Characterization of *Escherichia coli* MoeB and Its Involvement in the Activation of Molybdopterin Synthase for the Biosynthesis of the Molybdenum Cofactor*

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Silke Leimkühler, Margot M. Wuebbens, and K. V. Rajagopalan‡

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Amino acid sequence comparisons of *Escherichia coli* MoeB suggested that the MoeB-dependent formation of a C-terminal thiocarboxylate on the MoaD subunit of molybdopterin synthase might resemble the ubiquitin-activating step in the ubiquitin-targeted degradation of proteins in eukaryotes. To determine the exact role of MoeB in molybdopterin biosynthesis, the protein was purified after homologous overexpression. Using purified proteins, we have demonstrated the ATP-dependent formation of a complex of MoeB and MoaD adenylate that is stable to gel filtration. Mass spectrometry of the complex revealed a peak of a molecular mass of 9,073 Da, the expected mass of MoaD adenylate. However, unlike the ubiquitin activation reaction, the formation of a thioester intermediate between MoeB and MoaD could not be observed. There was also no evidence for a MoeB-bound sulfur during the sulfuration of MoaD. Amino acid substitutions were generated in every cysteine residue in MoeB. All of these exhibited activity comparable to the wild type, with the exception of mutations in cysteine residues located in putative Zn-binding motifs. For these cysteines, loss of activity correlated with loss of metal binding.

In *Escherichia coli*, several loci (*moa*, *mob*, *mod*, *moe*, and *mog*) have been implicated in the pleiotropy of the molybdenum enzymes. With the exception of *mod*, all of these are involved in the biosynthesis of the molybdenum cofactor (1). Molybdenum cofactor contains a tricyclic pterin derivative termed molybdopterin (MPT) that bears the cis-dithiolene group essential for molybdenum ligation. The dithiolene group is generated by MPT synthase, a heterotetrameric protein consisting of two large MoaE subunits (16.9 kDa) and two small MoaD subunits (8.8 kDa) (2). Mass spectrometry has identified a thiocarboxylate in the activated form of MoaD that serves as MoaD subunits (8.8 kDa) (2). Mass spectrometry has identified proteins, we have demonstrated the ATP-dependent formation of a complex of MoeB and MoaD adenylate that is stable to gel filtration. Mass spectrometry of the complex revealed a peak of a molecular mass of 9,073 Da, the expected mass of MoaD adenylate. However, unlike the ubiquitin activation reaction, the formation of a thioester intermediate between MoeB and MoaD could not be observed. There was also no evidence for a MoeB-bound sulfur during the sulfuration of MoaD. Amino acid substitutions were generated in every cysteine residue in MoeB. All of these exhibited activity comparable to the wild type, with the exception of mutations in cysteine residues located in putative Zn-binding motifs. For these cysteines, loss of activity correlated with loss of metal binding.

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Significant sequence similarities between MoeB and a number of other proteins have been identified (6). Particularly noteworthy is the amino acid sequence identity of 23% between MoeB and the eukaryotic ubiquitin-activating enzyme E1, encoded by *Uba1* (5). As part of the process for ubiquitin-targeted degradation of proteins, Uba1 was shown to activate ubiquitin in an ATP-dependent reaction with the initial formation of an Uba1-ubiquitin adenylate complex. This reaction is followed by generation of a thioester linkage between the C-terminal glycine of ubiquitin and a cysteine residue of Uba1 (7). Ubiquitin is subsequently transferred to one of the ubiquitin carrier proteins (ubiquitin carrier protein (E2)/ubiquitin-protein isopeptide ligase (E3)) before its attachment to the target protein by the formation of an isopeptide bond with a lysine residue.

The functional similarity of MoeB to Uba1 (5, 6, 8) and of MPT synthase to ubiquitin (2) has led to the proposal that the mechanism of activation of MPT synthase by MoeB might resemble the process of ubiquitin activation by Uba1. It has been proposed that the initial formation of a MoaD-adenylate leads to a thioester linkage with MoeB before sulfuration of the C-terminal carboxyl group of MoaD to create the reactive thio-carboxylate (5, 6). Significantly, MoeB contains several conserved cysteine residues, one of which has been postulated to be involved in the formation of a thioester linkage between MoaD and MoeB (5, 6, 8). It has been shown recently that the physiological sulfur donor for the formation of the dithiolene group of MPT is likely to be L-cysteine and that a NifS-like protein is involved in the mobilization and transfer of sulfur from cysteine to MPT synthase (9).

