Thiocarboxylation of Molybdopterin Synthase Provides Evidence for the Mechanism of Dithiolene Formation in Metal-binding Pterins*

Molybdopterin (MPT) is a pyranopterin with a unique dithiolene group coordinating molybdenum (Mo) or tungsten (W) in all Mo- and W-enzymes except nitrogenase. In *Escherichia coli*, MPT is formed by incorporation of two sulfur atoms into precursor Z, which is catalyzed by MPT synthase. The recently solved crystal structure of MPT synthase (Rudolph, M. J., Wuebbens, M. M., Rajagopalan, K. V., and Schindelin, H. (2000) *Nat. Struct. Biol.* 8, 42–46) shows the heterotetrameric nature of the enzyme that is composed of two small (MoaD) and two large subunits (MoaE). According to sequence and structural similarities among MoaD, ubiquitin, and ThiS, a thiocarboxylation of the C terminus of MoaD is proposed that would serve as the source of sulfur that is transferred to precursor Z. Here, we describe the in vitro generation of carboxylated and thiocarboxylated MoaD. Both forms of MoaD are monomeric and are able to form a heterotetrameric complex after coinubcation in equimolar ratios with MoaE. Only the thiocarboxylated MPT synthase complex was found to be able to convert precursor Z in vitro to MPT. Slight but significant differences between the carboxylated and the thiocarboxylated MPT synthase can be seen using size exclusion chromatography. A two-step reaction of MPT synthesis is proposed where the dithiolene is generated by two thiocarboxylates derived from a single tetrameric MPT synthase.

Molybdenum (Mo) plays an important role as the active center in many Mo-enzymes that catalyze essential redox reactions in the global C-, N-, and S-cycles (1). Mo-enzymes are important for such diverse metabolic processes like sulfur detoxification and purine catabolism in mammals (2), nitrate assimilation in autotrophs, and phytohormone synthesis in plants (2). With the exception of nitrogenase, in all Mo-enzymes studied so far, Mo is activated and chelated by the so-called molybdenum cofactor (Moco). In general, Moco consists of Mo covalently bound via a dithiolene group to the first coordination sphere of the metal. In addition, the variations in the number and nature of the substituents in the first coordination sphere of the metal. In addition, the unique pterin compound molybdopterin (MPT) (3), a tetrahydropyranopterin (4) that is highly conserved in eukaryotes, Eubacteria, and Archaeabacteria. The basic structure of Moco shows further modifications like the attachment of nucleotide monophosphates to the terminal phosphate of MPT and the coordination of molybdenum by two pterins in prokaryotes, or variations in the number and nature of the substituents in the first coordination sphere of the metal. In addition, the same pterin compound was found to coordinate tungsten (W) in W-enzymes (5, 6). Biosynthesis of Moco requires the multistep synthesis of the MPT moiety followed by the subsequent transfer of molybdenum (2, 7, 8). A mutational block in Moco biosynthesis leads to the combined loss of function of all Mo-enzymes. In humans, Moco deficiency as a rare inborn error is characterized by the loss of activity of sulfite oxidase, xanthine oxidase, and aldehyde oxidase. Affected patients die early postnataally because no therapy is yet available (9).

Genes encoding for Moco biosynthesis proteins have been identified in bacteria (10), fungi (11, 12) and recently in humans (13–15) and plants (16, 17). In this pathway, the proposed starting compound GTP is first converted to the sulfurfree precursor Z (18, 19). In the second step, two sulfur atoms are incorporated into the precursor Z thereby forming MPT as structural moiety of the cofactor. In *Escherichia coli*, three gene products are essential for the synthesis of MPT from precursor Z. MoaD (small subunit) and MoaE (large subunit) form the MPT synthase (20) that stoichiometrically converts precursor Z to MPT (21, 22). MPT synthase is activated (sulfurated) by MoeB (MPT synthase sulfurase). Pitterle et al. (22) postulated that it is the small subunit of MPT synthase that carries the sulfur because a 16 Da difference was observed in the molecular mass between MoaD derived from active MPT synthase and MoaD derived from inactive MPT synthase purified from *E. coli* moeB mutants. This difference was interpreted to be a MoeB-catalyzed replacement of an OH group for a SH group.

