Mechanistic and Mutational Studies of *Escherichia coli* Molybdopterin Synthase Clarify the Final Step of Molybdopterin Biosynthesis*

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Bio synthesis of the molybdenum cofactor, a chelate of molybdenum or tungsten with a novel pterin, occurs in virtually all organisms including humans. In the cofactor, the metal is complexed to the unique cis-dithiolene moiety located on the pyran ring of molybdopterin. *Escherichia coli* molybdopterin synthase, the protein responsible for adding the dithiolene to a desulfos precursor termed precursor Z, is a dimer of dimers containing the MoaD and MoaE proteins. The sulfur used for dithiolene formation is carried in the form of a thio carbamoyltransferase at the MoaD C terminus. Using an intein expression system for preparation of thio carbamoylated MoaD, the mechanism of the molybdopterin synthase reaction was examined. A stoichiometry of 2 molecules of thio carbamoylated MoaD per conversion of a single precursor Z molecule to molybdopterin was observed. Examination of several synthase variants bearing mutations in the MoaE subunit identified Lys-119 as a residue essential for activity and Arg-39 and Lys-126 as other residues critical for the reaction. An intermediate of the synthase reaction was identified and characterized. This intermediate remains tightly associated with the protein and is the predominant product formed by synthase containing the K126A variant of MoaE. Mass spectral data obtained from protein-bound intermediate are consistent with a monosulfurated structure that contains a terminal phosphate group similar to that present in molybdopterin.

Molybdopterin (MPT)1 syntheses catalyze the final step in the biosynthesis of MPT, the metal-binding organic portion of the molybdenum cofactor (Moco). Subsequent attachment of molybdenum or tungsten to MPT, with or without additional modifications, produces the various members of the Moco family (1, 2). These cofactors are present in a large family of diverse enzymes involved in electron transfer reactions (3). Although various dinucleotide derivatives of Moco have been identified, in all cases, it is the unique cis-dithiolene moiety of MPT that chelates molybdenum or tungsten within Moco. Genes encoding highly homologous proteins involved in MPT biosynthesis have been identified in virtually all organisms from archaea to humans, indicating that the biosynthetic pathway for MPT is highly conserved (2, 4). In *Escherichia coli*, biosynthesis of MPT begins with a guanosine derivative that is converted to the pterin intermediate, precursor Z, through the action of the moaA and moaC gene products (5, 6). Subsequent conversion of precursor Z to MPT by MPT synthase involves the addition of the dithiolene sulfur to the C-1′- and C-2′-positions of precursor Z and linearization of its cyclic phosphate.

*E. coli* MPT synthase is a heterotetramer composed of two MoaE (−16,850-Da) subunits and two MoaD (−8750-Da) subunits (7). The sulfur used to form the MPT dithiolene moiety is carried on the MoaD subunit in the form of a C-terminal thio carbamoyltransferase that must be regenerated after each round of MPT biosynthesis (8–10). In the MPT synthase crystal structure, the two MoaE subunits form a central dimer, and the MoaD subunits are located at opposite ends of this dimer. Each MoaD monomer contacts only one of the two MoaE monomers, and the most striking feature of this interaction is the insertion of the C terminus of each MoaD subunit into a pocket in one of the MoaE subunits to form one active site at each MoaE-MoaD interface (7). A preliminary association constant of 2.2 × 1010 M−1 has been obtained by isothermal titration calorimetry experiments for the interaction between MoaD and MoaE.5

MoaD shares a high degree of structural similarity with both ThiS and ubiquitin (11, 12). All three of these proteins contain a C-terminal Gly-Gly motif, where the terminal glycine forms a thio carbamoylate in the case of MoaD and ThiS or a thioester in the case of ubiquitin. Comparison of MoaD sequence conservation with the three-dimensional structure of the protein localized a number of highly conserved MoaE residues to the central cavity that embraces the MoaD C terminus in each half of the MPT synthase tetramer. These include the invariant or highly conserved residues Phe-34, Arg-39, Met-115, Lys-119, Lys-126, and Arg-140 (7). Additionally, loss-of-function mutations in MOCS2B, the human equivalent of MoaE, have been identified in cofactor-deficient patients. Two of these mutations correspond to an E128K substitution and to premature termination at Glu-141 in *E. coli* MoaE (13).

The observation that MPT synthase isolated from *E. coli* moeB− cells is inactive led to the proposal that the MoeB protein is essential for the formation of the MoaD thio carbamoylate and that it might serve as the donor of the dithiolo sulfurs atoms (8). Recent evidence, however, indicates that MoeB does not carry the transferable sulfur atom and that its role in thio carbamoylate regeneration is limited solely to adenylation of the inactivated MoaD C terminus (14, 15). A Nif-S like sulfurltransferase protein is the actual donor of a cysteine-derived sulfur to the activated MoaD C terminus to regenerate the thio carbamoylate (14). Crystal structures of the MoaD-MoeB complex in the apo, ATP-bound, and adenylated MoaD forms

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1 The abbreviations used are: MPT, molybdopterin; Moco, molybdenum cofactor; MoaD-SH, thio carbamoylated MoaD; HPLC, high performance liquid chromatography.