This article describes the characterization of the mechanism of action of *E. coli* MoeB. Studies on cysteine to alanine mutants of MoeB revealed that all of them retained activity, with the exception of mutations in the cysteine residues of the two conserved Zn-binding sequences. No evidence was found for the formation of a thioester intermediate between MPT synthase and MoeB or for the covalent binding of the cysteine-derived sulfur to MoeB. We propose a model for the role of MoeB in the activation of MPT synthase in which MoeB is only involved in the formation of an acyl-adenylate of MoaD, a mechanism that only partially resembles the first step of the ubiquitin-targeted degradation of proteins in eukaryotes.
EXPERIMENTAL PROCEDURES

**Bacterial Strains, Media, and Growth Conditions**—*E. coli* moeB(DE3) and moaD−mutant strains used in this study are isogenic mutants described previously (9, 10). *E. coli* BL21(DE3) cells and pET15b were obtained from Novagen. Cell strains containing expression plasmids were grown aerobically at 30 °C in LB medium in the presence of 150 μg/ml ampicillin or 50 μg/ml carbenicillin. Superdex 75, Superose 12, Phenyl-Sepharose, and Q-Sepharose resins were purchased from Amersham Pharma Biotech, and Q-Sepharose resin was purchased from Sigma. Inductively coupled plasma emission spectroscopy was performed by Garratt Callahan Company (Millbrae, CA).

**Purification of the Reaction Components**—Precursor Z was isolated from *E. coli* moeB−cells by high-performance liquid chromatography (HPLC) using reverse phase and anion exchange columns (11). Cloned MPT synthase was expressed from a pET15b vector (Novagen) in *E. coli* moeB(DE3) cells, and the protein was purified by ammonium sulfate precipitation and gel filtration as described by Rudolph et al. (2). *E. coli* cysteine sulfinate desulfinase (CSD) was expressed in a pET15b vector and purified by nickel-nitriothiocetic acid chromatography (9).

**Cloning, Expression, and Purification of Wild Type MoeB**—The gene encoding *E. coli* moeB was cloned from genomic DH5α DNA by polymerase chain reaction. The published gene sequence (12) was used to design primers that permitted cloning into the *Nco*I and *Ban*HI sites of the multiple cloning region of the pET15b expression vector. The resulting plasmid was designated pMW15eB. For expression, 6-liter cultures of *E. coli* BL21(DE3) cells transformed with pMW15eB were grown in LB medium containing carbenicillin to an A600 of 0.6. The cultures were then induced by the addition of 0.1 mM isopropyl-β-d-thiogalactopyranoside. After 4 h of aerobic growth at 30 °C, the cells were harvested, resuspended with a total of 60 ml of 50 mM Tris, 2 mM EDTA, pH 8.0, and frozen at −20 °C.

The cell suspension was lysed by two passes through a French pressure cell, and the resulting extract was centrifuged at 17,000 × g. All subsequent steps were carried out in buffers containing 5 mM DTT. The volume of the extract was increased by the addition of 0.1 mM isopropyl-β-d-thiogalactopyranoside. After centrifugation, solid ammonium sulfate (243 g/liter) was slowly added to the supernatant. The precipitate was removed by centrifugation, and a second aliquot of ammonium sulfate (48.5 g/liter) was added to the supernatant. After centrifugation, the pellet was suspended in 50 mM Tris, pH 8.0, and dialyzed overnight against the same buffer. The dialyzed sample was applied to a Q-Sepharose column (25-ml bed volume) equilibrated with 50 mM Tris, pH 8.0, and dialyzed overnight against the same buffer. The dialyzed sample was applied to a Q-Sepharose column (25-ml bed volume) equilibrated with 50 mM Tris, pH 8.0, and MoeB was eluted with a linear gradient of 0.25–0.7 M NaCl using an Amersham Pharmacia Biotech FPLC system. Fractons containing MoeB were pooled and dialyzed overnight against 50 mM Tris, pH 7.5. The sample was then divided in half, and each half was brought to 15% saturation with ammonium sulfate immediately before injection onto a Phenyl-Sepha-
rose C18 HPLC column equilibrated with the same buffer. MoeB was subsequently eluted from the column with 15% to 0% saturated ammonium sulfate gradient. After concentration, the protein was chromatographed on a 100-ml Superose 12 column. Fractions containing pure MoeB were pooled and dialyzed against 25 mM Tris, 10 mM NaCl, and 5 mM DTT, pH 7.5.

