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Reconstitution of a new Cysteine biosynthetic pathway in *Mycobacterium tuberculosis*

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Abstract

A new pathway for cysteine biosynthesis has been elucidated in *Mycobacterium tuberculosis*. This pathway involves a protein bound thiocarboxylate (CysO-SH) as the sulfide donor, similar to thiamin biosynthesis. Cysteine synthase M (CysM) catalyzes the addition of cysteine to the carboxy terminus of the protein bound thiocarboxylate to generate a CysO-cysteine adduct. A protease, Mec^+, hydrolyzes the CysO-cysteine adduct to release cysteine and regenerate CysO. Mec^+ contains a JAMM motif and this work provides the first functional characterization of the JAMM motif in prokaryotes. MoeZ, a paralog of ThiF, has been shown to transfer sulfur onto CysO.

In the biosynthesis of thiamin and molybdopterin, a small sulfide carrier protein (ThiS **1** and MoaD **4**) is converted to a carboxy terminal thiocarboxylate (**3**, **6**) which then functions as the sulfide donor for cofactor biosynthesis.[1] Analogous chemistry, involving formation of thioester **9**, occurs in the ubiquitin targeting of doomed proteins to the proteasome (Scheme 1).[2]

These observations suggested that sulfide carrier proteins may play a role in the biosynthesis of other sulfur-containing natural products. This was supported by the analysis of sequenced genomes for homologs of ThiS and MoaD, which revealed that ThiS-like proteins not only cluster with thiamin and molybdopterin biosynthetic genes, but also with the biosynthetic genes of the sulfur-containing siderophores pyridine-2,6-dithiocarboxylate and quinolobactin.[3] In addition, in *Mycobacterium tuberculosis* and other actinomycetes, a gene similar to *thiS* and *moaD* (Rv1335; herein called *cysO*) was found adjacent to a gene annotated as cysteine synthase (Rv1336; herein called *cysM*). This raised the possibility that CysO could function as the sulfide carrier **12** for cysteine biosynthesis in *M. tuberculosis* by displacing the acetate of O-acetylserine (Scheme 2).

The *mec*$^+$ gene (Rv1334), which codes for a putative hydrolase (JAMM; Jab1/MPN domain metalloenzyme motif), is also clustered with the *cysO* and *cysM* genes in *M. tuberculosis*.[4] This gene was originally identified by its ability to restore a nutritional requirement for cysteine and methionine in a mutant strain of *Streptomyces kasugaensis*, however, no function was assigned.[5] Based on its clustering with CysO and CysM and its predicted hydrolase activity, we proposed that Mec^+ might hydrolyze CysO-cysteine **14** generated by an N-S acyl shift of the initially formed adduct **13** (Scheme 2).[6] In this communication, we describe the experimental confirmation of this new variation of the cysteine biosynthetic pathway.

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Supporting Information **Available:** Cloning, expression, and purification of CysO-SH, His_{10}-CysO-cys, CysO, CysM, and Mec^+, MS protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

CysO-thiocarboxylate was prepared by expressing a CysO-Intein fusion containing a chitin binding domain using the pTYB1 vector. Binding of the fusion protein to a chitin column, followed by soaking in ammonium sulfide (30mM; 40hrs.) resulted in the release of pure CysO-thiocarboxylate.[7] His-tagged CysM was overexpressed using the pET16b vector and purified by Ni-Nta chromatography. MS analysis of a reaction mixture containing CysO-thiocarboxylate, CysM, and O-acetylserine demonstrated the formation of a new CysO derived species with a mass increase of 88 Da consistent with the expected mass of CysO-cysteine **14** (Figure 1). MS/MS analysis localized the adduct to the carboxy terminus of CysO. Neither ThiS-thiocarboxylate **3** from *Bacillus subtilis* nor CysK (Rv2334), the other cysteine synthase from *M. tuberculosis*, were functional in the reaction mixture demonstrating that adduct formation is specific for CysO/CysM.