2 H. Schindelin, personal communication.
have been determined (16). The two proteins form a heterotetrameric complex, as the central dimer. The MoaD fold in this complex is quite similar to that observed in the synthase complex, and the MoaD C terminus is again extended into a pocket on the Moeb surface to form the active site. Thus, MoaD is capable of forming two different stable, yet reversible, heterotetrameric complexes that perform biochemically distinct reactions involving the C terminus of MoaD.

Mutations causing a defect in any step of molybdenum cofactor biosynthesis result in the combined loss of activities of all cofactor-containing enzymes. In humans, cofactor deficiency is a rare inborn disease that generally results in death in early infancy (17). Whereas the activities of all three human cofactor-containing enzymes (aldehyde oxidase, xanthine oxidase, and sulfite oxidase) are impaired by cofactor deficiency, the devastating consequences of the disease can be traced to the loss of sulfite oxidase activity, since isolated sulfite oxidase deficiency produces similarly dire consequences. Mutations in either MPT synthase subunit or in Moeb result in accumulation of precursor Z within the affected organism. This has been observed in bacteria (18), humans (19), and Neurospora (20), and such mutants can serve as experimental sources of precursor Z (21).

Early attempts to study the E. coli MPT synthase reaction were hampered by the fact that enzyme generated by coexpression of the MoaD and MoaE subunits is almost completely inactive. Co-expression of the subunits with the E. coli Moeb protein resulted in synthase that was maximally 50% activated, and it was such a sample that was employed for the initial crystallographic studies of holo-MPT synthase (7). In vitro production of fully activated synthase was recently accomplished using an intein-based expression system to produce activated MoaD monomer (MoaD-SH) (10). This method was originally employed for the generation of activated ThiS, the MoaD-equivalent protein in the thiamine biosynthetic pathway (22). Using this system for MoaD-SH expression, Gutzke et al. (10) demonstrated the both MoaD and MoaD-SH form the MPT synthase heterotetrameric complex upon incubation with MoaE and that the MPT synthase formed with MoaD-SH is capable of converting precursor Z to MPT in vitro.

With the intein system as a source of MoaD-SH, we have further investigated the MPT synthase reaction using an in vitro, three-component assay consisting of MoaE, MoaD-SH, and precursor Z. By varying the ratios of the assay components, a stoichiometry of 2 molecules of MoaD-SH for the conversion of each precursor Z molecule to MPT was established. This assay was also used to explore the role of specific MoaD residues in the reaction. When taken together with the crystallographic data for MPT synthase and MoaE presented in the accompanying paper (23), these results further an understanding of the mechanism of the E. coli MPT synthase reaction.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—The genes encoding E. coli MoaD and MoaE were cloned by PCR from pSJE100 using the published sequence for the plasmid (24). PCR primers were designed to allow cloning into the Ncol and BamHI sites of the multiple cloning region of the pET15b expression vector (Novagen) to generate pMW15aD and pMW15aE, respectively. The following single amino acid substitutions in pMW15aE were then created using the Transformer kit from Clontech: F34A, R39A, M115A, K119A, K126A, E128K, R140A, and E141A. For expression of MoaD-SH, moaD was also cloned into the Ncol and Kpn1 sites of the pTYB3 expression vector (New England Biolabs) using a 3′ PCR primer designed to place the C-terminal glycine of MoaD immediately prior to the intein cleavage site in the vector. The resulting expression vector was designated pMWTY BaD-SH. An expression vector for the 813a truncation variant of MoaD-SH was similarly generated by PCR using a 3′ PCR primer with the appropriate bases deleted. All nucleic acid sequences were verified by automated sequencing. During the course of cloning, the second amino acid of all variants of expressed MoaD was changed from isoleucine to valine.

Expression and Purification of Proteins—For expression of all proteins, 1-liter cultures of E. coli BL21(DE3) cells carrying the appropriate expression plasmid were induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.1 mM when the cultures had attained an A600 of 0.6. Following 4–5 h of aerobic growth at 30 °C, cultures were harvested by centrifugation, suspended in 10 ml of 20 mM Tris-HCl, 2 mM EDTA, pH 8.0, per liter of culture and frozen at –20 °C.

For MoaE and MoaD purification, the cell pellet from 1 liter of the appropriate culture was thawed, passed twice through a French press at 7,000 × g. The supernatant volume was increased to 150 ml with suspension buffer prior to the addition of 16.5 ml of 20% (w/v) streptomycin sulfate, and precipitated nucleic acids were removed by centrifugation at 10,400 × g. For MoaD purification, solid ammonium sulfate (351 g/liter) was slowly added to the cold solution, which was centrifuged prior to the addition of a second aliquot of 33 g/liter ammonium sulfate. The precipitated MoaD was then pelleted by centrifugation. The protein was suspended in and dialyzed overnight against 50 mM Tris-HCl, 50 mM NaCl, pH 8.0. MoaE was precipitated from solution by the addition of 144 g/liter of solid ammonium sulfate and similarly dialyzed. For both MoaD and MoaE, all precipitation and dialysis steps were performed at 4 °C. FInal purification of both proteins was achieved by chromatography on a 100-ml bed volume Superdex 75 (Amersham Biosciences) column equilibrated with dialysis buffer. Final yield for both proteins was ~30 mg/liter of culture.