**Site-directed Mutagenesis of MoeB**—The transformer site-directed mutagenesis kit (CLONTECH) was employed for the generation of the single and double amino acid substitutions C44A, C128A, C128Y, C142A, C172A, C175A, C187A, C142A/C187A, C231A, C244A, and C247A within MoeB expressed from pMW15eB. The nucleotide sequence substitutions were verified by automated sequencing. *E. coli* moeB(DE3) cells containing the mutant forms of pMW15eB were grown in 3 liters of LB medium containing ampicillin and induced with isopropyl-β-d-thiogalactopyranoside as described for the wild type. For the purification of the MoeB variants, the cell pellet was resuspended in 50 mM Tris and 1 mM EDTA, pH 7.5, and lysed as described above. After streptomyacin sulfate treatment, each MoeB variant was precipitated by the addition of 277 g/liter ammonium sulfate. After centrifugation, the protein was resolubilized and dialyzed against 50 mM Tris, 1 mM EDTA, and 5 mM DTT, pH 7.5. The dialyzed sample was applied to a 25-ml Q-Sepharose FPLC column equilibrated with 50 mM Tris, 1 mM EDTA, and 5 mM DTT, pH 7.5, and the mutant protein was eluted with a linear gradient of 0–1 M NaCl. The pool of fractions containing MoeB was concentrated to 1 ml and chromatographed on a Superose 12 column equilibrated and eluted with 50 mM Tris, 1 mM EDTA, 5 mM DTT, and 100 mM NaCl, pH 7.5.

**In Vitro Activation of Inactive MPT Synthase with MoeB**—For the in vitro formation of MPT, inactive MPT synthase, MoeB, MgCl2, ATP, precursor Z, and CSD-bound persulfide were incubated in a total volume of 400 μl of 100 mM Tris, pH 7.2, as described previously (9). For the generation of CSD-bound persulfide, CSD was incubated for 5 min with 1-cysteine at 4 °C, followed by passage through a PD10 gel filtration column (Amersham Pharmacia Biotech) to remove excess L-cysteine as described by Leimkühler and Rajagopalan (9). In vitro production of MPT was quantitated by conversion to form A, its stable, fluorescent degradation product. For this conversion, the incubation mixtures were adjusted to pH 2.5 and excess iodine was added (13, 14). Form A was quantitated by subsequent HPLC analysis with an Alltech C18 HPLC column equilibrated with 50 mM ammonium acetate and 10% methanol, pH 6.8 (9).

**Polycyramide Gel Electrophoresis**—For polycrylamide gel electrophoresis, eluates of a Superdex 75 gel filtration column were heated at 95 °C in buffer containing 2% SDS and 5% β-mercaptoethanol. Perfect Protein Markers from Novagen were used as molecular mass standards. Electrophoresis was carried out on 15% polyacrylamide Ready Gels (Bio-Rad), and the gels were stained with Coomassie Brilliant Blue R (Sigma).

**Mass Spectrometry**—Mass spectrometric data were acquired on a Micromass Quattro LC triple quadrupole mass spectrometer (Altricham) equipped with a pneumatically assisted electrospray ion source operating at atmospheric pressure and in a positive ion mode. The protein samples in 25 mM Tris, pH 7.2, were analyzed in 50% aqueous acetonitrile containing 1% formic acid by loop injection into a stream of 50% aqueous acetonitrile flowing at 10 μl/min. The native protein samples were electrosprayed in 0.01M ammonium acetate. Spectra were acquired in the multichannel analyzer mode from m/z 1000–1800 (scan time, 5 s). The mass scale was calibrated using the multiply charged envelope of myoglobin (16,951.48 Da). The raw mass spectra were transformed to a molecular mass scale using a maximum entropy-based method (MaxEnt) that uses the MemSys5 program (MaxEnt Solutions Ltd., Cambridge, United Kingdom) and is part of the MassLynx software suite.