His-tagged Mec^+, fused to the maltose binding protein to improve solubility, was overexpressed using a modified pET vector and purified by Ni-Nta chromatography. MS analysis of a reaction mixture containing CysO-thiocarboxylate **12**, CysM, O-acetylserine and Mec^+ demonstrated the reformation of CysO **10**. In addition, the formation of cysteine **16** in the reaction mixture could be detected with ninhydrin.[8] When 2.6 nmoles of CysO-thiocarboxylate was added to CysM and Mec^+ in the presence of O-acetylserine, 2.7±0.16 nmoles of cysteine were produced, indicating full conversion to cysteine. When Mec^+ is pre-incubated with EDTA, no hydrolysis is observed. Addition of Zn^{2+} to the gel filtered, EDTA containing reaction restored the hydrolytic activity. This suggests that Mec^+ is a Zn^{2+} dependent CysO-cysteine carboxypeptidase.[9] To provide additional support for Mec^+ hydrolysis of amide **14** rather than thioester **13**, His-tagged CysO with an additional cysteine or alanine at the carboxy terminus (His_{10}-CysO-cys; His_{10}-CysO-ala) was overexpressed. Upon incubation of His_{10}-CysO-cys with Mec^+, we observed the production of cysteine by the ninhydrin assay and hydrolysis by ESI-FTMS (Figure 2). His_{10}-CysO-ala was also hydrolyzed, but at a reduced rate.

The reaction catalyzed by Mec^+ resembles the deubiquitination reaction of the 26S proteasomal subunit Rpn11, which sends doomed proteins into the proteasome for degradation and regenerates ubiquitin (Scheme 1). Both Rpn11 and Mec^+ contain the JAMM motif, which is found in bacteria, archaea, and eukaryotes.[10] Due to the complexity of the proteasome, it has been difficult to study Rpn11 activity on its own. Our demonstration of Mec^+ activity represents the first functional assignment for a JAMM motif protein in prokaryotes and may be a useful model for Rpn11 and the other deubiquitinating enzymes.

We have not yet identified the sulfur donor for CysO. In the formation of ThiS-thiocarboxylate **3** in thiamin biosynthesis, ThiS is activated as an acyl adenylate **2** in a reaction catalyzed by ThiF. The resulting ThiS-COOAMP then reacts with IscS persulfide. Since cysteine is the sulfur donor for IscS persulfide, it is unlikely that this species is the sulfur donor for CysO-thiocarboxylate formation.[11] We have identified and cloned a ThiF paralog in *M. tuberculosis* (*moeZ*, Rv3206) that may catalyze the CysO adenylation and sulfur transfer reaction. MoeZ was expressed from a pET16b vector and purified by Ni-Nta chromatography. When MoeZ was combined with pure CysO, we were unable to observe thiocarboxylate formation on CysO using various sulfur sources (sulfide, thiosulfate). However, when CysO and MoeZ overexperssion strains were co-lysed and co-purified from *E. coli* crude cell lysate, we observed CysO-thiocarboxylate (Figure 3). This indicates that MoeZ is able to catalyze sulfur transfer from an unidentified sulfur source onto CysO.

Analysis of the *M. tuberculosis* genome suggests that there are three routes to cysteine in this microorganism: the sulfide dependent pathway, the cystathionine pathway and the CysO-thiocarboxylate pathway.[12] The relative importance of these pathways is currently unknown. Two observations, however, may point to a function for the CysO dependent pathway. First,

transcriptional profile analysis of *M. tuberculosis* demonstrates that *mec*$^+$, *cysO*, *cysM* and *moeZ* are all upregulated under oxidative stress conditions.[13] Second, thiocarboxylates are much more resistant to oxidation than thiols. These observations suggest that when *M. tuberculosis* is in the highly oxidizing environment of the macrophage, CysO-thiocarboxylate may be used as an oxidation-resistant form of sulfide for cysteine biosynthesis.