Recently the high resolution crystal structure of *E. coli* MPT synthase has been determined demonstrating the heterotetrameric nature of the enzyme (23). Heterodimers are formed between the small and large subunits that dimerize via the large subunits to form the functional heterotetramer. Sequence homologies of the small subunit MoaD to ubiquitin and of the sulfurase MoeB to the ubiquitin-activating enzyme Uba1 from *Saccharomyces cerevisiae* (24, 25) suggested a functional role of the highly conserved C terminus of MoaD containing a double glycine motif (10). Furthermore, MoaD shows a three-dimensional fold very similar to ubiquitin, which interacts via its C terminus with MoeA thereby forming two distinct hypothetical active sites in the heterotetramer. (23). In addition, partially inactive MPT synthase shows a covalent linkage between the C terminus of MoaD and a lysine residue of MoeA. Therefore, it has been
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proposed that MoaD is thiocarboxylated, which serves as the sulfur donor for the synthesis of the diithiolene in MPT. For ThiS, a protein involved in thiamin biosynthesis in *E. coli* (26) that shows striking homologies to MoaD and ubiquitin, the formation of a thiocarboxylate at the C-terminal glycine residue was demonstrated (27). This glycine was shown to be essential for the transfer of a single sulfur atom in thiamin biosynthesis. MPT synthases identified in fungi (12), plants (2) and humans (15) show the same high degree of conservation in the C terminus with the typical double glycine motif. MOCS2A and MOCS2B are the human homologs for MoaD and MoaE (16). The observed bicistronic expression of MOCS2A and MOCS2B (15) indicates the functional pressure for maintaining the free C terminus in MOCS2A.

Here we present direct functional evidence that the C-terminal glycine residue of MPT synthase carries the sulfur that is transferred to precursor Z. Using an intein-based expression system, we generated preparative amounts of thiocarboxylated MoaD and show that this thiocarboxylate is essential and sufficient for the assembly of active MPT synthase. The two subunits of MPT synthase are monomeric when they are expressed and purified separately. Both the thiocarboxylated and the carboxylated form of MoaD are able to assemble a heterotetrameric MPT synthase complex. Finally, a two-step reaction for the synthesis of the diithiolene group in MPT is proposed.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were from the highest grade available. Restriction enzymes were purchased from Promega (Madison, WI) and the DNA polymerase from PegaLab. The *E. coli* strain ER2566, plasmid pTYB2 and the chitin matrix were obtained from New England Biolabs. Nickel-nitritriacetic acid superflow matrix, plasmids pQE30 and pQE60 as well as the *E. coli* M15 strain were purchased from Qiagen.

Construction of Expression Vectors—*E. coli* MPT synthase was cloned into pQE60 (Nco/I) by amplifying moaD and moaE from pJR11 plasmid (28) resulting in pGS100 for expression of MoaE with a C-terminal His tag (polymersase chain reaction deletion of the stop codon) or resulting in pGS101 for expression of unmodified MoaE. Both constructs were bicistronic expression vectors allowing the isopropyl-1-thiogalactoside-induced recombinant expression of MoaD and MoaE in *E. coli*. Expression and expressing exclusively MoaE as His-tagged protein (pGG110) was generated in the same way as described above. For recombinant expression of the small subunit as an intein fusion protein, *E. coli* moaD was polymerase chain reaction-cloned into pTYB2 (Nde/I) yielding pGG100.

Expression and Purification of His-tagged Proteins—His-tagged proteins were recombinantly expressed and purified according to the QIA-expressionist manual (Qiagen). Eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and pure protein fractions were subsequently rebuffed into nit-1 buffer (50 mM sodium phosphate, 250 mM NaCl, 5 mM EDTA, pH 7.2) using Nick columns (Amerham Pharmacia Biotech). The identity of the purified proteins was determined by colony overlay assay (31).

Site Exclusion Chromatography—Size exclusion chromatography was performed at room temperature on a Sephadex 200 column (Amersham Pharmacia Biotech) using an FPLC system (Amersham Pharmacia Biotech). The column was equilibrated in nit-1 buffer, and separation was run at a 0.3 ml/min flow rate. Samples of 200 μl (0.5–5 μg protein) were loaded and 0.5-ml fractions were collected. Small and large subunits were coincubated in molar ratios ranging from 1:4 to 4:1 for a 5 min prior separation. Where indicated, standard deviations of retention times were calculated from 5–7 independent experiments.