**RESULTS**

**Expression and Purification of MoeB**—Homologous expression of MoeB cloned from genomic *E. coli* DNA yielded a protein with an approximate monoclonic mass of 25 kDa as the major soluble protein after cell lysis (Fig. 1). This value corresponds closely to the calculated molecular mass of 26,719 Da for MoeB. The protein was purified by fractionated ammonium sulfate precipitation followed by chromatography on Q-Sepharose, phenyl-Sepharose, and Superose 12 columns as shown in Fig. 1. By this procedure, ~50 mg of MoeB can be obtained from...
MoeB cysteine mutants generated in this report are indicated with 
boxed acids are 
boxed.

Aspergillus nidulans 
mutation of residue Cys-263 in 
an alanine (Fig. 1). Appleyard 
estasis was performed to replace each MoeB cysteine residue with 
putative thioester linkage with MoaD, site-directed mutagen-
thesis during heterocyst formation (16), and 
Saccharomyces 
Uba4, a protein sharing similarities to the ubiquitin-
cerevisiae 
conserved in ThiF, HesA, and 
Uba4 (Fig. 2) but are not present 
E. coli 
comparison of the amino acid sequences of 
proteins.

Comparison of the amino acid sequences of E. coli MoeB, 
E. coli ThiF, Anaabaena HesA, and 
CnxF (the 
metal-binding 
motif (6). 

a 6-liter E. coli culture. After Superose 12 gel filtration chromatography, the purified protein displayed a single band on 
Coomassie Brilliant Blue R-stained SDS gels, and gel filtration 
experiments showed that in its native state, MoeB is a dimer with a molecular mass of about 52 kDa (data not shown).

Site-directed Mutagenesis of the E. coli MoeB Protein—From 
the amino acid sequence alignment shown in Fig. 2, it can be 
seen that E. coli MoeB shares significant sequence identities with 
E. coli ThiF, a protein involved in thiamine biosynthesis (15), Anaabaena HesA, a protein involved in Fe/S protein synthesis 
during heterocyst formation (16), and 
Saccharomyces cerevisiae Uba1 (8). These proteins and S. cerevisiae 
Uba1 from a 
E. coli moeB strain.

The sequence similarity of 
E. coli 
variants were expressed and purified from an E. coli moeB 
strain. 
The activities of the purified proteins were analyzed by their 
ability to activate inactive MPT synthase in vitro in mixtures 
containing precursor Z, Mg-ATP, and CSD-bound persulfide 
(9). CSD, described as sulfinate desulfinase (17), has been 
shown to form an internal persulfide group in CSD were carried out as 
described by Leinkühler and Rajagopalan (9).

The sequence similarity of 
E. coli 
variants but are also highly 
conserved in ThiF, HesA, and 
Uba4 (Fig. 2) but are not present in 
Uba1 (8).

The sequence similarity of 
E. coli 
variants identified near the C terminus of MoeB, which are also highly 
conserved in ThiF, HesA, and Uba4 (Fig. 2) but are not present 
in Uba1 (8).

Several conserved cysteine residues have been identified in the 
E. coli 
protein (Fig. 2). To ascertain which E. coli 
MoeB cysteine residue was involved in the formation of the 
putative thioester linkage with MoaD, site-directed mutagen-


![Fig. 2. Amino acid sequence comparison of MoeB and related proteins. Comparison of the amino acid sequences of E. coli MoeB, E. coli ThiF, Anaabaena HesA, and S. cerevisiae Uba4. Identical amino acids are boxed. Conserved cysteine residues are shaded in gray. The MoeB cysteine mutants generated in this report are indicated with small arrows.](http://www.jbc.org/)

Fig. 3. Analysis of the ability of MoeB variants to activate MPT synthase in vitro. Formation of MPT was measured after the in vitro incubation of 3.5 μM Moeb or a MoeB variant, 5.4 μM inactive MPT synthase, 4.2 μM CSD-bound persulfide, 2.5 mM Mg-ATP, and 200 μM precursor Z. Quantitation of the amount of MPT produced in vitro and generation of an internal persulfide group in CSD were carried out as described by Leinkühler and Rajagopalan (9).