Herein we show that a sulfide carrier protein from *M. tuberculosis*, CysO (Rv1335), in its thiocarboxylate form **12**, is alkylated by O-acetylserine to give **13**. This reaction is catalyzed by CysM. Following an S-N acyl rearrangement to give CysO-cys **14**, Mec$^+$ (Rv1334) hydrolyzes CysO-cys **14**, releasing cysteine and regenerating CysO **10**. We have also demonstrated that MoeZ (Rv3206), in crude cell lysates, can catalyze the conversion of CysO **10** to CysO-thiocarboxylate **12** (Scheme 2) using an unidentified sulfur source. We propose that this novel pathway, involving a protein bound thiocarboxylate instead of sulfide, could be necessary for cysteine biosynthesis in *M. tuberculosis* in the oxidizing environment of the macrophage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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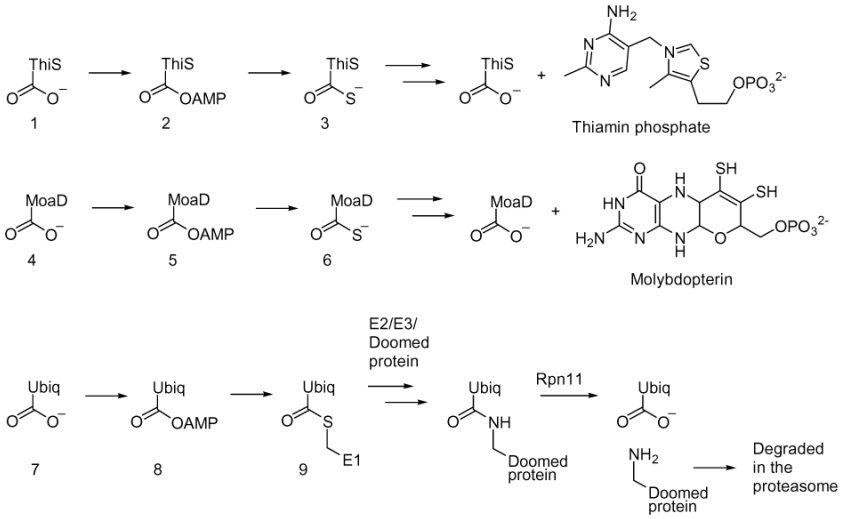
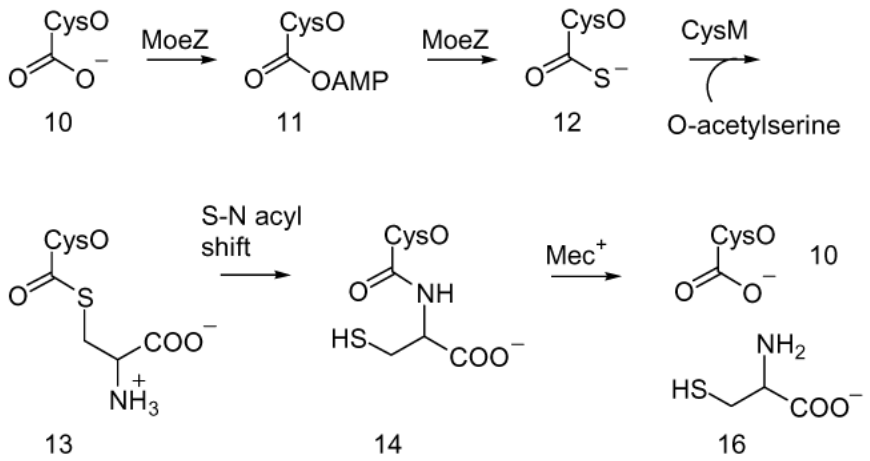


Scheme 1.



Scheme 2.