N-1 Reconstitution—Neurospora crassa nit-1 extract was prepared as described (32) and stored in aliquots at −70 °C. All reconstitutions were performed in nit-1 buffer (100-μl reaction volume) containing 50 μl of nit-1 crude extract in the presence of 4 mM reduced glutathione and 10 mM sodium molybdate. Where indicated, precursor Z was removed from the nit-1 crude extract by gel filtration on Nick columns. Supplement was carried out anerobically for two hours at room temperature. After addition of 20 mM NADPH for 10 min, reconstituted NADPH-nitrate reductase activity was determined as described (32).

Purification of Precursor Z and HPLC Form A Analysis—Precursor Z was prepared according to Ref. 19 and 1.84 pmol (18.4 μM) of purified precursor Z were incubated for 1 h either with assembled MPT synthase (55 pmol) or with thiocarboxylated MoaD alone (55 pmol) in a total reaction volume of 100 μl. MPT was detected and quantified by HPLC analysis of A-dephospho (33). Oxidation of the entire reaction mixture (100 μl), dephosphorylation, QAE chromatography, and reversed-phase HPLC analysis were performed as described (34).

RESULTS

Expression of MoaD and MoaE and Generation of Carboxylated and Thiocarboxylated Small Subunits—The large subunit of MPT synthase, MoaE, as well as MPT synthase were expressed and purified as His-tagged proteins (see “Experimental Procedures”). The activity of the expressed proteins was proven by functional complementation of *E. coli* moaD and moaE mutants (data not shown). In *E. coli* MoeB has been proposed to activate MoaD by forming a thiocarboxylate at the C-terminal glycine residue. To simulate the predicted reaction, MoaD was expressed as an intein fusion protein by cloning moaD into the pTYB2 vector allowing expression of the small subunit with a C-terminal intein tag and a chitin binding domain for affinity purification. The intein catalyzed self-cleavage reaction results in an N-S acyl shift at the target junction followed by transesterification of the resulting thioester with a cleavage reaction (Fig. 1A). For elution of MoaD we used two different chemicals for cleavage reaction (Fig. 1A). DTT results in the cleavage of a carboxylated unmodified protein while ammonium sulfide generates a thiocarboxylated cleavage product. DTT was previously described for ThiS (27). Analysis of both cleavage products by mass spectrometry proved this system to work correctly for MoaD (Fig. 1C). MoaD eluted with DTT showed a mass peak of 57577 Da while MoaD eluted with ammonium sulfide was 16.3 Da larger, indicating the exchange of an oxygen atom to a sulfur atom. The 0.2-Da difference of the theoretical and experimentally measured masses can be neglected. Hence after cleavage the primary structures of the small subunits were identical to native MoaD carrying the C-terminal double glycine motif. They only differed in the modification of the C terminus. MoaD was purified from 1 liter of *E. coli* culture resulting in 6 mg of carboxylated and 2 mg of...
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Fig. 1. Recombinant expression of E. coli MoaE and MoaD and generation of thiocarboxylated small subunit. A, purification and self-splicing of intein fusion proteins by generating carboxylated and thiocarboxylated small subunits. CDB, chitin binding domain. B, SDS-polyacrylamide gel (14% tricine gel) of purified MoaE and MoaD. MoaE was expressed and purified as C-terminal His-tagged protein (lane 1). MoaD was expressed as intein fusion protein and purified on a chitin affinity column. The intein fusion was cleaved either by addition of DTT or ammonium sulfide resulting in carboxylated (lane 2) or thiocarboxylated (lane 3) small subunits, respectively. In each lane 300 pmol of protein were loaded. C, mass spectrometry of carboxylated and thiocarboxylated MoaD. Proteins were diluted in water to a concentration of 10 µM and subjected to MALDI spectrometry. The molecular masses of the detected peaks are shown and are compared with the calculated masses.

thiocarboxylated protein (Fig. 1B; lanes 2 and 3), respectively.