For MoaD-SH purification, the cell pellets from 6 liters of culture were thawed, lyzed, and centrifuged as described above. The supernatant was combined with 30 ml of chitin affinity resin (New England Biolabs) equilibrated with 20 mM Tris-HCl, 0.5 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, pH 8.0, and stirred for 3 h at 4 °C. The resin was then poured into a column and washed with 150 ml of equilibration buffer followed by 100 ml of a wash buffer containing 20 mM Tris-HCl, 50 mM NaCl, 0.1 mM EDTA, pH 8.0. For MoaD purification, solid ammonium sulfate (351 g/liter) was added to the cold solution, which was centrifuged prior to the addition of a second aliquot of 30 g/liter ammonium sulfate. The precipitated MoaD was then pelleted by centrifugation. The protein was suspended in and dialyzed overnight against 50 mM Tris-HCl, 50 mM NaCl, pH 8.0. MoaD was precipitated from solution by the addition of 144 g/liter of solid ammonium sulfate and similarly dialyzed. For both MoaD and MoaE, all precipitation and dialysis steps were performed at 4 °C. Final purification of both proteins was achieved by chromatography on a 100-ml bed volume Superdex 75 column (Amersham Biosciences) column equilibrated with dialysis buffer. The yield of MoaD-SH was 1.3 mg/liter culture. All protein concentrations were determined based on their calculated extinction coefficients at 280 nm, and purified proteins were exchanged into 0.1 M Tris-HCl, pH 7.2, prior to storage at –80 °C in small aliquots.

Size Exclusion Chromatography—Size exclusion chromatography of the purified proteins was accomplished by high performance liquid chromatography (HPLC) using a Zorbax GF-250 analytical column (Agilent). All HPLC analyses were performed at room temperature with a Hewlett Packard HP1090 liquid chromatograph. Absorbance was measured with an HP10475 diode array detector. The purified MoaD-SH was finally diluted to 30 mg/ml wash buffer and concentrated prior to final purification by chromatography on the Superdex 75 column as described above. The yield of MoaD-SH was 1.3 mg/liter culture. All protein concentrations were determined based on their calculated extinction coefficients at 280 nm, and purified proteins were exchanged into 0.1 M Tris-HCl, pH 7.2, prior to storage at –80 °C in small aliquots.

MPT Synthase Reaction—Precursor Z was purified from E. coli moaD cells and quantitated as previously described (21). Standard MPT synthase reactions were performed at room temperature in a total volume of 0.4 ml of 100 mM Tris, pH 7.2. MoaD-SH and MoaE were combined and preincubated for 10 min on ice and then transferred to the reaction tube. After adding buffer, the reaction was started by precursor Z addition. Whereas the final precursor Z concentration varied from 1 to 12 μM, the values of precursor Z in 10 mM Tris-HCl, pH 8.0, added to the reaction was always less than 50 μM. At the specified time, the reaction was terminated by the addition of 50 μl of acid iodine to convert precursor Z to compound Z and MPT to Form A (25). Both of the latter molecules are stable, fluorescent compounds that can be easily quantitated by HPLC analysis with fluorescent detection.

Temperature for Measurements—10 μM acid iodine was reduced by the addition of 55 μl of 1% ascorbic acid, and the sample was made basic (pH 8–9) with NaOH. The phosphate monooester of Form A was cleaved by the addition of MgCl2 (final concentration of 33 mM) and 3 units of calf intestine alkaline phosphatase (Roche Molecular Biochemicals). Reactions were analyzed by subsequent injection of the elution fraction onto a C-18 reverse phase HPLC column (Alltima, 3 μm, 18×250 mm) equilibrated with 50 mM ammonium acetate containing 10% methanol with a flow rate of 2 ml/min. In-line fluorescence was monitored by an Agilent 1100 series detector with excitation at 295 nm and emission at 448 nm.
RESULTS

It has been demonstrated that both the carboxylated (MoaD) and thiocarboxylated (MoaD-SH) forms of MoaD readily associate with MoaE in vitro to form the heterotetrameric MPT synthase complex but that only the complex containing MoaD-SH is active (8, 10). To examine this association and the MPT synthase reaction further, MoaD-SH was prepared by expressing MoaD with a C-terminal fusion to an intein and a chitin-binding domain following the procedure previously employed for both MoaD and ThiS (10, 22). Induction of intein self-cleavage with ammonium sulfide released MoaD-SH from the chitin affinity resin, and mass spectrometry verified complete incorporation of the C-terminal thiocarboxylate on the purified MoaD-SH (data not shown). An SDS-polyacrylamide gel of the purified proteins used in this work is shown in Fig. 1A, and the absorption spectra of MoaD and MoaD-SH are shown in Fig. 1B. The presence of the thiocarboxylate moiety on MoaD significantly alters its absorption spectrum at lower wavelengths, resulting in a strong increase in absorbance below 280 nm. The spectrum of MoaD-SH was unaltered by either dialysis or passage through a sizing column.