| MoeB variant | Zn content (%) |
|--------------|----------------|
| MoeB         | 75             |
| C172A        |                |
| C175A        |                |
| C244A        |                |
| C247A        |                |

Table I Analysis of the Zn content of MoeB and MoeB variants by inductively coupled plasma emission spectroscopy

- Inductively coupled plasma emission spectroscopy was performed by Garrat Callahan Company.
- Below the limit of detection.

For the in vitro production of MPT, the assays contained 
precursor Z, inactive MPT synthase, CSD-persulfide, Mg-ATP, and wild type or mutated MoeB. As shown in Fig. 3, the majority of the MoeB mutants exhibit activities comparable to that of wild type MoeB. The exceptions are mutations C172A, C175A, C244A, and C247A in the highly conserved Zn-binding motifs. Because all other cysteine to alanine mutations showed 
MPT production equivalent to that of the wild type, it can be concluded that none of the cysteine residues within MoeB are required for the sulfur transfer reaction for the activation of 
MPT synthase. The C128Y variant exhibited no activity as seen
for the equivalent mutant in A. nidulans (8). However, because the C128A mutant was completely active, it can be postulated that insertion of a tyrosine at this position disrupts the structure of the protein.

Inductively coupled plasma emission spectroscopy was performed on purified MoeB and the mutated variants C172A, C175A, C244A, and C247A to determine the Zn content of the proteins. Table I shows that whereas the wild type protein contains a near stoichiometric amount of Zn, no Zn was identified in the mutated variants analyzed. These results agree with the previous suggestion that the two conserved CXXC motifs are responsible for the binding of Zn (5). The residual activities exhibited by the C244A and C247A variants (Fig. 3) are probably a reflection of the traces of other metals (e.g. Cu) found in the purified proteins. This suggests that other metals may be able to at least partially fulfill the role of Zn in the activity of MoeB.

**TABLE II**

| Treatment                             | Production of MPT (nmol) |
|---------------------------------------|-------------------------|
| MoeB + sulfide                        | 0                       |
| MoeB + sulfide + Mg-ATP               | 0                       |
| MPTS + sulfide + Mg-ATP               | 0                       |
| MoeB + MPTS + sulfide                 | 0                       |
| MoeB + MPTS + sulfide + Mg-ATP        | 1283                    |

*82 μM MoeB, 34 μM MPT synthase, 50 mM sodium sulfide, or 20 mM Mg-ATP was incubated for 30 min at room temperature. After gel filtration, 200 μM precursor Z, 2.7 μM MPT synthase, or 2.5 mM Mg-ATP was added and incubated for 30 min at room temperature before the addition of acidic iodine. The amount of MPT produced in vitro was determined as described by Leimkühler and Rajagopalan (9).*

**Fig. 4.** Identification of a MoeB-MoaD adenylate complex by SDS-PAGE analysis of gel filtration eluates. A mixture of 130 μM MoeB and 74 μM inactive MPT synthase was incubated (A) with 10 mM Mg-ATP and 25 units of inorganic pyrophosphatase (Sigma), (B) without any additions, (C) with 10 mM Mg-ATP and 40 mM sodium pyrophosphate, and (D) with 10 mM Mg-ATP, 25 units of inorganic pyrophosphatase, and 50 mM sodium sulfide. The mixtures were incubated for 30 min at room temperature before they were applied to a Superdex 75 FPLC gel filtration column. Appropriate fractions were then subjected to SDS-PAGE on 15% polyacrylamide gels. The positions of MoeB, MoaD, and MoaE are indicated on the right.
filtration, MPT was formed after the addition of precursor Z (Table II). In addition, the sulfide-dependent activation of MPT synthase by MoeB is ATP-dependent since MPT formation was only observed when Mg-ATP was included in the mixture before gel filtration (Table II).