Assembly of E. coli MPT Synthase from Single Subunits—E. coli MPT synthase was recombinantly expressed and purified using a His-tagged fusion at MoaE. The expressed protein was fully active, and after affinity purification of MoaE also the small subunit MoaD was copurified (data not shown). Therefore, the interaction between both subunits of MPT synthase seems to be of high affinity. We studied the assembly of E. coli MPT synthase from its subunits using analytical size exclusion chromatography. The large subunit MoaE, separately purified as His-tagged protein (Fig. 1B, lane 1), eluted at 16.00 ± 0.02 ml, whereas the carboxylated and thiocarboxylated small subunits (MoaD) showed similar chromatograms with a single peak at 17.35 ± 0.06 ml elution volume (Fig. 2, A and B, inset). According to the elution volumes of standard proteins, the experimentally determined molecular masses of MoaD and MoaE are 6.9 and 16.5 kDa, respectively. Because the theoretical molecular masses of MoaD and MoaE are 8.8 and 18.8 kDa, respectively, we conclude that both subunits of MPT synthase are monomers in solution (Fig. 2D). Coincubation of MoaE with carboxylated or thiocarboxylated MoaD in equimolar amounts resulted in the formation of protein complexes eluting between 14 and 15 ml (Fig. 2, A–C, bold lines), while the peaks of the separated subunits simultaneously disappeared, demonstrating the formation of a heterotetramer. Prior to size exclusion chromatography, both subunits were coincubated for 5 min. Longer incubation times did not increase the amount of tetramers formed (data not shown) suggesting a rapid and high affinity interaction between MoaD and MoaE. Furthermore, mixtures of MoaD and MoaE in submolar ratios resulted in the formation of a tetrameric complex and the appearance of one unbound monomeric subunit that was provided in excess (Fig. 2, A and B, thin and dashed lines) illustrating the high affinity of interaction and conformational behavior of unbound monomers.

Comparison of the elution profiles of MPT synthase complexes formed either with a carboxylated (Fig. 2A) or a thiocarboxylated small subunit (Fig. 2B), revealed a significant shift of 0.19 ml in the elution volume between both forms (Fig. 2C). While thiocarboxylated MPT synthase eluted at 14.45 ± 0.03 ml, the carboxylated form eluted at 14.64 ± 0.02 ml, corresponding to molecular masses of 52.8 and 46.1 kDa, respectively. The determined molecular weights give rise to the conclusion that both carboxylated and thiocarboxylated MoaD are able to form a heterotetrameric complex (53.6 kDa) with MoaE. In addition, purified active MPT synthase (Fig. 2C, control) eluted at 14.49 ± 0.02 ml from the column (Fig. 2C), which is identical to the elution volume of the MPT synthase complex formed by MoaE and thiocarboxylated MoaD. Based on these findings one can argue that the nature of the C terminus of MoaD influences the overall shape of the heterotetrameric complex, resulting in a different Stokes radius. Slight conformational changes or different hydrations near the active site might result in the observed different retention times on size exclusion chromatography between the thiocarboxylated and carboxylated form. However, different interaction of both MPT synthase forms with the gel filtration matrix are rather unlikely because in the crystal structure of MPT synthase the C terminus of MoaD is not exposed to the surface of the molecule (23) and size exclusion chromatography was performed in the
presence of 250 mM salt. Because the elution profiles of active in vivo-assembled and -purified MPT synthase as well as in vitro assembled MPT synthase with thiocarboxylated small subunits were identical and close to the theoretical molecular weight, we conclude that both enzymes have similar conformations.

In Vitro Synthesis of MPT Using Reassembled MPT Synthase—To analyze if the in vitro assembled MPT synthase complexes are active, in vitro synthesis of MPT was shown in two ways: 1) by using the nit-1 reconstitution assay and 2) by using purified precursor Z and demonstrating MPT formation by HPLC analysis. The N. crassa mutant nit-1 is defective in the second step of Moco biosynthesis and accumulates precursor Z that can be converted to MPT by MPT synthase, which is exogenously added to the crude nit-1 protein extract (20). In the presence of 1 mM molybdate, the resulting MPT is non-catalytically converted to Moco that reconstitutes N. crassa apo-nitrate reductase (20, 22).

