reaction.

Since the biosynthesis of Moco has been most extensively studied in E. coli, the identification of a novel protein component separate from the products of the previously identified mo loci involved in MPT formation was a somewhat surprising observation. The well characterized genetic loci moa, mob, mod, moe, and mog were identified in E. coli by selection for chlorate resistance. During this selection, mutant strains were obtained that are deficient in nitrate reductase activity (22). Since a sulfurtransferase involved in the mobilization of L-cysteine-bound sulfur for the biosynthesis of Moco was not identified by selecting for chlorate-resistant mutants, it must be concluded that either a mutation in the sulfurtransferase impairs the viability of the cell or that a number of sulfurtransferases within the cell are capable of this activity.

In order to identify the sulfurtransferase required for MPT formation in vivo, a mutant strain with an in-frame deletion in the iscS gene was tested for its ability to form MPT. This iscS mutant strain was reported to have decreased levels of the activity of Fe-S-containing enzymes (10). Additionally, it lacks 4-thiouridine in its tRNA and requires thiamin and nicotinic acid for growth in minimal media (11). These observations implied that IscS has a general role in sulfur mobilization for the biosynthesis of Fe-S clusters, 4-thiouridine tRNA, and thiamine (11). However, it was reported that several Fe-S cluster-containing enzymes tested in the iscS mutant exhibited some residual activity, indicating that other proteins are at least partially involved in replacing IscS in its role for Fe-S cluster formation in vivo (10). Our data have shown that nitrate reductase, a molybdoenzyme requiring Fe-S clusters for its activity, is completely inactive in the iscS mutant strain. However, expression of recombinant sulfate oxidase in an iscS mutant strain yielded 10% of the activity in comparison with the corresponding parental strain. Correspondingly, the cofactor content of the iscS mutant strain was determined to be very low, and no precursor Z accumulation could be demonstrated in this strain. It is concluded that the low level of MPT in this strain is due to the inability of MoaA, an Fe-S cluster-containing enzyme involved in the synthesis of precursor Z (23), and not due to an inability to convert precursor Z to MPT. Since the crude extract of the iscS mutant exhibited the ability to convert externally added precursor Z to MPT to the same level as the parental strain, it was apparent that the other sulfurtransferases in the cell are fully capable of providing the sulfurs for the sulfuration of MPT synthase. These findings also indicate that IscS is not involved in the sulfur transfer process for MPT formation in vivo.

The exact physiological functions of the two E. coli NifS-like proteins CSD and CsdB are not known to date. It has been assumed that like IscS, they are involved in iron-cluster formation or the biosynthesis of selenophosphate in the cell (9). To determine the role of CSD and CsdB in the biosynthesis of MPT in vivo, the ability of mutant strains in csdB and csdA to produce MPT must be investigated. Since CSD showed the highest catalytic activity for MPT formation in vitro and CsdB is considered more as a selenocysteine lyase than as a cysteine desulfurase, CSD appears more likely to be involved in the mobilization of sulfur from L-cysteine for the synthesis of MPT. In sum, the data presented here show that the utilization of the sulfur atom of cysteine for MPT synthesis requires a persulfide-containing protein. While all NifS-like proteins tested in this study can serve the purpose in the established in vitro system, it is possible that also other persulfide-containing proteins are able to serve the same function. Nevertheless, the data presented in this study establish the requirement for a persulfide-containing protein, which acts as the sulfur donor for MPT biosynthesis. In future studies, it has to be investigated which persulfide-containing protein is the physiological sulfur donor for MPT formation in the cell.
Recently, we could show that MoeB does not serve the function as the immediate sulfur donor for the formation of the thiocarboxylate group in MoaD. The proposed mechanism of MPT synthase activation by sulfur transfer suggests that MoeB primes the small subunit of MPT synthase by the formation of a MoeB-MoaD adenylate complex for subsequent sulfuration but is itself not a carrier of the sulfur atom derived from L-cysteine.

The results shown above using 35S-labeled L-cysteine demonstrated that the same sulfur transfer pathway is involved in the incorporation of both sulfurs of the dithiolene group of MPT. It was shown previously that purified active MPT synthase and precursor Z are sufficient for the formation of MPT in vitro (17, 19). In the activated form of MPT synthase, the C terminus of the small MoaD subunit is present as a thiocarboxylate, which serves as the sulfur donor for MPT formation. The high resolution crystal structure of MPT synthase has shown that in the heterotetrameric protein, the C terminus of each MoaD subunit is inserted into one of the MoaE subunits to form the active site (3). The newly formed MPT remains tightly bound to the synthase in the absence of proteins that are able to bind MPT with higher affinity (17). However, details of the mechanism catalyzed by MPT synthase, including the insertion of two sulfur atoms into precursor Z for the formation of MPT without the need for resulfuration of the MoaD subunit, are unknown at present. One possibility is that the two active sites of an MPT synthase tetramer are able to act cooperatively and that each MoaD subunit of the tetramer provides one sulfur atom for the formation of the MPT dithiolene group. This reaction would require that the precursor is getting transferred between the two active sites of each MoaE subunit. In future studies, the reconstitution system presented here should help in understanding the mechanism of sulfur incorporation carried out by MPT synthase.

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