Purification of the MoeB-MoaD Adenylate Complex by Gel Filtration—The data presented above suggested that MoeB itself is not sulfurated during the sulfur transfer reaction for the activation of MPT synthase and that its role is limited to...
Involvement of MoeB in the Biosynthesis of MPT

generation of the acyl adenylate of MoaD. To purify the proposed adenylate complex between MoeB and MPT synthase, MoeB was incubated with inactive MPT synthase and Mg-ATP for 30 min at room temperature before the mixture was applied to a gel filtration column, and the eluting fractions were analyzed by SDS-PAGE. Fig. 4A shows that MoeB and MoaD coelute and are well separated from the MoaE subunit during gel filtration, indicating that the MoaD subunit is released from the MPT synthase tetramer during the formation of the MoeB-MoaD adenylate complex. The clear separation of the MoeB-MoaD adenylate complex from the 33.9-kDa MoaE dimer indicates that the complex is a tetramer with a mass of 71.8 kDa. We propose that the two MoaD bands visualized in SDS-PAGE (Fig. 2A) represent the difference in molecular mass between the adenylated (9.1 kDa) and the nonadenylated (8.8 kDa) forms of MoaD. In the absence of Mg-ATP, no separation of the MoaD subunit from the MoaE subunit was observed, revealing that the formation of the MoeB-MoaD complex is ATP-dependent (Fig. 4B). Because of the similar molecular masses of the MPT synthase tetramer (51.6 kDa) and the MoaD dimer (53.4 kDa), these proteins have similar elution profiles during gel filtration (Fig. 4B).

To obtain further evidence for the formation of the MoeB-MoaD adenylate complex, the effect of excess pyrophosphate on the formation of the complex was examined. For this purpose, MoeB, MPT synthase, and Mg-ATP were incubated for 15 min at room temperature before the addition of pyrophosphate. The mixture was incubated for another 15 min before it was applied to a gel filtration column. As shown in Fig. 4C, the inclusion of pyrophosphate in the ATP-containing incubation mixture gave an elution profile virtually identical to that observed from an incubation mixture containing no ATP. This indicates that the MPT synthase tetramer was reformed under these conditions. Thus, pyrophosphate is able to reverse the formation of the MoeB-MoaD adenylate complex by reversing the adenylation step. In accordance with the ability of sulfide to serve as the in vitro sulfur donor for the production of the activated MPT synthase (shown above), the addition of sulfide to the incubation mixture consisting of MoeB, MPT synthase, and Mg-ATP also resulted in the breakage of the adenylate bond between MoaD and MoeB (Fig. 4D). After the formation of the C-terminal MoaD thiocarboxylate group, the MPT synthase tetramer is formed and thus is not well separated from the MoeB dimer by gel filtration. (Fig. 4D).

The MoeB-MoaD Adenylate Complex Forms MPT in the Absence of Added Mg-ATP—In extension of these findings, it was of further interest to examine the ability of the gel-filtered MoeB-MoaD adenylate complex to form MPT in vitro in the absence of Mg-ATP. For this purpose, a sample of gel-filtered MoeB-MoaD adenylate complex including the MoaE dimer was incubated with CSD, L-cysteine, and precursor Z. After acidic iodine treatment of the mixture, form A was readily detected (Fig. 5A), indicating that the MoeB-MoaD adenylate complex does not require the presence of additional Mg-ATP for the conversion of precursor Z to MPT. As expected, when MoeB and MPT synthase were incubated without the addition of Mg-ATP before gel filtration, no MPT was formed (Fig. 5B). In addition, the pyrophosphate-treated incubation mixture also produced no MPT (Fig. 5C), providing further evidence that the formation of the MoeB-MoaD adenylate complex is reversed by pyrophosphate. As already shown in Table II, the inclusion of sulfide in the incubation mixture results in active MPT synthase capable of converting precursor Z to MPT (Fig. 5D).