Bicistronically expressed and purified E. coli MPT synthase (data not shown) was capable of forming MPT and showed an activity of 8.4 units/pmol of purified protein (Fig. 3A, control). MPT synthase that was assembled from separately expressed and purified MoaE and thiocarboxylated MoaD exhibited an even higher activity in MPT synthesis (Fig. 3A, 12.3 units/pmol of protein). In contrast, MPT synthase containing carboxylated MoaD was not active under the same conditions. Only when using 1000 times higher concentrations of carboxylated MPT synthase, a low activity was observed that might be due to an activation of the enzyme within the nit-1 crude extract by a MoeB-like sulfurase. Interestingly, the addition of free small subunit (MoaD) in concentrations 100 times higher than the active enzyme, resulted also in the formation of MPT, suggesting that MoaD forms a chimeric complex with the endogenous large subunit of nit-1 MPT synthase or that MoaD has an activity by itself, which however is rather unlikely. In addition, like carboxylated MPT synthase also carboxylated MoaD alone exhibited nit-1 activities when used in high concentrations, supporting the possibility that a weak sulfuration of the small subunits takes place within the nit-1 crude extract. MoaE alone never showed any activity in the nit-1 assay, suggesting a lesion of the MoaD-homologous protein in the nit-1 mutant. Finally, no activity was observed with all MPT synthases when the protein fraction of the nit-1 extract (gel filtrated) was used or when molybdate

**Fig. 2.** Size exclusion chromatography analysis of MoaD, MoaE, and of their reassembled MPT synthase complexes. A, size exclusion chromatography of 2.0 μM MoaE and 10.6 μM carboxylated MoaD (inset) as well as an equimolar mixture (4 μM, bold line) and submolar mixtures of MoaE and carboxylated MoaD (thin line, 4 μM MoaE + 1 μM MoaD; dotted line, 2 μM MoaE + 8 μM MoaD). B, size exclusion chromatography of 2.0 μM MoaE and 2.2 μM thiocarboxylated MoaD (inset) as well as an equimolar mixture (2 μM, bold line) and submolar mixtures of MoaE and thiocarboxylated MoaD (dotted line, 2 μM MoaE + 0.5 μM MoaD; hair line, 1 μM MoaE + 4 μM MoaD). C, comparison of the size exclusion chromatography elution profiles of 2 μM active MPT synthase (control, dotted line), thiocarboxylated (MoaDE-SH, bold line) and carboxylated (MoaDE-OH, thin line) reconstituted MPT synthase. D, determination of the molecular mass of MoaD, MoaE, as well as thiocarboxylated (E-SH) and carboxylated MPT synthase (E-OH) by plotting their elution volume against that of standard proteins (ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa; equine myoglobin, 17 kDa; ribonuclease A, 14 kDa; vitamin B12, 1.35 kDa).
was depleted from the reaction mixture (data not shown).

To further analyze the activity of reassembled thiocarboxylated MPT synthase, the conversion of precursor Z to MPT was demonstrated using a fully defined system containing either MPT synthase, thiocarboxylated MoaD, or no protein (control) and purified precursor Z. When 55 pmol of reassembled MPT synthase was coincubated with an excess of purified precursor Z (1.84 nmol) the formation of 10.4 pmol of MPT (Fig. 3B, middle chromatogram; form A) could be shown, while in the absence of any protein (Fig. 3B, control) no form A was detectable. However, the low but significantly detectable MPT synthesis by the thiocarboxylated small subunit itself in the nit-1 reconstitution assay was not found when using this fully defined in vitro system (Fig. 3B, lower chromatogram) where 55 pmol of thiocarboxylated MoaD (MoaD-SH) was not sufficient to generate any detectable amounts of MPT.

**DISCUSSION**

MoaD and small subunits of MPT synthases from other organisms (12, 15) show homologies to ubiquitin (24) and ThiS (26), proteins with similar small size and a highly conserved double glycine motif at the C terminus. It was shown that ThiS in the thiamin biosynthetic pathway in *E. coli* (35) contains a thiocarboxylate at the C terminus that is the source of the incorporated sulfur (26). Comparing thiamin biosynthesis with Moco biosynthesis, parallels in sulfur transfer and activation can be seen. However, in contrast to ThiS, the small subunit of MPT synthase needs to interact with the large subunit before gaining its activity. For synthesis of the dithiolene group in MPT two sulfur atoms must be incorporated, while in thiamin pathway only one sulfur per reaction is needed. Further evidence for the functional importance of the C-terminal glycine residue of the small subunit is the finding that in humans both subunits of MPT synthase are expressed by a single bicistronic mRNA (15), which allows the coexpression of both subunits and maintains the free C terminus of the small subunit.