HPLC size exclusion chromatography was employed to verify that the expressed MoaD-SH was capable of forming the MPT synthase complex with MoaE. As seen in Fig. 2C, in the presence of a 0.5 molar ratio of MoaD-SH, half of the MoaE present was shifted into the MPT synthase complex. Although MoaD-SH and MoaE both eluted from the column at ~11 min (Fig. 2, A and B), identification of these peaks was easily accomplished in light of the distinctive nature of the MoaD-SH absorption spectrum (Fig. 1B). Incubation of equimolar amounts of MoaE and MoaD-SH resulted in quantitative formation of the MPT synthase complex (Fig. 2D), and the addition of increasing ratios of MoaD-SH did not alter the elution parameters of the synthase complex peak (Fig. 2, E and F). These results are comparable with those observed previously for MoaD-SH produced by the intein expression method (10), and they confirm the strong propensity of mixtures of MoaD-SH and MoaE to rapidly form the stable &alpha;β₂ MPT synthase complex. The elution position of MoaE in these experiments (Fig. 2, B and C) is clearly not consistent with its molecular weight. Since the observed elution positions of MoaD-SH and MPT synthase were within 20% of that expected by comparison with known protein standards, the behavior of MoaE cannot be attributed to lack of resolution by the HPLC column. Rather, the anomalous elution behavior of MoaE is more likely caused by interactions between the protein and the column matrix involving the hydrophobic surface of MoaE that interacts with MoaD to form the synthase complex (23).

Using purified MoaD-SH and MoaE, the MPT synthase reaction was examined. For these reactions, precursor Z was added to a mixture of the two synthase subunits, and the MPT produced was quantitated by conversion to its fluorescent derivative, Form A. An examination of buffer requirements for the assay revealed that MPT synthase exhibited near maximal activity in a variety of buffers between pH 5.5 and 7.5 but that its activity dropped off sharply above pH 7.5 (data not shown). Time courses for the MPT synthase reaction at MoaD-SH/precursor Z ratios of 1× and 2× are shown in Fig. 3A. From these data, the t₁/₂ for the reaction at room temperature was determined to be 0.24 min for the reaction containing a 2-fold excess of MoaD-SH over precursor Z and 0.30 min for the reaction with equimolar concentrations of MoaD-SH and precursor Z.

Although the rate of MPT production in these two reactions was similar, the maximum amount of MPT produced in the reaction with a 2-fold excess of MoaD-SH was significantly higher than in that containing a stoichiometric amount of MoaD-SH. To explore this difference, MPT production and precursor Z depletion were quantitated in reactions containing
various ratios of MoaD-SH and MoaE relative to precursor Z. For this experiment, MoaD-SH/precursor Z ratios ranging from 0.1 to 2.25 were assayed at MoaE/precursor Z ratios of 0.5, 1.0, and 2.0. Both the decrease in precursor Z (measured as compound Z in Fig. 3B) and the increase in MPT (measured as Form A in Fig. 3C) reached their maximum level at a MoaD/precursor Z ratio of 2, correlating with the addition of two sulfur atoms to each precursor Z side chain. Altering the MoaE/precursor Z ratio from 2 to 0.5 decreased MPT production less than 28% at any MoaD-SH/precursor Z ratio, suggesting that the MoaE subunit acts in a catalytic manner during MPT production.

Standard reaction conditions were employed to evaluate the functionality of MoaE proteins with mutations in invariant or conserved residues. The positions of these residues, all located near the MoaD thiocarboxylate, are illustrated in Fig. 4 along with the locations of two mutations corresponding to ones identified in human MOCS2B. As shown in Table I, when compared with the wild type rate, MPT synthase containing any of the MoaE mutants exhibited an increased \( t_{1/2} \) for MPT production. In the case of the F34A, M115A, and R140A MoaE variants, this increase was less than 5-fold, indicating that mutations at these positions only moderately affected the activity of the resulting MPT synthase. Both of the MoaE mutations corresponding to human mutations exhibited markedly slower rates. MPT synthase containing E128K MoaE was 16.6 times slower than the wild type protein, whereas synthase containing MoaE truncated after Arg-140 was 12.3 times slower. These greatly decreased reaction rates could account for the in vivo loss of function observed in patients with these mutations (13). Con-
version of the invariant, positively charged residues Arg-39 and Lys-126 to alanine severely decreased the rate of MPT production. MoaE containing the R39A mutation was 23.7 times slower than wild type, whereas K126A MoaE was 57.8 times slower. The arginine at position 39 of MoaE has been postulated to stabilize the negatively charged phosphate moiety of precursor Z (23), and the terminal amino group of Lys-126 forms a salt bridge with one of the MoaD terminal oxygens in the MPT synthase crystal structure (7). Thus, it is not surprising that a mutation at either of these positions drastically attenuated the rate of MPT production.

K119A was the only MoaE mutation that completely abolished MPT synthase activity. Lys-119 is an invariant residue located in close proximity to the MoaD C-terminal glycine (Gly-81) in the crystal structure of holo MPT synthase (23). To explore this possibility, an equimolar mixture of G81Δ MoaD-SH and wild type MoaE was also subjected to HPLC size exclusion chromatography. When compared with the peak of the wild type complex in Fig. 5A, the complex formed with G81Δ MoaD-SH in Fig. 5D exhibited a substantial shift in elution position as well as a broadening of peak width. Thus, destabilization of the synthase complex is likely to be a significant contributing factor to the loss of activity observed with this variant.