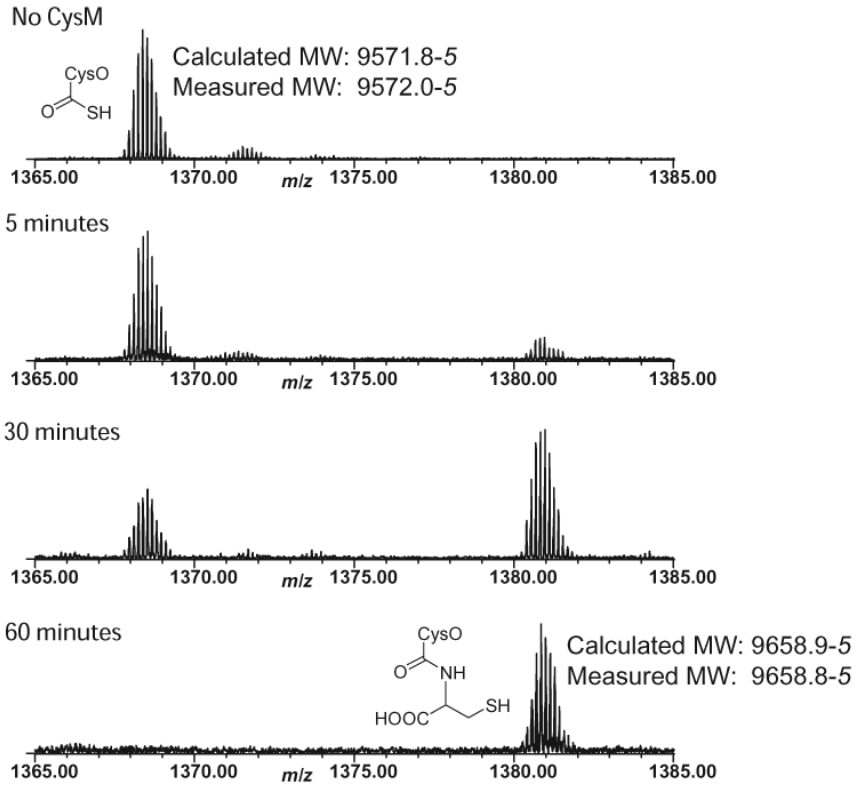


Figure 1.
ESI-FTMS analysis of the reaction between CysM and CysO-thiocarboxylate in the presence of O-acetylserine (+7 charge state).

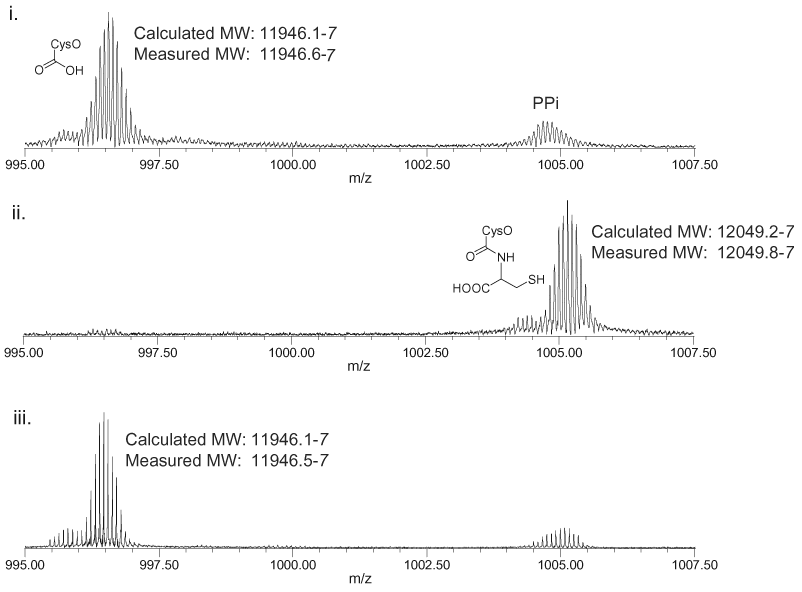


Figure 2.

ESI-FTMS analysis of the Mec^{+} catalyzed hydrolysis of His_{10}-CysO-cys (+12 charge state). i. His_{10}-CysO-cys treated with Mec^{+}; ii. His_{10}-CysO-cys, treated with Mec^{+} that was pre-incubated with EDTA; iii. Reaction ii + Zn^{2+}.

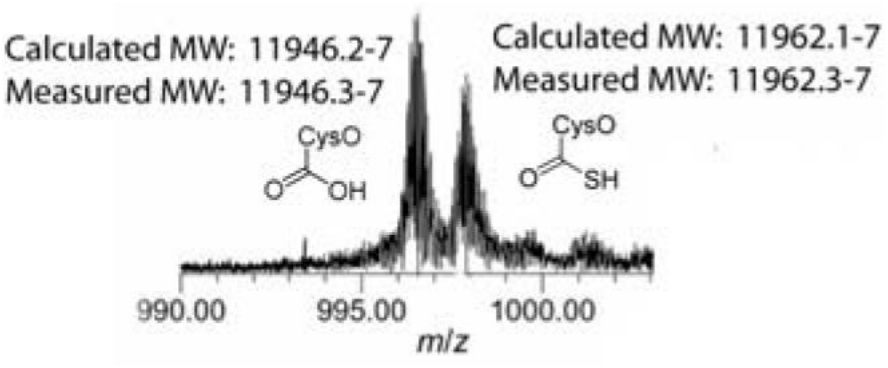


Figure 3.
ESI-FTMS analysis of the MoeZ catalyzed sulfur transfer onto CysO (+12 charge state).