Our experiments to synthesize MPT with *in vitro* assembled MPT synthases demonstrate that the C-terminal glycine residue of the small subunit carries the sulfur that is incorporated into MPT. Furthermore, we show that the *in vitro* formation of a thiocarboxylated subunit and its subsequent assembly with the large subunit generates active MPT synthase. Based on these data and the conservation of MPT synthases among the phyli we propose that also the reaction catalyzed by MPT synthase and its substrate precursor Z are conserved in prokaryotes and eukaryotes. Another line of evidence for the functional importance of the thiocarboxylate of the small subunit comes from size exclusion chromatography experiments where different retention times were observed for active and inactive MPT synthase indicating slight but significant conformational differences between thiocarboxylated and carboxylated MPT synthase. Both kinds of subunits induce the formation of a heterotetrameric MPT synthase whereas the separated subunits (MoaD and MoaE) behaved clearly as monomers under our experimental conditions. Interestingly, the crystal structure of MPT synthase (25) shows that heterotetramer formation is exclusively mediated by the dimerization of MoaE, while the small subunits have no contact to each other; rather they bind to the large subunits thereby forming two independent active sites. However, the dimerization of MoaE seems to be MoaD-dependent because we have found MoaE monomers only in the absence of MoaD suggesting that the large subunit MoaE undergoes large conformational changes during the binding of MoaD resulting in the heterotetramer formation. In the crystal structure of MPT synthase (23) a ~50% active enzyme (50% thiocarboxylated) was analyzed showing a region of high mobility between residues 39 and 45 that are disordered in MoaE. These residues are not in direct contact with the C terminus of MoaD but they are in close proximity. In addition, it is important to note that the terminal glycine Gly-81 of MoaD is forming a strong salt bridge to Glu-126 of MoaE, which is altered after formation of an artificial covalent complex between MoaD and MoaE (23). Therefore it might be reasonable to argue that thiocarboxylation of MoaD influences the conformation of the active site within the MPT synthase complex, a phenomenon that might be reflected by our size exclusion chromatography results.

The main advantage for the cell to possess a MPT synthase
The starting compound precursor Z of MPT synthase is shown in white, while the carboxylated dimer is black, and the dimer with an intermediate conformation (induced fit) is shown in gray.

complex would be the donation of two sulfur atoms by a single heterotetrameric complex with two independent active sites harboring two reactive sulfurs as thiocarboxylates. Assuming that both sulfur atoms in MPT originate from the two small subunits within the heterotetrameric MPT synthase we present the following model for the reaction mechanism of MPT synthase (Fig. 4). A two-step reaction with the formation of a hypothetical intermediate (precursor G) carrying one sulfur atom as a thione is proposed. First, the carboxyl group of the C2 atom in the side chain of precursor Z is protonated followed by a nucleophilic attack of the sulfur from the first thiocarboxylate of MPT synthase. After carboxylation of the small subunit and formation of a thione, precursor G is released. Carboxylation of the first small subunit results in a conformational change of the MPT synthase heterodimer (Fig. 4, shaded), as observed in this study for the fully carboxylated enzyme. The conformational change of the first dimer would result in an induced fit of the second heterodimer (Fig. 4, gray) generating an active site with an increased affinity for the intermediate and a decreased affinity for precursor Z as compared with the starting enzyme. According to this model an equimolar ratio exists between high affinity binding sites and the intermediate allowing a rapid processing of the intermediate without any detectable accumulation of precursor G. In the second half-reaction, the oxygen atom of the cyclic phosphate could be protonated followed by nucleophilic attack of the thiocarboxylate from the second small subunit. The phosphate ring is opened, and a thioester bond is formed. For carboxylation of the second small subunit a water molecule is needed. Finally MPT is released and a fully carboxylated MPT synthase is formed that can be recharged by the sulfurase.

The data presented here confirmed the postulated thiocarboxylation of the small subunit of MPT synthase. Thiocarboxylation seems to be an evolutionary old feature for sulfur mobilization and activation to perform complex C–S bond chemistry. Our proposed model for the reaction mechanism of MPT synthesis provides a first hint to explain how the diithiolene is formed by a single MPT synthase with two active sites.

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