During the course of the activity experiments described above, variable but minor amounts of a fluorescent peak eluting shortly before the Form A peak were often observed in the HPLC chromatographs. To determine whether this material represented an intermediate in the MPT synthase reaction, the appearance of this peak in reactions containing various ratios of the three components in the wild type synthase reaction was examined. As can be seen in Fig. 6A, this peak was maximal in reactions where the MoaD-SH concentration was lower and the MoaE concentration was higher than the precursor Z concentration, suggesting that it could be derived from an intermediate of the synthase reaction. A minor peak at this position was also observed in chromatographs obtained from synthase reactions containing all of the mutant forms of MoaE with two exceptions. The peak was completely absent from any chromatograph derived from synthase reactions containing K119A MoaE, and large quantities of the material were present in all reactions containing K126A MoaE.

Fig. 6B shows a time course for the reaction of MPT synthase containing K126A MoaE. From this figure, it appears that production of MPT and the putative intermediate increase in parallel and that the concentration of both molecules is maximal after approximately 1 h. However, when the initial time points of this reaction are examined more closely (Fig. 6C), it can be seen that the appearance of the earlier peak precedes the beginning of MPT production, supporting the hypothesis that this peak corresponds to a synthase reaction intermediate. From the absolute fluorescence values shown in Fig. 6B, it appears that MPT production in the K126A synthase reaction (measured as Form A) is 2–3-fold higher than that of the intermediate (measured as the oxidized derivative). However, as seen in Fig. 7A, Form A has a fluorescence/A290 ratio at least 12-fold higher than the oxidized intermediate under the same conditions. Thus, the intermediate, rather than MPT, is the major product of the K126A synthase reaction. Periodate treatment of the oxidized intermediate resulted in its complete conversion to pterin-6-carboxylic acid (data not shown), verifying that the intermediate is a pterin with a 6-alkyl substituent (25, 26) and providing further evidence that the intermediate is related to precursor Z and MPT.

The presence and nature of a phosphate moiety on the oxidized intermediate was examined. In the absence of alkaline phosphatase treatment, none of the fluorescent components produced by iodine oxidation of a K126A MoaE synthase reac-
tion are retained on a C-18 column as indicated by the solid trace in Fig. 7B. However, following alkaline phosphatase treatment of the sample, both the oxidized intermediate and Form A were retained on the reverse phase HPLC column as seen in the dashed trace of Fig. 7B.

As expected, the elution position of compound Z was unaltered, since its cyclic phosphodiester is insensitive to alkaline phosphatase cleavage (27). These results indicate that the intermediate is phosphorylated and that the phosphate is a monoester similar to that present in MPT rather than a cyclic phosphodiester as in precursor Z (25, 27).

The majority of the intermediate formed by the K126A synthase reaction remained protein-bound even after extended incubation times. Since tight binding of MPT to MoaE had been observed previously (9, 28), this result was not completely unexpected. To quantitate the extent of intermediate binding, MPT synthase reactions containing K126A MoaE were passed through a 3-kDa filter, the retained protein samples and filtrates were acid iodine-treated under the same conditions, and the samples were analyzed by HPLC to quantitate the compound Z, oxidized intermediate, and Form A present in each sample. A comparison of these values for the protein and filtrate samples revealed that 72.3 ± 0.4% of precursor Z, 96.4 ± 0.3% of the intermediate, and 89.8 ± 0.4% of the MPT had remained bound to the synthase. Attempts to separate the intermediate from the synthase without oxidizing or otherwise altering its structure were unsuccessful; however, the protein-bound intermediate proved to be amenable to characterization by mass spectrometry. For this analysis, reactions containing either inactive mutant (K126A MoaE-MoaD), active mutant (K126A MoaE-MoaD-SH), or active wild type MPT synthase

| MPT synthase variant | Wild type | MoaE | MoaD |
|----------------------|-----------|------|------|
| α/τ of maximal MPT production (min)<sup>a</sup> | 0.30 | 1.33 | 7.11 | 1.17 | ND<sup>b</sup> | 17.32 | 4.98 | 0.67 | 3.68 | ND |
| α/τ variant/α/τ wild type | 1.00 | 4.43 | 23.70 | 3.90 | 57.73 | 16.60 | 2.23 | 12.27 |

<sup>a</sup> Reaction rates were determined by direct fit of the data to a single exponential.
<sup>b</sup> ND, none detected.
containing no precursor Z. Also present in a control spectrum obtained from a reaction spectra is unrelated to either of these molecules since it was at 356 Da observed in both the precursor Z and intermediate reactions that contained only MPT (data not shown). The peak was observed. This was also the case for the MPT synthase contained MPT, no peak at 394 Da, the expected mass of MPT, precursor Z peaks at 344 and 362. Although this sample also surprising that the intermediate spectrum exhibited minor increase of 34 Da. Since all active K126A synthase reactions lacking precursor Z. The expected negative ion mass for precursor Z is 344, and a peak with this mass was present in the spectrum of the inactive K126A synthase reaction shown in Fig. 8A. A hydrated (+18) derivative of precursor Z at mass 362 was the only other major peak in this sample in the range of 220–410 Da. The mass spectrum of the intermediate-containing reaction, shown in Fig. 8B, exhibited a similar set of peaks 18 Da apart at molecular masses of 378 and 396 Da, indicating that conversion of precursor Z to the intermediate is accompanied by a mass increase of 34 Da. Since all active K126A synthase reactions contain some amount of unreacted precursor Z, it was not surprising that the intermediate spectrum exhibited minor precursor Z peaks at 344 and 362. Although this sample also contained MPT, no peak at 394 Da, the expected mass of MPT, was observed. This was also the case for the MPT synthase reactions that contained only MPT (data not shown). The peak at 356 Da observed in both the precursor Z and intermediate spectra is unrelated to either of these molecules since it was also present in a control spectrum obtained from a reaction containing no precursor Z.