Mass Spectrometry of the Adenylated MoaD Subunit—To obtain direct evidence for the MoeB-catalyzed formation of MoaD adenylate and the sulfide-dependent generation of MoaD thiocarboxylate from the adenylate, the reaction mixtures were analyzed by mass spectrometry. The mass spectrum of an incubation mixture of MoeB with inactive MPT synthase in the presence of Mg-ATP yielded masses for MoaE and MoeB of 16,852 and 26,563 Da, respectively (data not shown). Two other peaks with molecular masses of 8,744 and 9,073 Da were also observed (Fig. 6A). Whereas the 8,744-Da peak corresponds to the inactive form of MoaD with the C-terminal carboxyl group, the 9,073-Da peak corresponds to the adenylated form of MoaD. The difference in molecular mass of 329 Da corresponds exactly to the expected molecular mass of the adenylate group. However, the majority of MoaD was determined to be present in its inactive nonadenylated form, indicating that the MoaD-adenylate complex is not stable and that the majority of the complex is hydrolyzed under the acid conditions used during mass spectrometry. These data validate the conclusion that the two MoaD bands seen in Fig. 2A represent free MoaD and its acyl adenylate. The acyl adenylate is stable when bound to MoeB but is susceptible to hydrolysis upon release.

The mass spectrum of the reaction mixture incubated in the absence of ATP provided further support that the 9,073-Da species is the adenylated form of MoaD. Fig. 6B shows clearly that only the 8,744-Da peak of inactive MoaD was present in this incubation mixture. However, after the addition of sulfide to a mixture of inactive MPT synthase, MoeB, and Mg-ATP, the molecular mass of the major peak was determined to be 8,759.5 Da (Fig. 6C). The 16-Da increase in the mass of MoaD corresponds to that expected for the substitution of a single sulfur for an oxygen, as reported previously for activated MPT synthase (4). The complete absence of the 8,744-Da peak in this incubation mixture shows that the addition of sulfide converts all of the MoaD to the thiocarboxylated species. In contrast, no thiocarboxylated MoaD is formed when inactive MPT synthase, MoeB, and sulfide are incubated in the absence of Mg-ATP (Fig. 6D). The data presented in Fig. 6C also show that in the presence of Mg-ATP, all of the MoaD in the mixture was present as the adenylate since, as shown in Fig. 6D, sulfide does not form thiocarboxylate from nonadenylated MoaD. In sum, the results clearly show that in the presence of Mg-ATP, MoeB catalyzes the formation of a MoaD adenylate complex with a molecular mass of 9073 Da that can be detected by mass spectrometry.

DISCUSSION

The fact that E. coli moeB mutant strains contain an inactive, desulfo form of MPT synthase led to the proposal that MoeB is involved in sulfuration of MPT synthase. Accordingly, MoeB has been designated as MPT synthase sulfursase (4, 5). The sequence homology between MoeB and the ubiquitin-activating enzyme Uba1 (5) and the structural similarity between MoaD and ubiquitin (2) suggested that MoeB might catalyze the adenylation of MoaD, followed by the formation of a thioester linkage between

FIG. 7. Model for the involvement of MoeB in the activation of MPT synthase.
the two proteins. Cleavage of this bond by sulfur would then lead to the formation of the reactive thio-carboxylate group of MoaD that is required for MPT biosynthesis (5, 6, 8). In general, E1-like enzymes of the ubiquitin pathway in eukaryotes contain an active site cysteine residue involved in the formation of the thioester linkage to ubiquitin (6).

The present studies on the mechanism of action of the E. coli MoeB protein show that MoeB binds the MoaD subunit of MPT synthase and catalyzes the formation of an adenylation MoaD species, activating the C-terminal glycine carboxylate group for thiolation. Based on the data obtained from cysteine to alanine substitutions within MoeB, we suggest that MoeB itself is not a carrier of sulfur in the sulfur transfer process for the formation of the thio-carboxylate group in MoaD. Residue Cys-187 in E. coli MoeB corresponded to the location of a possible active site cysteine residue (6). However, analysis of the activity of the generated MoeB variants C187A and C142A/C187A showed that these exhibited an in vitro enzyme activity comparable to that of the wild type protein, excluding the possibility of involvement of Cys-187 in the sulfur transfer reaction. To mimic the C263Y mutant in A. nidulans cmnF (8), the equivalent E. coli MoeB residue Cys-128 was mutated to tyrosine or alanine. Whereas the C128Y mutation inactivated MoeB completely, the C128A mutation retained full activity, indicating that Cys-128 is not essential for enzyme activity and that substitution with a tyrosine disrupts the structure of the protein in a manner resulting in loss of protein activity. Substitution of all other MoeB cysteine residues with alanine generated variants that retained full enzyme activity, with the exception of cysteine to alanine substitutions in the two CXXC Zn-binding motifs, which abolished the ability of MoeB to bind Zn. Although the exact role of Zn for MoeB function is unknown, it is likely that the metal is primarily involved in structural stabilization of the protein as opposed to playing a direct role in enzyme catalysis, a statement supported by the observation that other metals can partially replace Zn for enzyme activity. In addition, the structure of the heterotetrameric MoeB-MoaD adenylyl complex has been recently solved.\(^2\) The crystal structure revealed that the Zn-binding site is quite distant from the active site, supporting the suggestion of a structural role for the metal. In agreement with our data, the most striking feature of the structure is the presence of a MoaD acyl adenylate at the active site. Formation of a thioester intermediate was also not observed.