**DISCUSSION**

MPT synthase catalyzes the formation of MPT, the essential building block of the members of the molybdenum cofactor family, and is composed of two evolutionarily conserved sub-units. In *E. coli*, the enzyme is an \( \alpha_2\beta_2 \) heterotetramer of the smaller MoaD and larger MoaE proteins encoded by the last two open reading frames of the *moa* operon with a single base separating the two open reading frames (24). In human MPT synthase, the smaller MOCS2A and larger MOCS2B subunits are encoded on a single bicistronic mRNA (MOCS2), where the open reading frames for the two proteins overlap by 77 nucleotides (29). In its active form, the small subunit of *E. coli* MPT synthase carries a C-terminal thio-carboxylate that serves as the sulfur donor for the formation of the MPT dithiolene moiety. In *vivo* formation of this thio-carboxylate requires the action of two other proteins: MoeB, which preactivates the MoaD C terminus by adenylation (15, 16), and a sulfurtransferase that subsequently transfers a sulfur from cysteine to form the thio-carboxylate on MoaD (14).

MoaD, ThiS, and ubiquitin share limited sequence similarities that include a C-terminal Gly-Gly motif. Activation of each of these proteins involves the formation of an acyl adenylate intermediate at the C-terminal glycine (15, 30, 31), and their activating proteins (MoeB, ThiF, and E1, respectively) also share sequence homologies (16). MoaD, ThiS, and ubiquitin display a high degree of structural similarity, the most noticeable feature in each case being the protrusion of the C terminus from the compact structure of the remainder of the protein. The activity of all three proteins depends on the presence of the C-terminal Gly-Gly motif, since deletion of this motif from either ThiS (32) or ubiquitin (33) deactivates the protein, and MoaD with either one more or one less glycine at the C terminus cannot complement a *moaD*– strain (34). Additionally, sequencing of the MoaD gene in the *moaD*– strain (MJ7chIM (20)) used here as a source of precursor Z revealed a single base change that results in conversion of the penultimate glycine to a glutamate (data not shown).

In the crystal structures of both *E. coli* MPT synthase and the MoaD-MoeB complex, the MoaD C-terminal tail extends into a pocket in the larger protein to form the active site for adenylation in the case of MoeB and sulfur transfer to precursor Z in the case of MoaE. Thus, it would be expected that mutations or deletions at the MoaD C terminus might affect both processes. The observation that *E. coli* MoaD lacking the terminal glycine (G81 Δ MoaD) could not restore molybdoprotein activity in *moaD*– cells could not differentiate between impaired MoaD activation or inhibition of sulfur transfer at the...
MPT synthase step as the reason for lack of complementation (34). The intein fusion expression system for the generation of fully thiocarboxylated MoaD bypasses the normal MoEB-mediated activation route, allowing for characterization of the effect of MoaD mutations specifically on the MPT synthase reaction. The complete lack of activity of G81Δ MoaD-SH observed in this work indicates that the lack of complementation by this variant is not due solely to a defect in MoaD activation, since sulfur transfer to precursor Z is not supported by this variant. Although it is possible that G81Δ MoaD could still be adenylated by Moeb, the high degree of structural similarity between the MPT synthase and the MoaD-MoeB crystal structures makes this a remote possibility. The G80E MoaD variant is probably similarly inactive in both reactions due to the introduction of a bulky side chain at this position. Information gained on the activity of MoaD-SH variants using the system described here is relevant to an understanding of the mechanism of action of both ThiS and ubiquitin, since cocryystals of these proteins with their corresponding modifying proteins have not yet been reported.

An examination of the stoichiometry of the MPT synthase reaction (Fig. 3, B and C) revealed that two molecules of MoaD-SH are required for the conversion of each precursor Z molecule to MPT. This stoichiometry is not surprising, since each MoaD-SH carries a single thiocarboxylate at its C terminus, and the complex reaction requires the addition of two sulfur atoms to precursor Z to form a single MPT dithiolene. Additionally, previous studies using a combined MoaD activation/MPT synthase reaction had determined that both MoaD-SH were derived from cysteine via the same MoeB/sulfurtransferase pathway (14). Thus, one MoaD-SH molecule is required for each sulfur added to precursor Z. Unlike the MoaD-SH/precursor Z ratio, altering the MoaE/precursor Z ratio in the MPT synthase reaction from 0.5 to 2.0 had little effect on the amount of MPT produced (Fig. 3C), indicating that substoichiometric amounts of MoaE are sufficient for the MPT synthase reaction and that MoaE is not altered in the reaction.

During the course of these experiments, a potential intermediate of the E. coli MPT synthase reaction was identified. Minor amounts of this molecule were detected in MPT synthase reactions containing all of the MoaE variants with the exception of the K119A mutant, which produced no intermediate, and the K126A variant, which produced large quantities of this intermediate. Definitive evidence that this molecule was a reaction intermediate was obtained by further characterization of the molecule. These experiments determined that 1) the potential intermediate was a 6-alkyl pterin, 2) the extent of its production was directly related to the ratios of the three components in the synthase reaction, 3) it remained tightly bound to the synthase, and 4) its formation preceded the generation of MPT in the K126A synthase reaction. A careful examination of the extent of intermediate production in wild type MPT synthase reactions containing various ratios of the three reaction components provided further clues to its identity. As seen in Fig. 6A, maximum production of the molecule occurred when substoichiometric amounts of MoaD-SH were present in the reaction, but as the MoaD-SH/precursor Z ratio approached 2.0, the amount of intermediate in the reaction decreased drastically. Conversely, intermediate production was increased at higher MoaE/precursor Z ratios.

These results are compatible with the behavior of a reaction intermediate that contains a single sulfur and remains bound to MoaE during the formation of MPT. Thus, at any given MoaE concentration, MoaD-SH/precursor Z ratios less than 1.0 increase the likelihood of a single sulfur being added to each precursor Z molecule, since there is not enough sulfur to finish the conversion of all precursor Z molecules. As the MoaD-SH/precursor Z ratio increases above 1, a decrease in intermediate concentration is observed as sufficient thiocarboxylate to fully convert all precursor Z molecules to MPT is added. While an inverse relationship exists between the MoaD-SH/precursor Z ratio and the amount of intermediate present in a reaction, there is a direct relationship between the MoaE/precursor Z ratio and the intermediate concentration. Increasing this ratio at any particular MoaD-SH concentration increases the proportion of precursor Z bound and sequestered at the active sites, thus increasing the proportion of hemisulfurated molecules under limiting MoaD-SH conditions. Decreasing the MoaE/precursor Z ratio has the opposite effect. Under these conditions, fewer precursor Z molecules are bound to the synthase, so the limited MoaD-SH pool will be used to preferentially convert all intermediate molecules to MPT. Direct evidence that the intermediate is tightly bound to the synthase supports these conclusions.

The strong affinity of the intermediate for the synthase complicated structural studies of the molecule in that all attempts to remove it from the protein resulted in its oxidation or destruction. Hence, mass spectral analysis of protein-bound intermediate was explored as a means of obtaining structural information for the molecule. Negative ion analysis of a control sample containing protein-bound precursor Z detected a molecule with the expected mass of 344 Da and a hydrated adduct of precursor Z at 362 Da (Fig. 8A). A similarly spaced set of peaks at 378 and 396 Da was the main feature of the spectrum of the intermediate-containing sample (Fig. 8B), indicating a mass difference of 34 Da between precursor Z and the intermediate.

In addition to dithiolene formation, the conversion of precursor Z to MPT by MPT synthase involves cleavage of the precursor Z phosphodiester bond at C-2. Since chemical evidence indicated that the intermediate contains a phosphomonoester (Fig. 7B), the most plausible interpretation of the mass spectra data is that the intermediate contains a terminal phosphomonoester and a single sulfur atom. The 34-Da increase upon conversion of precursor Z to the intermediate could then be explained by two different reaction sequences as seen in Fig. 9. In the first, the addition of H2S(+) (+34 Da) at the second carbon cleaves the cyclic phosphate, resulting in an intermediate with a sulphydryl group on C-2. The hydroxyl from a water molecule would replace the thiocarboxylate on the first MoaD-SH, and the thiocarboxylate from the second MoaD-SH would then exchange with the C-1 hydroxyl to form MPT (top reactions of Fig. 9). In the second possible sequence, the first MoaD-SH sulphydryl is exchanged with the C-1 hydroxyl of precursor Z, resulting in a mass increase of 16 Da. The addition of water at C-2 to cleave the phosphodiester bond and place a hydroxyl at C-2′ would account for the remaining 18-Da difference between the masses of precursor Z and the intermediate. In this case, the addition of the second dithiolene sulfur would occur at C-2′ (bottom reactions of Fig. 9). The two possible intermediate structures shown in Fig. 9 differ only by the relative positions of their side chain hydroxyl and sulphydryl groups at C-1′ or C-2′ and thus have identical masses. Although mass spectral data cannot distinguish between the two intermediate structures, introduction of the first sulfur atom at C-2′ would be more straightforward, since it could proceed in a single step.

Determination of the crystal structure of MPT synthase identified a pair of symmetrical active sites in each heterotetramer that are located at opposite ends of the molecule at the point of insertion of each MoaD C terminus into its corresponding MoeB subunit. The presence of two active sites in a protein responsible for two similar, yet mechanistically distinct, sulfur
Molybdopterin Synthase Reaction

**MPT SYNTHASE REACTION**

![Diagram of MPT Synthase Reaction]

**Fig. 9. Possible reaction mechanisms for the conversion of precursor Z to MPT by MPT synthase.** Initial attack by the first MoaD thiocarboxylate could occur at either the C-1'- or C-2'-positions of precursor Z to produce one of two hemisulfurated intermediates with a negative ion molecular mass of 378 Da. Both of these reactions would require a molecule of water for completion (see “Discussion”). For either intermediate structure, MPT formation would be completed by replacement of the remaining side chain hydroxyl by the sulfhydryl from the second MoaD thiocarboxylate.