MoeB shares significant sequence similarities to the ThiF protein in the E. coli thiamine biosynthetic pathway. In this pathway, ThiF, ThiS, ThiI, and IscS participate in the generation of the thiazole moiety of thiamine, and l-cysteine has been identified as the physiological sulfur donor for the thiolation of MPT (15). ThiS thiocarboxylate synthesis requires adenylation by ThiF (19), and its C-terminal thiocarboxylate acts as the direct sulfur donor for the formation of the thiazole thio-carboxylate moiety. ThiS thio-carboxylate synthesis requires adenylation by ThiF (20), and ThiI appears to act as a sulfurtransferase, accepting sulfur from IscS and transferring it further on to ThiS (15). During the generation of ThiS thio-carboxylate, sulfur transfer to ThiF was not observed (18). Given the mechanistic similarities between the synthesis of the thiazole moiety of thiamin and the dithiolene group of MPT, the pathway of sulfur transfer in both systems is likely to be similar. Recently, our laboratory reported that l-cysteine is the likely physiological sulfur donor for the formation of the dithiolene group of MPT and that NiS-like sulfurtransferases are capable of mobilizing sulfur from cysteine for the sulfuration of MPT synthase in an in vitro system (9). However, in contrast to the role of IscS in thiamine biosynthesis (18), the protein is not essential for the biosynthesis of MPT since an iscS mutant strain produces active MPT synthase (9). Unlike the thiamine pathway, in which Thi appears to act as an intermediary between IscS and ThiS, no such requirement has been identified for the biosynthesis of MPT.

In summary, the data presented above indicate that the transfer of sulfur for the activation of MPT synthase proceeds as shown in Fig. 7. MoeB activates MPT synthase by adenylylating the C-terminal carboxylate group of MoaD. During this process, MoaD must dissociate from its complex with MoaE to form a stable adenylyl complex with MoeB (Fig. 5A). In the adenylyl complex, MoaD is susceptible to sulfuration, which most likely proceeds in vivo by the action of a NiS-like sulfurtransferase (9), transferring sulfur from l-cysteine to MoaD. However, it is not yet known how the sulfurtransferase interacts with the MoeB-MoaD complex to perform the sulfur transfer reaction. The nonspecificity of this reaction is somewhat surprising. In vitro, inorganic sulfide is able to serve the same function, suggesting the possibility that nascent sulfide is produced in the active site for addition to the activated MoaD intermediate. After the formation of the reactive MoaD thio-carboxylate group, MoaD thio-carboxylates dissociates from MoeB and reassociates with MoaE to form active MPT synthase, which is able to convert precursor Z to MPT. These observations suggest that the partitioning of MoaD between MoaE and MoaB is governed by the carboxylate versus thio-carboxylate status of the C-terminal Gly of MoaD. The basis for this selectivity is under investigation.

The data presented here show that the interaction of MoeB with MoaD resembles only the first step of the ubiquitin-targeted degradation of proteins in eukaryotes, i.e. the ATP-dependent activation of ubiquitin by Uba1. The similarity in the mechanism of ATP-dependent co-factor sulfuration and ATP-dependent protein conjugation mirrors the structural similarities seen between the components of the two systems (2, 19). This implies that the eukaryotic system has evolved further to include the formation of thioester intermediates with proteins involved in the transfer of ubiquitin to target proteins.

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