Additions to a single precursor molecule raises an intriguing question. Does addition of both dithiolene sulfurs occur independently at both active sites, or is a hemisulfurated intermediate transferred from one active site to the other for the addition of the second sulfur atom? Based on evidence that purified MoaE is monomeric, that the presence of either inactive or thiocarboxylated MoaD is required for MoaE dimerization, and that there is an apparent size difference between active and inactive MPT synthase, Gutzke et al. (10) have proposed the second option. They suggested that precursor Z binds to one of the two active sites of fully activated MPT synthase where conversion to an intermediate with a thione moiety at C-1 occurs. The presence of carboxylated MoaD at this site then induces a conformational change within the synthase that results in the release of the intermediate from the first active site and its preferential binding to the second, where sulfur transfer and phosphoryl ring cleavage subsequently occur (10). Our studies have shown that precursor Z, the intermediate, and MPT are all tightly associated with the synthase. Furthermore, the accompanying paper (23) indicates that MoaE exists as a dimer in the crystal state in the absence of MoaD. Therefore, we believe it likely that each precursor Z molecule remains bound at a single active site until conversion to MPT is completed and that an exchange of carboxylated for thiocarboxylated MoaD occurs while the intermediate is bound at that same active site. If this is true, then each active site must be capable of carrying out both stages of the MPT synthase reaction: cleavage of the cyclic phosphate with the concomitant addition of the first sulfur and the addition of the second sulfur to form the dithiolene.

The relative activities of the MoaE variants studied in this work help to clarify the role of individual active site residues in the two-stage conversion of precursor Z to MPT. Mutation of the lysine residue at position 119 to an alanine completely abolished production of both the intermediate and MPT. The side chain of Lys-119 is located within 3.5 Å of the MoaD C terminus in the crystal structure of MPT synthase (7), and it is this residue that forms an intersubunit isopeptide bond with MoaD-SH over time in preparations of active MPT synthase. Therefore, loss of activity with this mutation was not unexpected. However, the complete lack of both MPT and intermediate production by synthase containing this mutation implies that Lys-119 is directly involved in and absolutely essential for the initial stage of the reaction. Since thioformic acid is ~8 kcal/mol more acidic than formic acid (35), it is likely that the incoming MoaD thiocarboxylate is already deprotonated. The basic Lys-119 side chain may be responsible for properly orienting and positioning the first of these two negatively charged thiocarboxylates for its attack on C-2’ of precursor Z. In addition to Lys-119, it is probable that Arg-39 is also involved in the first stage of the reaction, since 1) the rate of MPT production by synthase containing the R39A variant is 24-fold slower than the wild type protein, 2) the reaction stops well before complete conversion of all precursor Z (data not shown), and 3) there is little accumulation of the intermediate. Due to its position in the active site, Arg-39 is postulated to be involved in the binding and orientation of the precursor Z cyclic phosphodiester (7, 23), and that role is consistent with the activity results obtained with this variant.

Cleavage of the precursor Z cyclic phosphate to the linear monophosphate during the first step in MPT synthesis could shift the position of the intermediate within the active site, resulting in displacement of the carboxylated MoaD C terminus. This would facilitate its exchange for the second MoaD-SH needed for formation of the MPT dithiolene. The terminal amino group of Lys-126 forms a salt bridge with the MoaD C terminus in the MPT synthase crystal structure (7), and the rate of MPT production by synthase containing K126A MoaE is very slow. This residue must be involved in either the structural rearrangement of the active site or in the transfer of the second sulfur to the intermediate side chain, since large quantities of the intermediate accumulate in reactions containing K126A MoaE. It is possible that the function of this residue in the second half of the reaction resembles that of Lys-119 in the first half (thiocarboxylate orientation or formation of a covalent adduct with the intermediate). If this is the case, then the relatively small amount of MPT produced by synthase containing this variant may be due to the ability of Lys-119 to partially substitute for the function of Lys-126 in the second stage of the reaction.

The rates of MPT production in synthase reactions containing the F34A, M115A, or R140A variants of MoaE were only slightly slower than the wild type reaction, indicating that whereas these residues may be involved in the reaction, they are not essential. Two MoaE variants corresponding to naturally occurring mutations identified in the human equivalent of MoaE were also expressed and assayed for their ability to support MPT synthase function. Truncation of the E. coli pro-
tein after residue 140 results in loss of the final α-helix of the protein and decreases the rate of MPT production 12-fold. In the MPT synthase crystal structure, this helix is preceded by a loop from residues 130–140 that lies over the extended MoaD C terminus and forms one wall of the putative pterin-binding pocket (7). The crystal structure of this variant is described in the accompanying paper (22). Since it is likely that the C-terminal helix serves to anchor that loop, it is not surprising that loss of this helix greatly disrupts synthase activity. It was somewhat surprising, however, that the decrease of function observed with this variant is not due to a gross disruption of heterotetramer formation (Fig. 5C).

MPT synthase containing the E128K variant of MoaE is 17-fold slower than wild type synthase. The reason for the sharp decline in activity observed with this variant is unclear. Although Glu-128 is located in the vicinity of the active site, it is far enough away to make direct involvement in the reaction unlikely. It is possible that the substitution of a positive lysine nucleotide of the final exon, it is also possible that a defect in RNA processing contributes to the severe consequences of this mutation in humans (13). Future crystallographic studies on this other mutant MPT synthase variants should shed light on the functions of the corresponding residues and lead to a better understanding of the reaction mechanism of MPT synthase in all organisms.

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